

Drug-resistant colon cancer cells produce high carcinoembryonic antigen and might not be cancer-initiating cells

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Purpose: We evaluated the higher levels of carcinoembryonic antigen (CEA) secreted by the LoVo human colon carcinoma cells in a medium containing anticancer drugs. Drug-resistant LoVo cells were analyzed by subcutaneously xenotransplanting them into mice. The aim of this study was to evaluate whether the drug-resistant cells isolated in this study were cancer-initiating cells, known also as cancer stem cells (CSCs).

Methods: The production of CEA was investigated in LoVo cells that were cultured with 0–10 mM of anticancer drugs, and we evaluated the increase in CEA production by the LoVo cells that were stimulated by anticancer drug treatment. The expression of several CSC markers in LoVo cells treated with anticancer drugs was also evaluated. Following anticancer drug treatment, LoVo cells were injected subcutaneously into the flanks of severe combined immunodeficiency mice in order to evaluate the CSC fraction.

Results: Production of CEA by LoVo cells was stimulated by the addition of anticancer drugs. Drug-resistant LoVo cells expressed lower levels of CSC markers, and LoVo cells treated with any of the anticancer drugs tested did not generate tumors within 8 weeks from when the cells were injected subcutaneously into severe combined immunodeficiency mice. These results suggest that the drug-resistant LoVo cells have a smaller population of CSCs than the untreated LoVo cells.

Conclusion: Production of CEA by LoVo cells can be stimulated by the addition of anticancer drugs. The drug-resistant subpopulation of LoVo colon cancer cells could stimulate the production of CEA, but these cells did not act as CSCs in *in vivo* tumor generation experiments.

Keywords: drug treatment, colon cancer cell, 5-fluorouracil, stem cell, CD133

Introduction

Tumors contain a small subpopulation of cancer-initiating cells, known as cancer stem cells (CSCs), which exhibit a self-renewing capacity and are responsible for tumor generation.¹ CSCs are reputed not to be typical cancer cells, and they may persist in tumors as a distinct population, causing relapse and metastasis by giving rise to new tumors. The first evidence for CSCs was reported in 1997 by Bonnet and Dick² in a study in which they isolated a subpopulation of leukemic cells that expressed a specific surface marker, CD34, but lacked the CD38 marker. The authors established that the CD34⁺/CD38⁻ subpopulation was capable of initiating tumors in non-obese diabetic/severe combined immunodeficiency (SCID) mice and that these tumors were histologically similar to the primary leukemic tumors.

CSCs can form tumors while having stem cell properties such as self-renewal and the ability to differentiate into multiple cell types. It has been suggested that CSCs

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persist in tumors as a distinct population and cause relapse and metastasis by giving rise to new tumors.³⁻⁵ The development of specific therapies that target CSCs could improve the survival and quality of life of cancer patients, especially for those suffering with metastatic disease.

Colon carcinoma was the third most common cause of death in the United States in men and women in 2009.^{4,6} The hypothesis that stem cells drive tumorigenesis in colon cancer raises the question of whether current anticancer therapies can efficiently target the tumorigenic cell population that is responsible for tumor growth and maintenance.⁴ Current therapies mostly fail to eradicate CSC clones and instead favor expansion of the CSC pool and/or select for drug-resistant CSC clones, leading to a fatal outcome.⁷ The isolation and characterization of tumorigenic colon CSCs should enable the development of novel diagnostic and therapeutic procedures.

Specific surface markers for colon CSCs have been reported, and CD133 is the most studied surface marker for colon CSCs.⁸⁻¹¹ CD133 is considered an important marker for identifying the subpopulation of CSCs in leukemia, brain tumors, retinoblastoma, renal tumors, pancreatic tumors, colon carcinoma, prostate carcinoma, and hepatocellular carcinoma.⁸⁻¹² Based on the immunohistochemical findings, Hilbe et al¹³ suggested that CD133-positive (CD133⁺) progenitor cells may play a role in the development of tumor vasculature in non-small-cell lung cancer patients. Ricci-Vitiani et al⁸ reported that CD133 can be used to identify and confirm expansion of human colon CSCs. They injected CD133⁺ colon cancer cells subcutaneously, which readily generated a tumor in SCID mice, whereas CD133⁻ cells did not form tumors.⁸ However, their results were controversial.¹⁴⁻²²

Shmelkov et al¹⁴ prepared a knock-in *lacZ* reporter mouse (CD133^{lacZ/+}) in which the expression of *lacZ* was driven by the endogenous CD133 promoters. Using these mice, CD133 expression in the colon was found not to be restricted to stem cells alone; CD133 was ubiquitously expressed on differentiated colonic epithelia in both adult mice and humans. An examination of CD133 expression did not reveal the entire population of CSCs in human metastatic colon cancer; both CD133⁺ and CD133⁻ metastatic tumor subpopulations were capable of long-term tumorigenesis in a non-obese diabetic/SCID xenotransplantation model.¹⁴

Several other colon CSC markers have been proposed: epithelial specific antigen (EpCAM, BerEp4; cell adhesion molecule), CD44 (CDW44; cell adhesion molecule, hyaluronic acid receptor), CD166 (ALCAM; cell adhesion

molecule), Msi-1 (Musashi-1; RNA-binding protein), CD29 (integrin β 1; cell adhesion molecule), CD24 (HSA; cell adhesion molecule), Lgr5 (GPR49; Wnt targeting gene), and ALDH-1 (ALDC; enzyme).^{4,5,9,22-30} However, exact and reliable surface markers of colon CSCs have not yet been identified. The only reliable method for identifying and quantifying CSCs is to observe tumor formation in a serial xenotransplantation model.

It is generally accepted that CSCs express active transmembrane ATP-binding cassette (ABC) transporter family members, such as the multidrug-resistant transporter 1 and ABC sub-family G member 2 (ABCG2),⁷ which render them drug resistant.³¹ In our previous study,³² drug-resistant cells from human colorectal adenocarcinoma tumors produced two orders higher than normal levels of carcinoembryonic antigen (CEA) per cell. Only 1% of cells treated with acetylsalicylic acid (aspirin) in their culture medium survived, compared with cells grown in the normal expansion medium. This experiment raised questions about whether the drug-resistant colorectal cells, which are increased by adding anticancer drugs into the culture medium, might be CSCs; if so, this method might be the simplest isolation method for CSCs. It will also be important to determine which anticancer drugs or chemotherapy treatments can efficiently deplete CSCs when colon cancer cells are subcutaneously xenotransplanted into mice after the cells have been treated with anticancer drugs.

