의학박사 학위논문

Anti-cancer effect of neural stem cells transfected with carboxylesterase and sTRAIL genes in animal model of metastatic brain tumor from lung cancer

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지도 교수 왕규창

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뇌신경과학

홍석호

홍석호의 의학박사 학위논문을 인준함

2014년 6월

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Abstract

Introduction: Metastatic brain tumor is the most common type of malignancy in the central nervous system, which is one of the leading causes of death in patients with lung cancer. The purpose of this study is to evaluate the efficacy of the novel treatment using neural stem cells (NSCs) encoding genes for rabbit carboxylesterase (rCE) and secretable form of tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) as a vector to deliver therapeutic materials into the brain in animal model of metastatic brain tumor with lung cancer and whether it augment the efficacy of the chemotherapeutic agent.

Methods: The therapeutic cells were immortalized human fetal NSCs (HB1.F3s) transduced with rCE and/or sTRAIL genes to express rCE (F3.CE), sTRAIL (F3.sTRAIL), or both (F3.CE.sTRAIL). The cytotoxic effects of the therapeutic cells on A549 and H460 human lung cancer cells were evaluated in vitro with a co-culture assay in the presence of CPT-11. The expression levels of DR4 and DR5,cell surface receptors for sTRAIL, and DcR1 and DcR2, decoy receptors for sTRAIL, were measured by ELISA in A549 and H460 cells after CPT-11 treatment in vitro. Immune-deficient mice were inoculated with lung cancer cells, intracardially injected with therapeutic cells, and then treated with CPT-11. Histology and survival were analyzed to determine the anti-cancer efficacy of each therapeutic cell type by measuring tumor volumes in brain sections.

Results: . Human NSCs encoding rCE (F3.CE and F3.CE.sTRAIL) significantly inhibited the growth of A549 and H460 cells in the presence of CPT-11 in vitro. F3.sTRAIL cells also showed a mild cytotoxic effect on lung cancer cells that was enhanced by CPT-11 treatment. All therapeutic cells exerted cytotoxic effects on tumor cells in the co-culture assay, with more apoptosis induced as the proportion of therapeutic cells to tumor cells increased. In the resting state, over 70% of H460 cells expressed DR4, but A549 and NSCs showed low (<30%) rates of DR4 expression. For DcR2 expression, however, less than 5% of H460 cells expressed surface DcR2, whereas 40% and 71% of A549 and NSCs expressed DcR2. After CPT-11 addition, DR4 expression in A549 cells and DcR1 in NSCs increased up to 70% and 90%, respectively. Tumor volume in immune-deficient mice was significantly smaller when the mice were transplanted with F3.CE.sTRAIL cells and subsequently treated with CPT-11. Mice treated with F3.CE plus CPT-11 and with F3.sTRAIL alone showed a smaller tumor size than control mice or mice treated with F3.CE or CPT-11 alone. Survival was also significantly prolonged following treatment with F3.sTRAIL, F3.CE plus CPT-11, and F3.CE.sTRAIL plus CPT-11.

Conclusions: NSCs transduced with rCE and sTRAIL genes showed significant anti-cancer effects on brain metastatic lung cancer in vivo (in the brain) and in vitro and the effect may be synergistic when rCE/CPT-11 and sTRAIL are combined. This stem cell-based study using two

therapeutic genes with different biological effects could be translatable to clinical application.

Keywords: metastatic brain tumor, cell therapy, neural stem cell, carboxylesterase, CPT-11 (irinotecan), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

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CONTENTS

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Introduction

Metastatic brain tumors are the most common type of intracranial neoplasm in adults, arising in 10%–40% of all cancer patients (1). Brain metastasis is a principal cause of cancer-related morbidity and mortality because it may rapidly devastate the function of the central nervous system (2). Lung cancer has the highest incidence for brain metastasis, with 40% of all patients with lung cancer developing brain lesions during the course of the disease (3).

While a single metastatic lesion can be managed by local treatment such as surgery or radiosurgery, multiple lesions may need more wide-acting or systemic treatment, and the use of chemotherapy or whole-brain radiation is a clinically plausible solution (4). However, radiation therapy often results in unsatisfactory tumor control and may impair cognitive function. Chemotherapy is often insufficient because the blood-brain barrier hinders effective delivery of the therapeutic agents to the brain lesions and because it can cause unacceptable systemic toxicity(5).