In this study, we evaluated the higher levels of CEA secreted by the LoVo colon carcinoma cell line, which was cultured in serum-free and serum-containing media containing anticancer drugs. We also treated the cells with aspirin because only aspirin enhanced the expression of CEA in colon carcinoma cells in our previous study.³² Drug-resistant LoVo cells were analyzed to determine whether those cells had CSC characteristics, eg, small size of the cells/colonosphere and strong expression of CSC surface markers, as indicated by flow cytometry and immunohistochemistry analysis. Finally, in vivo tumorigenesis was examined by subcutaneously xenotransplanting the isolated drug-resistant LoVo cells into mice. We then evaluated whether the drug-resistant cells isolated in this study were CSCs.

Material and methods

Cell culture

The LoVo human colon cancer cell line purchased from Food Industry Research and Development Institute (BCRC 60148; Hsinchu, Taiwan) was cultured in (a) a serum-containing medium (Ham's F-12 nutrient mixture medium

[catalog # O135-500; Biowest, Nuaille, France] containing L-glutamine sodium pyruvate and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], with 20% fetal bovine serum [catalog # 04-001-1A; Biological Industries, Beit Haemek, Israel]) and (b) a serum-free medium (Dulbecco's Modified Eagle's Medium/F12 medium containing 10 ng/mL of human recombinant basic fibroblast growth factor and 10 ng/mL of epidermal growth factor) containing 0–10 mM of anticancer drugs. The medium was changed twice a week. The anticancer drugs used in this study were 5-fluorouracil (5-FU) (catalog # F6627; Sigma-Aldrich, St Louis, MO, USA), oxaliplatin, (catalog # O9512; Sigma-Aldrich), cisplatin, (catalog # P4394; Sigma-Aldrich), and acetylsalicylic acid (aspirin) (catalog # A5376; Sigma-Aldrich). A defined amount (0–10 mM) of each anticancer drug was added to the serum-containing or serum-free media. The drugs were dissolved using ultrasonic waves supplied by an ultrasonic cleaner (DC150H; Delta New Instrument, Bangkok, Thailand). The solution was filtered through a disposable 0.22 μm Millex filter (Merck Millipore, Billerica, MA, USA) and adjusted to pH 7.4. Penicillin-Streptomycin Amphotericin B Solution (catalog # 03-033-1B; Biological Industries) was also added to the culture medium, where concentrations of penicillin, streptomycin, and amphotericin B were 250 U/mL, 250 $\mu\text{g/mL}$, and 0.625 $\mu\text{g/mL}$, respectively.

The cell survival rate was defined as follows:

$$\text{Cell survival rate (\%)} = (D_{\text{drug}}/D_0) \times 100 \quad (1)$$

where D_{drug} and D_0 represent the density of the cells cultured in the presence or absence, respectively, of the anticancer therapy.

CEA production by LoVo cells

The concentration of CEA in the culture medium was measured using the enzyme-linked immunosorbent assay (ELISA) (catalog # 25-CEAHU-E01; ALPCO Diagnostics, Salem, NH, USA) and an ELISA plate reader.^{32,33} The concentration of CEA was measured by reading the optical density values obtained at 450 nm. The cell number was estimated by examining the cells on the dishes using an inverted microscope equipped with a charge-coupled devices video camera (MicroPublisher, 3.3RV, Qimaging, Surrey, BC, Canada). CEA production was calculated using the following equation:

$$\text{CEA production (pg/cell} \cdot \text{day)} = C_{\text{CEA}} \times V/N \times D \quad (2)$$

where C_{CEA} represents the concentration of CEA in the culture medium, V is the volume of culture medium (2 mL), N is the

number of cells in the culture medium, and D is number of days the cells were cultured after the addition of fresh culture medium (2 days).

The CEA production ratio was defined as follows:

$$\text{CEA production ratio} = \text{CEA}(\text{drug})/\text{CEA}(0) \quad (3)$$

where $\text{CEA}(\text{drug})$ and $\text{CEA}(0)$ are the concentration of CEA produced by LoVo cells in the culture medium in the presence and absence, respectively, of the anticancer drugs.

Flow cytometry and immunostaining

The CD133/2 (293C3)-phycoerythrin (PE) antibody (catalog # 130-090-853, Miltenyi Biotec, Auburn, CA, USA) and immunoglobulin (Ig)G2b-PE antibody (catalog # 130-092-215, Miltenyi Biotec) as an isotype control, were used for flow cytometric analysis³⁴ of LoVo cells. Conventional staining protocol^{35–39} was used. The expression of CD133, forward scattering intensity, and side scattering intensity of the LoVo cells were analyzed by flow cytometry (Coulter EPICS™ XL; Beckman-Coulter, Brea, CA, USA). Immunohistochemistry was performed after formalin fixation of LoVo cells cultured in tissue culture dishes. The dishes were incubated with antibodies to cell surface markers as follows: CD29 (rabbit anti-human CD29, catalog # NB100-92076; Novus Biologicals, Littleton, CO, USA), CD44 (mouse anti-human CD44, catalog # NBP1-47386; Novus), CD133 (rabbit anti-human CD133, catalog # PAB12663; Abnova, Taipei City, Taiwan), CD166 (rabbit anti-human CD166, catalog # BP1-96579; Novus), ALDH-1 (rabbit anti-human ALDH-1, catalog # PAB3093; Abnova), Lgr5 (goat anti-human Lgr5, catalog # SC-68580; Santa Cruz Biotechnology, Dallas, TX, USA), Msi-1 (mouse anti-human Msi-1, catalog # H00004440-M04, Abnova), as well as secondary antibodies: Alexa Fluor 488 (anti-rabbit IgG, catalog # A21206; Life Technologies, Carlsbad, CA, USA), Alexa Fluor 488 (anti-mouse IgG, catalog # A21202; Life Technologies), and Alexa Fluor 594 (donkey anti-goat IgG, catalog # A11058; Life Technologies). The stained LoVo cells were analyzed using a fluorescence inverted microscope (Eclipse Ti-U; Nikon Instruments, Melville, NY, USA).

MACS sorting

A CD133 micro-bead kit (catalog # 130-050-801, Miltenyi Biotec) was used for the positive and negative selection of LoVo cells using magnetic activated cell sorting (MACS) (MiniMACS™, Miltenyi Biotec). The cells were analyzed using the manufacturer's protocol.

Xenotransplantation with cancer cells

LoVo cells at concentrations of 10^5 were treated with either 0, 0.01, 1, or 10 mM concentrations of the anticancer drugs. The cells, unsorted or sorted by CD133 expression using MACS, were then injected subcutaneously into the flanks of SCID mice, with the approval of the Institutional Animal Care and Use Committee of the Cathay General Hospital and National Central University. Six mice were used to evaluate tumor generation on each drug-treated condition. The mice were sacrificed after 8 weeks, and their tumors were extracted, fixed in 10% neutral buffered formalin solution (catalog # HT501128-4L, Sigma-Aldrich), and paraffin embedded.⁸ The paraffin sectioning and hematoxylin-eosin staining were performed in the Department of Pathology and Medical Laboratory at the Landseed Hospital.

Statistical analysis

All of the quantitative results were obtained from four independent experiments. The data are expressed as the mean \pm standard deviation (SD).

Results

The survival of LoVo cells after treatment with anticancer drugs

LoVo colon cancer cells were cultured in dishes with serum-free and serum-containing media supplemented with 20% fetal bovine serum (FBS); the cell morphology and density were evaluated for 10 days. The LoVo cell morphology before and after 10 days of treatment with anticancer drugs is shown in Figure 1A. The treated LoVo cells shrank and showed more spherical morphology compared with the cells that did not receive treatment (Figure 1A). The LoVo cell density was lower after treatment with anticancer drugs, which is shown in Figure 1A. The cell survival rate was evaluated for LoVo cells treated with 0–10 mM of several anticancer drugs. The cell survival rate decreased as the concentration of the anticancer drugs increased in both the serum-free (Figure 1B) and serum-containing media (Figure 1C). The LoVo cell survival rate in the serum-containing medium was higher than that in the serum-free medium. The LoVo cell survival rate in

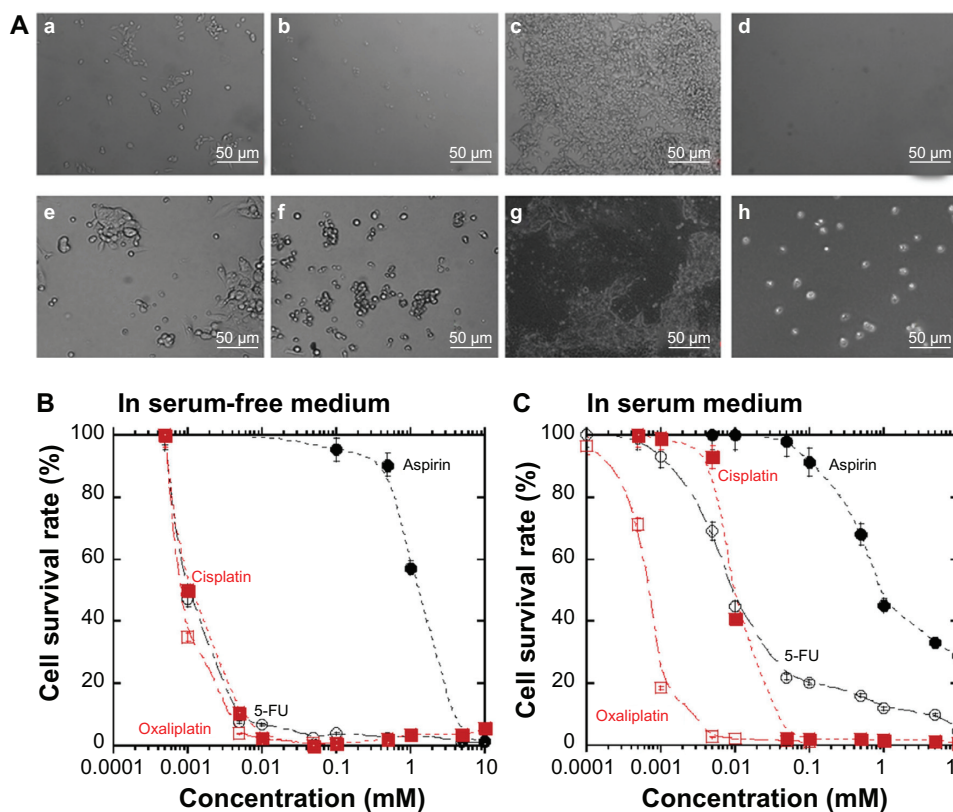


Figure 1 Decreased survival of LoVo cells grown in culture medium containing anticancer drugs.

Notes: (A) The morphology of LoVo cells grown in serum-free medium with no anticancer drugs after 2 days in culture (a), 10 mM of 5-FU after 2 days (b), no anticancer drugs after 10 days (c), and 10 mM of 5-FU after 10 days (d). Also shown here are LoVo cells in serum medium with no anticancer drugs after 2 days in culture (e), 10 mM of 5-FU after 2 days (f), no anticancer drugs after 10 days (g), and 10 mM of 5-FU after 5 days (h). The bar indicates 50 μm. (B) The dependence of the LoVo cell survival rate on the concentration of the following anticancer drugs after 10 days culture in serum-free medium: 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■). (C) The dependence of the LoVo cell survival rate on the concentration of the following anticancer drugs after 10 days culture in serum-containing medium: 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■).

Abbreviation: 5-FU, 5-fluorouracil.

the serum-containing medium was dependent on the specific anticancer drugs used, whereas the LoVo cells in the serum-free medium treated with 5-FU, oxaliplatin, and cisplatin had similar survival rates with the same concentrations of anticancer drugs. Cell viability, cultured in both the serum-free and serum-containing media, with or without anticancer drugs, was observed to be more than 98% in each case as determined by the trypan blue exclusion method.

LoVo cell size after treatment with anticancer drugs

The size of the LoVo cells treated with anticancer drugs was evaluated based on the intensity of the forward and side scatter flow cytometry measurements. Figure 2A shows typical examples of forward and side scatter plots of LoVo cells cultured in the serum-free and serum-containing media, each with or without 5-FU. The data were collected using

flow cytometry after 10 days of cell culture. We defined the small cell size parameters on the forward and side scatter plots as shown in the green box in Figure 2A, and the frequency of small LoVo cells was evaluated by flow cytometry; the same operation was used for the LoVo cells treated with and without anticancer drugs. Cell size decreased with increasing concentrations of 5-FU in both the serum-free and serum-containing media, as shown in Figure 2A.