To overcome current limitations in the treatment of metastatic brain tumors, novel modalities have been suggested, one of which is the use of neural stem cells (NSCs) as a vector to delivery therapeutic agents to brain lesions. NSCs have been reported to have inherent tumor-tropic properties as well as show self-renewal, differentiation to multiple cell types, and migratory potential, and recent studies have suggested that therapeutic strategies using the tumor tropism of NSCs are plausible in cancer

treatment (6). The use of NSCs as a delivery vehicle to treat disseminated intracranial lesions is highly attractive because therapeutic genes can be incorporated into NSCs, enabling them to specifically target invasive tumor cells (7). Numerous studies have reported that such a strategy could be effective in animal models of malignant glioma (7-9), and also be applicable for metastatic brain tumors from breast, prostate, and lung cancer (10-12).

NSCs carrying therapeutic suicide genes are useful in the treatment of primary or metastatic brain neoplasm (7,13,14). Carboxylesterase, which converts the prodrug CPT-11 (irinotecan) to SN-38 (7-ethyl-10-hydroxy-campotecin), a potent topoisomerase inhibitor I, is an effective therapeutic agent that can be harbored in NSCs and used to attack tumor cells in primary or metastatic brain tumors (9,15,16).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, APO2 ligand, or APO2L), a member of the TNF protein superfamily, is a promising chemotherapeutic agent because it damages and kills neoplastic cells but spares normal tissue (17,18). TRAIL protein is expressed on the cell surface or is present as a soluble form (19,20), and activates a p53-independent extrinsic pathway by binding to the transmembrane death receptors DR4 (TRAIL receptor 1) and DR5 (TRAIL receptor 2). DcR1 (TRAIL receptor 3) and DcR2 (TRAIL receptor 3) also bind to TRAIL but they lack the cytoplasmic death domain that activates apoptotic downstream signals and thus act as decoy receptors (21).

TRAIL protein on the cell surface is cleaved by a specific metalloprotease into soluble TRAIL (sTRAIL) to form a homotrimeric complex that has potent apoptosis-inducing activity (17,22). Systemic administration of recombinant sTRAIL proteins inhibits the growth of numerous tumor cells (18).

Since lung cancer is resistant to a number of chemotherapeutic agents, TRAIL receptor-targeting therapy has been attempted. However, a single use of TRAIL as an anti-cancer drug often produces insufficient clinical efficacy (23), and preclinical studies have shown that approximately half of all lung cancer cell lines are intrinsically resistant to the apoptotic activity of TRAIL receptor-targeting therapy (24), including A549 cells. To overcome the TRAIL resistance of tumor cells and enhance therapeutic efficacy, strategies to sensitize tumors to TRAIL-induced apoptosis have been suggested (25). Combined use of different chemotherapeutic drugs or irradiation has been explored for this purpose and are reported to considerably increase anti-tumor activity in preclinical studies (26,27). CPT-11 has a synergistic effect when used with TRAIL receptor-targeting therapy (28,29).

In the present study, HB1.F3 human NSCs were traduced with the genes of rabbit carboxylesterase (rCE) and the soluble form of TRAIL (sTRAIL) and transplanted systemically into animals bearing metastatic lung cancer in the brain; administration of the prodrug CPT-11 followed. The aim was to prove that metastatic tumors can be effectively targeted with this approach.

Materials and Methods

Cell culture

HB1.F3 (F3), a stably immortalized human NSC cell line, was derived from human fetal telencephalon at 15 weeks of gestation by introducing a retroviral vector encoding v-myc (30-33). F3 and F3 cells encoding rCE (F3.CE), sTRAIL (F3.sTRAIL), and both (F3.CE.sTRAIL) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY), 2 mmol L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (DMEM/10% FBS). A549 and H460 human non-small cell lung adenocarcinoma cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea) and American Type Culture Collection (ATCC, USA), respectively, and maintained in DMEM/10% FBS.

Genetic engineering of therapeutic cells

The clonal F3.CE, F3.sTRAIL, and F3.CE.sTRAIL human NSC lines were derived from the parental F3 NCS line.

For transduction and establishment of stable cell lines encoding rCE, an expression plasmid with rCE was created using retroviral pIRESneo (Clontech, Palo Alto, CA, USA) (30,34-36), and transduced with MV12 envelope-coding plasmid cDNA into pA317 cells. Parental HB1.F3 cells

were infected with retrovirus collected from the supernatant of packaging cell cultures and clonal cells were selected with neomycin for 7 days.