Figure 2 shows the dependence of the frequency of small cell size on the cell survival rate of LoVo cells cultured in the serum-free (Figure 2B) and serum-containing media (Figure 2C), containing 5-FU, aspirin, oxaliplatin, and cisplatin. Cell size decreased with increasing concentrations of the anticancer drugs in the serum-free as well as the serum-containing media. LoVo cells in the serum-containing medium were smaller, in parallel with the decrease in the cell survival rate of LoVo cells treated with any of the anticancer

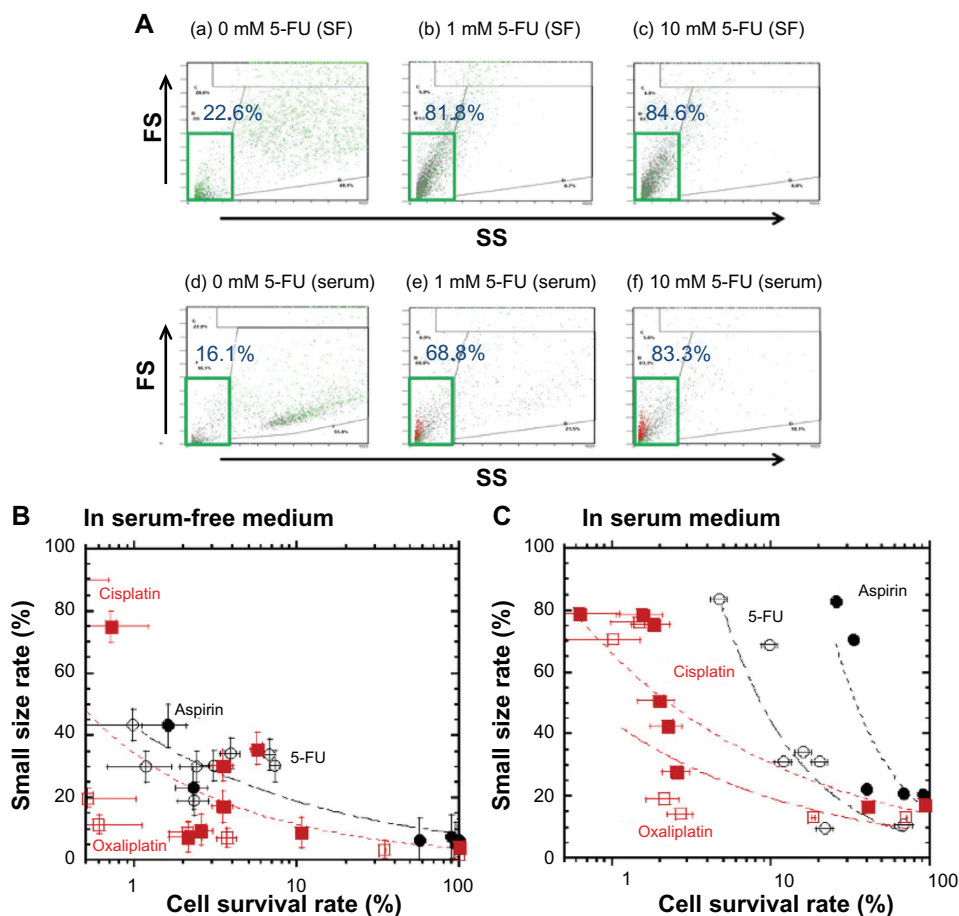


Figure 2 Reduction in LoVo cell size in both serum-free medium and serum-containing medium at high concentrations of anticancer drugs.

Notes: The cells were cultured for 10 days. (A) Flow cytometry scatterplots show the forward (FS) and side (SS) scatter of LoVo cells cultured in serum-free medium with no 5-FU (a), 1 mM of 5-FU (b), and 10 mM of 5-FU (c) and in the serum-containing medium with no 5-FU (d), 1 mM of 5-FU (e), and 10 mM of 5-FU (f). Small LoVo cells were defined as those cells located within the green box shown on the flow cytometry scatterplots. (B) The relationship between small size and the cell survival rate of the LoVo cells cultured in serum-free medium with 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■). (C) The relationship between small size and the cell survival rate of the LoVo cells cultured in serum-containing medium with 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■).

Abbreviations: 5-FU, 5-fluorouracil; FS, scatter forward; SS, side scatter; SF, serum free.

drugs used in this study. These results indicate that under conditions where no anticancer drugs are present, drug-resistant LoVo cells are smaller than normal LoVo cells.

Production of CEA by LoVo cells during treatment with anticancer drugs

We investigated the production of CEA in LoVo cells when they were cultured with 0–10 mM of anticancer drugs, and we evaluated an increase in CEA production which was stimulated by anticancer drug treatment. Figure 3A shows the dependence of the LoVo cell CEA production on the 5-FU concentration in the culture medium 8–10 days post-treatment. CEA production by LoVo cells increased with increasing 5-FU concentration in both the serum-free and serum-containing media. CEA production by the LoVo cells in the serum-containing medium was higher than that in the serum-free medium for the same concentration of 5-FU. We investigated the dependence of CEA production on cell

survival rate in LoVo cells in serum free (Figure 3B) and serum-containing media (Figure 3C), containing several anticancer drugs. The CEA production ratio was higher for LoVo cells with lower survival rates. These results suggest that drug-resistant LoVo cells produce CEA with high efficiency when LoVo cells are treated with anticancer drugs. This result is consistent with previous research in which the human colorectal adenocarcinoma tumor cell line expressed higher levels of CEA when cell growth was suppressed by the addition of aspirin to the serum medium.³² In the present study, aspirin and other anticancer drugs tested (5-FU, oxaliplatin, and cisplatin) were found to stimulate CEA production in LoVo cells at a much higher level than in LoVo cells grown in the absence of the anticancer drugs. When LoVo cells were cultured in the serum-free medium containing aspirin, cisplatin, or 5-FU, they produced higher levels of CEA, with the same cell survival rate, than when cultured with oxaliplatin; no significant difference was

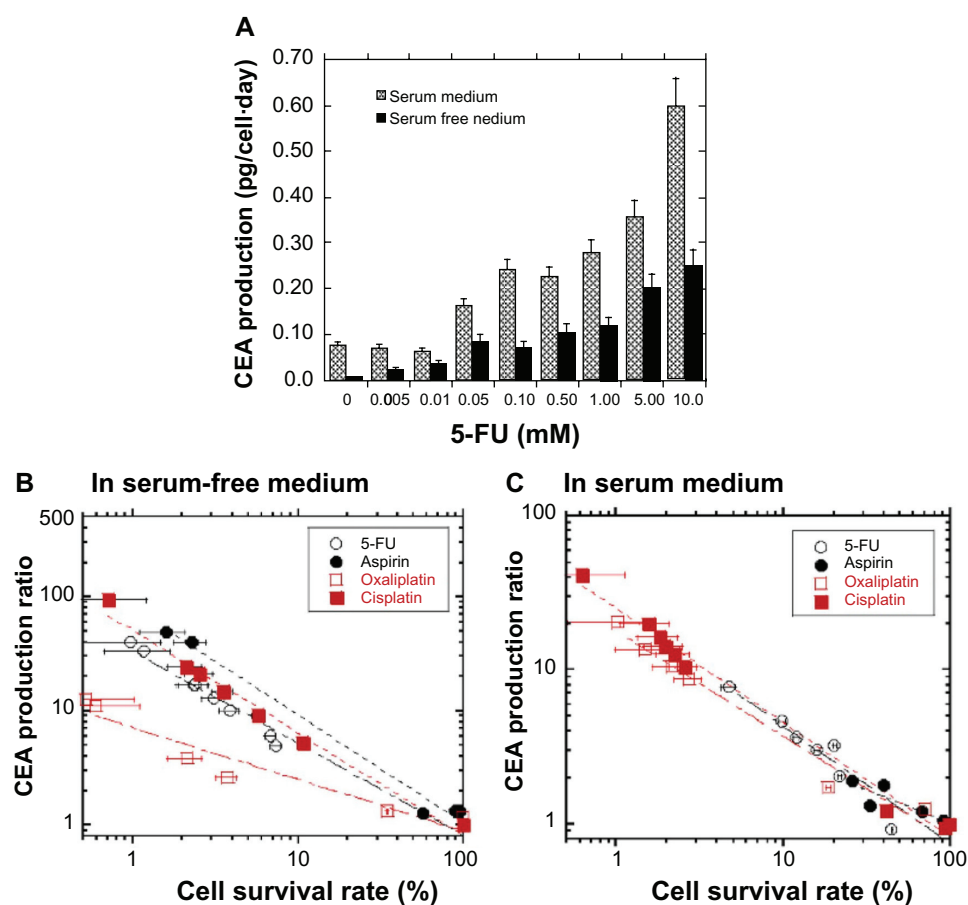


Figure 3 Production of CEA by LoVo cells was enhanced by the suppression of cell survival with the addition of anticancer drugs to the culture medium during cell culture for 8–10 days.

Notes: (A) The dependence of CEA production by LoVo cells on the concentration of 5-FU in the serum free and serum-containing media. (B) The dependence of the CEA production ratio on the cell survival rate of LoVo cells cultured in serum-free medium with 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■). (C) The dependence of the CEA production ratio on the cell survival rate of LoVo cells cultured in serum-containing medium with 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■).

Abbreviations: 5-FU, 5-fluorouracil; CEA, carcinoembryonic antigen.

observed between cells treated with aspirin, cisplatin, or 5-FU (Figure 3B). When LoVo cells were grown in the serum-containing medium, there was no significant difference in the CEA production ratio after treatment with any of the anticancer drugs for the same cell survival rate (Figure 3C). The cell survival rate appears to be an important determinant of the production of CEA by LoVo cells in the serum-containing medium with different anticancer drugs.

Expression of CSC markers by LoVo cells during treatment with anticancer drugs

We expected that a small fraction of LoVo cells, those that showed drug resistance and produced high levels of CEA, might contain a high proportion of CSCs. Therefore, we evaluated the expression of several CSC markers, including CD29, CD44, CD133, CD166, ALDH-1, Lgr5, and Msi-1 in LoVo cells treated with anticancer drugs.

Figure 4A shows typical flow cytometry analyses of CD133⁺ LoVo cells that were cultured in the serum-free and serum-containing media, each with or without 5-FU, after 10 days of cell culture. The population of CD133⁺ cells was dramatically lower after 5-FU treatment in both the serum-free and serum-containing media groups in this study.

Figure 4 shows the relationship between the percentage of CD133⁺ cells and the survival rate of LoVo cells treated with several anticancer drugs in the serum-free (Figure 4B) and serum-containing media (Figure 4C). The percentage of CD133⁺ cells decreased in parallel with the decrease in the cell survival rate in the serum-free as well as the serum-containing media. This indicates that the number of CD133⁺ cells decreased when the LoVo cells were cultured in medium containing higher concentrations of the anticancer drugs. The percentage of CD133⁺ cells was less than 15% when the LoVo cells were treated with any of the anticancer drugs, and the

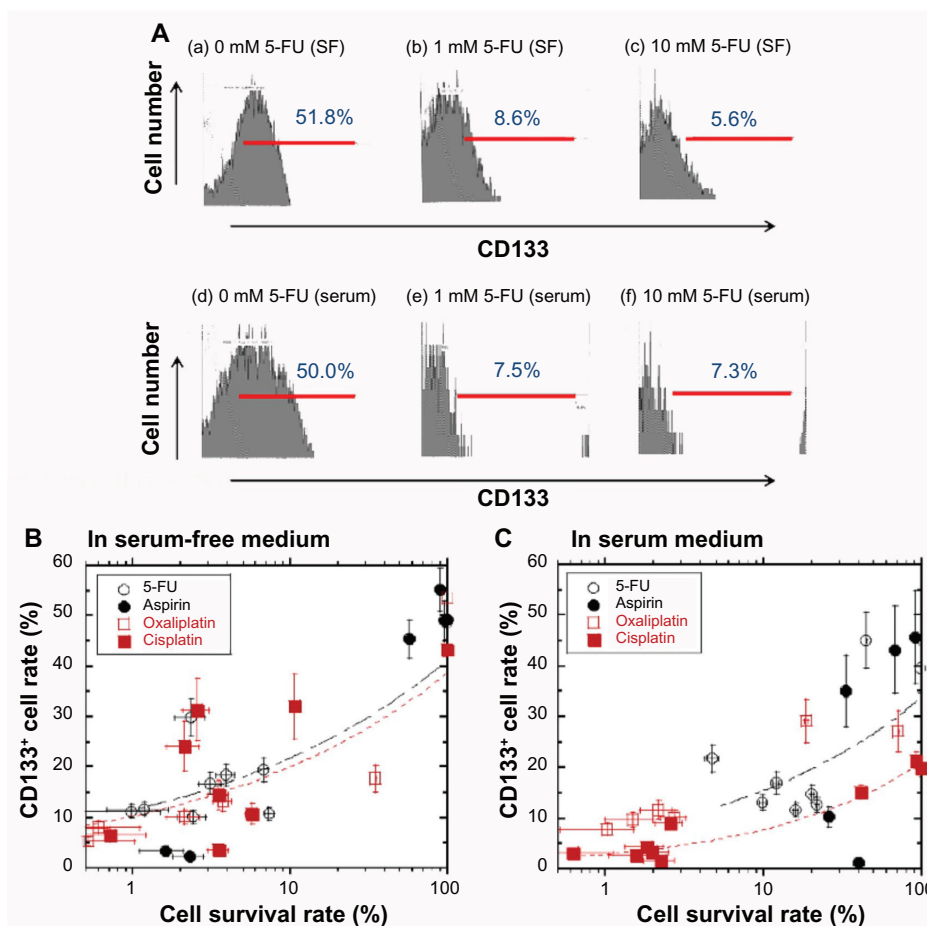


Figure 4 CD133 expression on LoVo cells was decreased by the suppression of cell survival with the addition of anticancer drugs to the culture medium after 10 days in cell culture.