For transduction of sTRAIL genes, lentivirus containing the secretable form of the TRAIL gene (sTRAIL) was engineered (37). The recombinant replication-deficient lentiviral vector encoding the gene for enhanced green fluorescent protein (GFP) was constructed using pLHCX.eGFP (Clontech, Palo Alto, CA, USA) (30), and transduction was performed as described above. Transduced cells were selected by fluorescence-activated cell sorting (FACS) using GFP expression (38).

Reverse transcription-polymerase chain reaction

Successful transduction of rCE and sTRAIL genes into the cells was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) using the primer pairs for rCE and sTRAIL genes (table 1). For RT-PCR, cells were collected by centrifugation and total RNA was isolated using Trizol according to the manufacturer's protocol (Promega). One microgram of total RNA was reverse-transcribed into first-strand cDNA using oligo-dT primer. Reverse transcription was performed with AMV reverse transcriptase (Takara) for 1 h at 42°C, inactivated for 10 min at 95°C, and cooled at 4°C. The cDNA was diluted to a final volume of 25 µl and a 2-µl aliquot was used in a PCR reaction containing 1 × DNA polymerase buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 10 pmol primers, and 2.5 units of Taq polymerase (Takara). The cDNA was amplified using 30 PCR cycles and RT-PCR products were separated electrophoretically on a

1.2% agarose gel containing ethidium bromide and visualized under UV light. Expression of topoisomerase and TRAIL ligands in A549 and H460 lung cancer cells was also confirmed using primer pairs for topoisomerase genes (TOP1, TOP2a, TOP2b, TOP3a, TOP3b, TOPBP1) and sTRAIL ligand genes (DR4, DR5, DcR1, DcR2).

Gene	Sense	Antisense
DR4	GCGGGGAGGATTGAACCAC	CGACGACAAACTTGAAGGTCTT
DR5	ATGGAACAACGGGGACAGAAC	CTGCTGGGGAGCTAGGTCT
DcR1	ACCAACGCTTCCAACAATGAA	CTAGGGCACCTGCTACACTTC
DcR2	GTTGGCTTTTCATGTCGGAAGA	CCCAGGAACTCGTGAAGGAC
Top1	CCAGACGGAAGCTCGGAAAC	GTCCAGGAGGCTCTATCTTGAA
Top2a	ACCATTGCAGCCTGTAAATGA	GGGCGGAGCAAAATATGTTCC
Top2b	GGTTCGTGTAGAGGGGTCAAG	GCCGTCCACCTTTTGTAGTTG
Top3a	CCCGAAGACCGTGCCTTTT	CTCATGCGACCGTTTGACAG
Top3b	TGGCGAGAAGACCGTGTTC	AATCACCGTATTTCCCCTGGA
Topbp1	TGAGTGTGCCAAGAGATGGAA	TGCTTCTGGTCTAGGTTCTGT
Top1mt	ACGAAGACGGGGTGAAGTG	CCGGAAAACCTCCTTTGTTGTG
rCE	ATGATGGCCTGGCTCTTTCT	TCTCGGAAAATTGCTCGATG
sTRAIL	GGAATCATCAAGGAGTGGGC	TGGACCATTTGTTTGTCGTT
GADPH	CATGACCACAGTCCATGCCATCACT	TGAGGTCCACCACCCTGTTGCTGTA

Table 1. Primer pairs for RT-PCR to confirm encoded gene expression in therapeutic cells (rCE, rabbit carboxylesterase; sTRAIL, secretable form of tumor necrosis factor-related apoptosis-inducing ligand), expression of the gene for sTRAIL ligand (DR4, DR5, DcR1, DcR2), and topoisomerase (TOP1, TOP2a, TOP2b, TOP3a, TOP3b, TOPbp1, TOP1mt) in tumor cells.

Enzyme-linked immunosorbent assay

Secretion of sTRAIL was measured by an enzyme-linked immunosorbent assay (ELISA), in vitro and in vivo, using a TRAIL ELISA kit (Abcam, Cambridge, UK). For measurement of sTRAIL in cell media, 1×10^6 cells of F3, F3sTRAIL, and F3.CE.sTRAIL were plated in 6-well plates and cultured for 72 h in 2 ml DMEM/10% FBS. Supernatant media was collected for ELISA. To measure sTRAIL concentration in mice brain, the animal was euthanized 3 days after injection of F3.sTRAIL and F3.CE.sTRAIL cells and the brain tissue within 1–2 mm of the tumor-brain border was acquired and transferred to 200 μl lysis buffer. Following homogenization with TissueLyser (Qiagen, Hilden, Germany), the supernatant layer collected after centrifugation at 10,000 rpm was transferred to a new tube for ELISA.

Cell viability assay

To confirm the activity of rCE in therapeutic cells, the cytotoxic effect of CPT-11 on F3, F3.CE, F3.sTRAIL, and F3.CE.sTRAIL cells was analyzed using a cell viability assay. Each cell type (1×10^4 per well) was plated in 96-well plates. Twenty-four hours after seeding, 0.5–10 μM of CPT-11 (Hanmi Pharmaceutical, Seoul, Korea) was applied for 48 h. Cell status was then analyzed using a microscope and viability and apoptosis were determined with a colorimetric assay (Cell Counting Kit-8; Dojindo

Molecular, Gaithersburg, MD) and Muse® Cell Analyzer (Millipore, Billerica, MA).

In vitro bystander effect experiments

A549 and H460 human non-small cell lung adenocarcinoma cells were plated in 96-well plates with F3, F3.CE, F3.sTRAIL, or F3.CE.sTRAIL cells (A549 or H460 cells:F3 or F3.CE or F3.sTRAIL or F3.CE.sTRAIL = 75:25, 50:50, 25:75, or 0:100). Cells were maintained in DMEM/10% FBS. After 24 h of cell growth, 10 μM CPT-11 was added to the co-cultures and cell viability was determined 48 h later as described above

FACS analysis of TRAIL receptors

Surface expression of the TRAIL receptors DR4, DR5, DcR1, and DcR2 was determined by FACS. A549 and H460 lung cancer cells and parental F3 NSCs were plated and incubated in the presence of 0, 0.1, or 1 μM CPT-11 for 24 h. After dissociation, cells were labeled with DR4, DR5, DcR1, and DcR2 antibodies conjugated with phycoerythrin (1:3.5 dilution) for 30 min according to the manufacturer's protocol. After washing with phosphate-buffered saline (PBS), the labeled cells were detected using a FACSAria II cell sorter (BD Biosciences, CA). Anti-DR4 (TRAIL R1), anti-DR5 (TRAIL R2), anti-DcR1 (TRAIL R3), and anti-DcR2 (TRAIL R4) antibodies were purchased from R&D Systems (Minneapolis, MN).

Animal model of lung cancer brain metastasis

All animal experiments were reviewed and approved by the Animal Care and Use Committee of Chung-Ang University (Certification CA11-0086). For direct tumor cell implantation into the brain, anesthetized SCID mice (7 weeks old) were secured in a rodent stereotaxic frame and a small drill hole was made at 2 mm left and 1 mm anterior to bregma; 1×10^6 A549 human lung cancer cells in 4 μl PBS were then injected into the brain via a Hamilton syringe at a depth of 2 mm [anterior/posterior (AP) +1.0 mm, medial/lateral (ML) +1.7 mm, dorsal/ventral (DV) –3.2 mm] over a period of 10 min.

In vivo therapeutic efficacy

The therapeutic efficacy of each cell type was evaluated in six study groups (control, F3.CE, CPT-11, F3.sTRAIL, F3.CE/CPT-11, and F3.CE.sTRAIL/CPT-11), each containing nine mice. Thirteen and twenty days after A549 cell injection, animals were administered an intracardiac arterial injection of 100 μl of PBS (control or CPT-11 group) or 1×10^6 therapeutic cells (F3.CE, F3.sTRAIL, F3.CE/CPT-11, and F3.CE.sTRAIL/CPT-11 groups). Forty-eight hours after the cell injection, the control, F3.CE, and F3.sTRAIL groups received intraperitoneal injection of normal saline (100 μl), whereas the CPT-11, F3.CE/CPT-11,

and F3.CE.sTRAIL/CPT-11 groups received CPT-11 (3.75 mg/kg in 100 μl normal saline) everyday for 5 days after each cell injection, for a total of 10 days. Two days after the last injection, brains were removed and cut into 4–6-mm-thick coronal slices. For tumor volume measurement (largest width^2 × largest length × 0.5), the brain slices were fixed in 10% formalin in PBS, embedded in paraffin, sectioned into 4-μm sections using a microtome, and stained with hematoxylin and eosin.