Notes: (A) Flow cytometry scatterplots showing CD133 expression on LoVo cells in serum-free (SF) medium with no 5-FU (a), 1 mM of 5-FU (b), and 10 mM of 5-FU (c) and in serum-containing medium with no 5-FU (d), 1 mM of 5-FU, and 10 mM of 5-FU. (B) The dependence of the frequency of CD133 expression on the cell survival rate of LoVo cells cultured in serum-free medium with 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■). (C) The dependence of the frequency of CD133 expression on the cell survival rate of LoVo cells cultured in serum-containing medium with 5-FU (○), aspirin (●), oxaliplatin (□), and cisplatin (■).

Abbreviation: 5-FU, 5-fluorouracil.

cell survival rate was less than 2% in both the serum-free and serum-containing media, whereas the rate of CD133⁺ cells was 50% ± 5% in both the serum-free and serum-containing media with no anticancer drugs. We found that fewer of the drug-resistant LoVo cells were CD133⁺ compared with the untreated LoVo cells in both the serum-free and serum-containing media.

In accordance with CD133, CD29, CD44, CD166, ALDH-1, Lgr5, and Msi-1 having all been reported to be cancer stem cell markers,^{4-7,9} the expression levels of these molecules were measured by immunohistochemistry on LoVo cells cultured in the serum-free medium with 0, 0.01, 1, and 10 mM of 5-FU, and the results are shown in Figures 5 and 6. No significant expression of the CSC markers was found on the LoVo cells in the serum-free medium containing and not containing 5-FU. However, the levels of the CSC markers CD29, CD44, CD133, CD166, ALDH-1, Lgr5, and Msi-1 were slightly lower for LoVo cells treated with 5-FU than for the untreated LoVo cells. Thus, the drug-resistant

LoVo cells expressed lower levels of the CSC markers in this study.

Tumor generation by LoVo cells receiving anticancer therapy

The induction of tumor generation *in vivo* by subcutaneously injecting LoVo cells into mice is the most direct method for qualitatively and quantitatively evaluating the tumorigenic potential of CSCs. Therefore, *in vivo* tumor generation was examined by subcutaneously injecting treated and untreated LoVo cells into SCID mice. Figure 7A shows the tumors generated in mice following the injection of LoVo cells cultured in serum-free and serum-containing media without any anticancer drugs. The size of the tumors in the SCID mice was larger when the cells had previously been cultured in the serum-containing medium than when they had been cultured in the serum-free medium. This indicates that the CSC subpopulation of the LoVo cells cultured in the serum-containing medium was higher than that of the

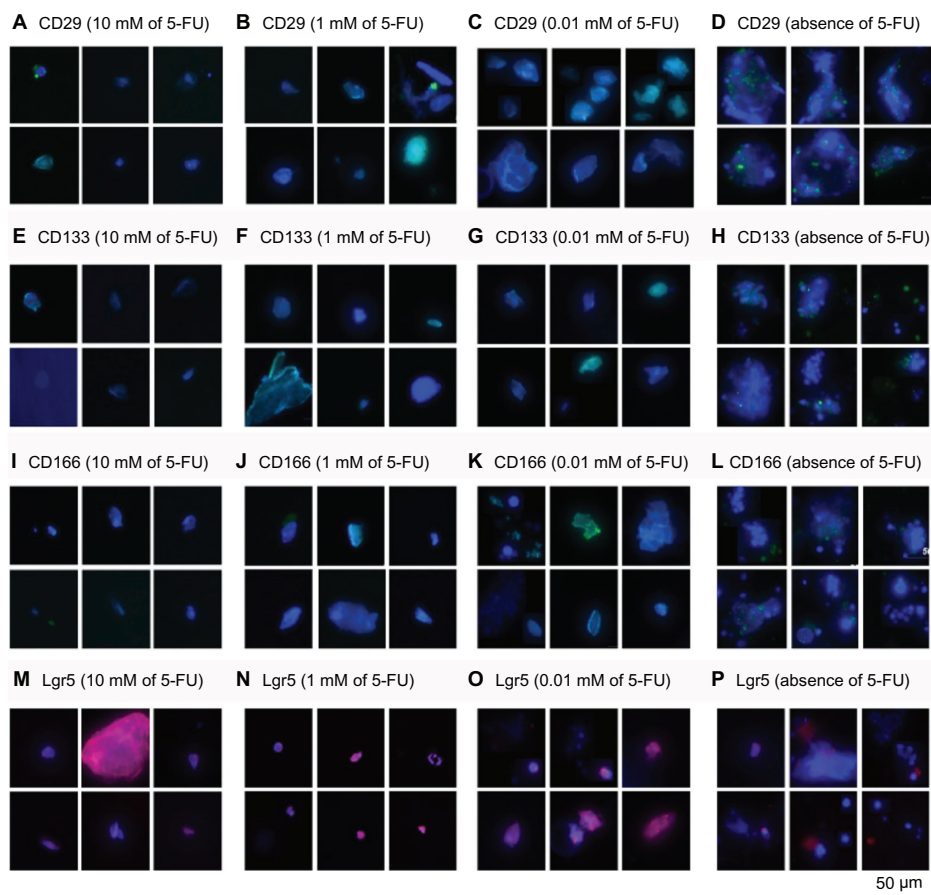


Figure 5 Immunohistochemical analysis of CSC surface markers on LoVo cells.

Notes: Immunohistochemical analysis of CD29 (A–D), CD133 (E–H), CD166 (I–L), and Lgr5 (M–P) expression (green and red) and Hoechst staining (blue) of LoVo cells cultured in serum-free medium in the absence (D, H, L and P) or presence of 0.01 mM (C, G, K and O), 1 mM (B, F, J and N) and 10 mM (A, E, I and M) of 5-FU.

Abbreviations: 5-FU, 5-fluorouracil; CSC, cancer-initiating cell; Lgr5, leucine-rich repeat-containing G protein-coupled receptor 5.

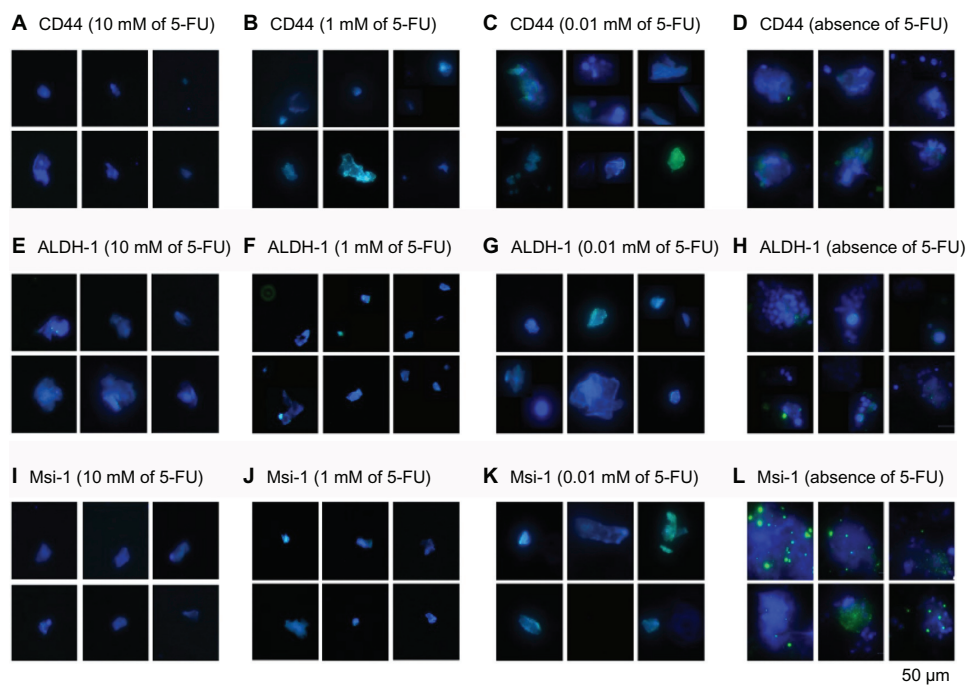


Figure 6 Immunohistochemical analysis of CSC surface markers on LoVo cells.

Notes: Immunohistochemical analysis of CD44 (A–D), ALDH-1 (E–H), and Msi-1 (I–L) expression (green) and Hoechst staining (blue) of LoVo cells cultured in serum-free medium in the absence (D, H and L) or presence of 0.01 mM (C, G and K), 1 mM (B, F and J) and 10 mM (A, E and I) of 5-FU.

Abbreviations: 5-FU, 5-fluorouracil; ALDH-1, aldehyde dehydrogenases-1; CSC, cancer-initiating cell; Msi-1, musashi-1.

LoVo cells cultured in the serum-free medium in these culture conditions. Figure 7B shows the time dependence of the tumor growth in SCID mice injected with LoVo cells that had been cultured in serum-free and serum-containing media, with or without anticancer drugs. LoVo cells in the serum-free or the serum-containing media, with 0.01, 1, or 10 mM of any anticancer drug (5-FU, aspirin, oxaliplatin, or cisplatin), did not generate tumors within 8 weeks of when the cells were injected subcutaneously into SCID mice. This indicates that 144 mice (3 [different concentration] × 4 [different drugs] × 2 [serum-free and serum medium] × 6 [6 mice on each condition]) generated no tumor within 8 weeks when the drug-treated LoVo cells were injected subcutaneously. These results suggest that the drug-resistant LoVo cells have a smaller population of CSCs than the untreated LoVo cells. Furthermore, all of the anticancer drugs used in this study effectively killed the CSCs within the LoVo cells, as observed in the cells cultured with anticancer drugs in vitro.

The CD133⁺ and CD133⁻ cells were sorted using the MACS method, and the tumorigenic potential of those cells was also evaluated (Figure 7B). The CD133⁺ population of LoVo cells sorted by CD133⁺ MACS was analyzed and determined to be 45.7% ± 9.2% and CD133⁻ population sorted by CD133⁻ selection was 2.4% ± 1.3%. Tumor

generation by the CD133⁺ cells was slightly higher than that for the CD133⁻ cells ($P < 0.05$), where the viability of both CD133⁺ cells and CD133⁻ cells was found to be more than 96%, but tumor generation by both CD133⁺ and CD133⁻ cells, which were cultured in the serum-containing medium, was lower than that of normal LoVo cells without MACS treatment. This result indicates that LoVo cells are damaged mechanically and/or chemically when they are sorted by MACS treatment, although the cell viability of sorted cells is more than 96%. Treating the LoVo cells with an anticancer drug decreased the CSC population more effectively compared with isolating CD133⁻ LoVo cells in this study.

Discussion

Colon cancer cell lines produced large amounts of CEA when the cell survival rates were decreased by the addition of aspirin and also several anticancer drugs (5-FU, aspirin, oxaliplatin, and cisplatin) in both the serum-free and serum-containing media. In previous studies,^{32,40,41} only aspirin and 5-FU were reported to stimulate the production of CEA in colorectal cancer cells.

Because CSCs are often reported to be drug-resistant cells,⁴⁻⁷ we hypothesized that the drug-resistant cells selected by adding anticancer drugs to the culture medium might be

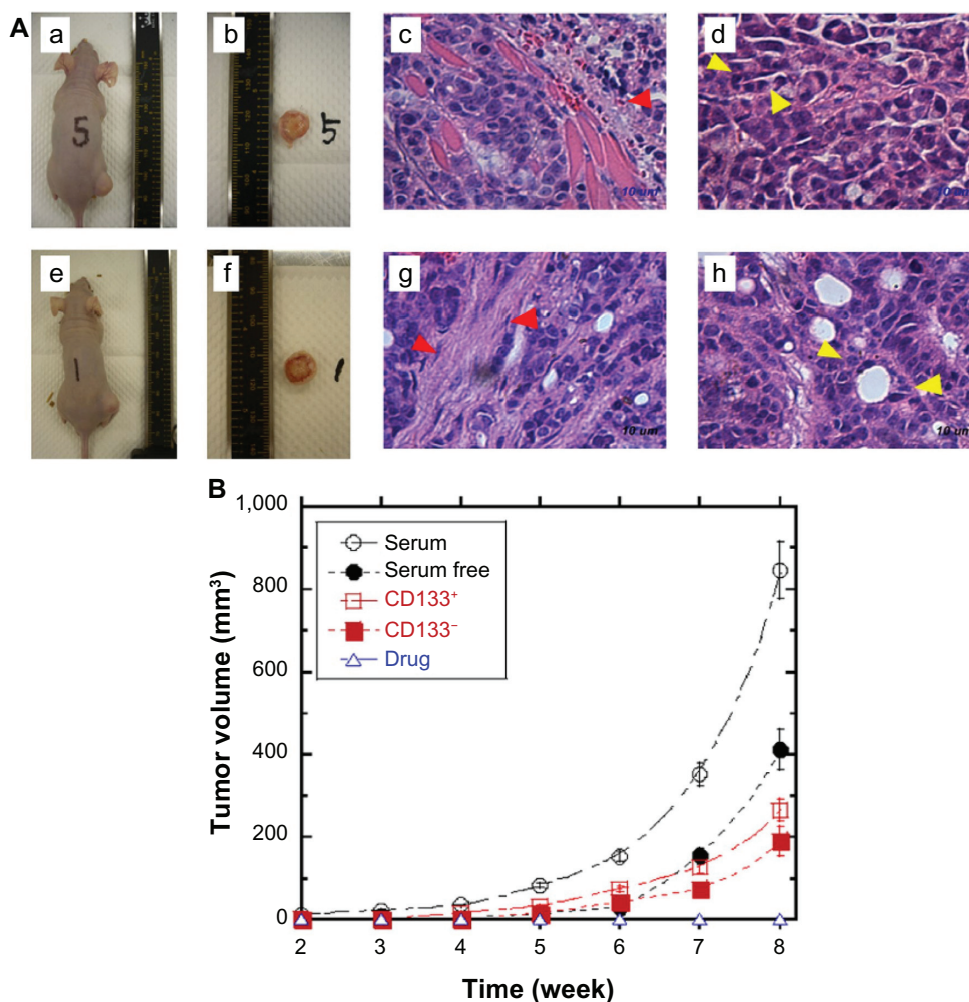


Figure 7 The tumor generation potential of LoVo cells in vivo.

Notes: (A) Tumor generation in mice induced by the subcutaneous xenotransplantation of 10^5 LoVo cells cultured in serum-free medium (a and b) or in serum-containing medium (e and f). Hematoxylin-eosin staining of the tumors generated by subcutaneous injection of LoVo cells cultured in serum-free medium (c and d) or serum-containing medium (g and h) for 10 days. Red and yellow arrowheads indicate the dead cell areas. (B) The time dependence of the tumor size (volume) generated by the subcutaneous injection of LoVo cells cultured in serum-free medium (●) or serum-containing medium (○) in the absence of the drugs. Tumor growth is also shown for MACS-sorted CD133⁺ cells (□) and CD133⁻ cells (■) in the absence of the drugs. LoVo cells treated with 0.01, 1, or 10 mM of anticancer drugs (△; 5-FU, aspirin, oxaliplatin, and cisplatin) did not generate tumors.

CSCs. The drug-resistant LoVo cells isolated in this study were smaller than the normal colon cancer cells (Figure 2) but contained fewer CD133⁺ cells. A future issue for us to evaluate is whether other types of drug-resistant colon cancer cells are smaller than their normal colon cancer cells. The CD133 marker is typically used to identify colon CSCs.^{8–11} In this study, the presence of several other CSC markers, including CD29, CD44, CD166, ALDH-1, Lgr5, and Msi-1^{4,5,9,22–25} was also evaluated in the drug-resistant LoVo cells using immunohistochemistry (Figures 5 and 6); however, drug-resistant LoVo cells expressed lower levels of CSC markers.

Currently, the only reliable method of identifying CSCs is by measuring tumor generation in vivo following the subcutaneous xenotransplantation of the cells into mice.

LoVo cells cultured in serum-free and serum-containing media, without anticancer drugs, can generate tumors, which implies that the LoVo colon cancer cell line contains a subpopulation of CSCs. However, drug-resistant LoVo cells selected by treatment with any of the tested anticancer drugs (5-FU, oxaliplatin, and cisplatin) or aspirin did not generate tumors in this study. We were unable to perform serial xenotransplantations on mice transplanted with LoVo cells treated with drugs, because these mice did not generate tumors. However, we performed xenotransplantation using 144 mice (3 [different concentration] × 4 [different drugs] × 2 [serum-free and serum medium] × 6 [6 mice on each condition]) and no tumor generation was observed on mice transplanted with LoVo cells treated with drugs. These results indicate that LoVo cells treated with drugs contain few or no CSCs in

this study. Recently, Yan et al⁴² reported that Du145 prostate cancer cells treated with drugs (ie, etoposide, paclitaxel, staurosporine, and 2-paclitaxel analogs) exhibited greatly reduced tumorigenicity or were nontumorigenic. This result indicates that drug-resistant Du145 cells are not CSCs, or contain less CSCs compared to Du145 cells without treatment of drugs. Their report also supports our findings in this study.

In the clinic, biomarkers are often measured to evaluate disease progression or responses to a therapeutic intervention such as drug treatment. Predictive biomarkers provide information on the response to a treatment, whereas prognostic biomarkers provide information about the outcome, independent of the treatment effect. CEA is one of the earliest studied biomarkers in colorectal cancer, and the preoperative serum CEA level is an independent negative prognostic factor.^{43,44} A CEA surge or flare has been observed as an early biochemical phenomenon in metastatic colorectal cancer during chemotherapy in approximately 10% of the patients who experience a clinical benefit.^{45–47} In light of this clinical evidence, we speculate that drug-resistant cancer cells are not CSCs because patients with CEA surges experience a clinical benefit, which is inconsistent with the CSC theory that predicts that these patients would have a worse prognosis.

Conclusion

Production of CEA by LoVo cells can be stimulated by the addition of anticancer drugs as well as aspirin in both serum-free and serum-containing media. Increased CEA production was also reported in the human colorectal adenocarcinoma tumor cell line, following treatment with aspirin in the serum-containing medium.³² CSCs are believed to be drug resistant cells;^{4–7} however, although the drug-resistant subpopulation of LoVo colon cancer cells, which were isolated by the addition of anticancer drugs to the culture medium, could stimulate the production of CEA in both serum-free and serum-containing media, these cells did not act as CSCs in in vivo tumor generation experiments.

Acknowledgments

This research was partially supported by the National Science Council of Taiwan under grant numbers NSC100-2120-M-008-004 and NSC101-2120-M-008-003. This work was also supported by the National Central University-Cathay General Hospital Project (CGH-MR-10025, 102NCU-CGH-02, and 101CGH-NCU-A2).

Disclosure

The authors report no conflicts of interest in this work.

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