For survival analysis, seven mice per group were treated as described and observed for evidence of a symptomatic intracranial mass such as incoordination, lethargy, or weight loss >25% of maximal body weight. Symptoms of an intracranial mass evident during observation were regarded as mortality and the animal was euthanized.

Statistics

Statistical comparisons were performed using the Student's t-test. Survival analysis was performed using the Kaplan–Meier and log rank tests. P-values <0.05 were considered statistically significant.

Results

Human NSC cells expressing rabbit CE and sTRAIL

Expression of rCE and sTRAIL genes in the therapeutic cells was demonstrated by RT-PCR (Figures 1a and b). As expected, rCE and sTRAIL transcripts were detected in F3.CE and F3.sTRAIL cells, respectively, but not in parental F3 cells. rCE and sTRAIL transcripts were also found in F3.CE.sTRAIL cells. sTRAIL secretion was measured by ELISA in the cell media collected following 72 h cell culture of F3, F3.sTRAIL, and F3.CE.sTRAIL cells (Figure 2). The amount of sTRAIL from F3.sTRAIL and F3.CE.sTRAIL cells was markedly higher (>1300 ng/ml) than that from parental F3 cells (<50 ng/ml). To confirm the function of rCE, F3.CE cells were exposed to the prodrug CPT-11 at concentrations of 0.01–1 μM for 48 h. F3.CE cell survival was considerably reduced by 48 h exposure to CPT-11 at concentrations of <0.1 μM (Figure 3a). When F3 control cells and F3.CE cells were exposed to 1 μM CPT-11 for 48 h, <10% of F3.CE cells survived, whereas >60% of control F3 cells survived, indicating that F3.CE cells efficiently processed CPT-11 into cytotoxic SN-38 and underwent suicidal cell death (Figures 3b and c). This result was also confirmed in F3.CE.sTRAIL cells.

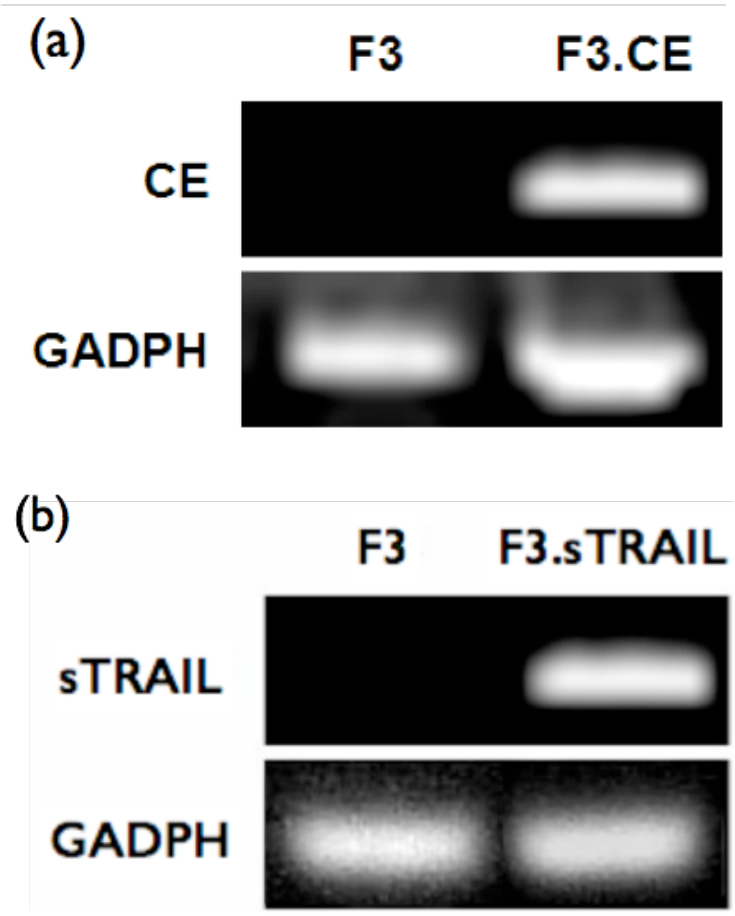

Figure 1. RT-PCR analysis of rCE and sTRAIL genes. (a) RT-PCR analysis of F3 and F3.CE cells for expression of the rCE gene. The HB1.F3 (F3) human NSC line was transduced with a retroviral vector encoding the rCE gene. (b) RT-PCR analysis of F3 and F3.sTRAIL cells for expression of the sTRAIL gene. The HB1.F3 (F3) human NSC line was transduced with a lentiviral vector encoding sTRAIL.

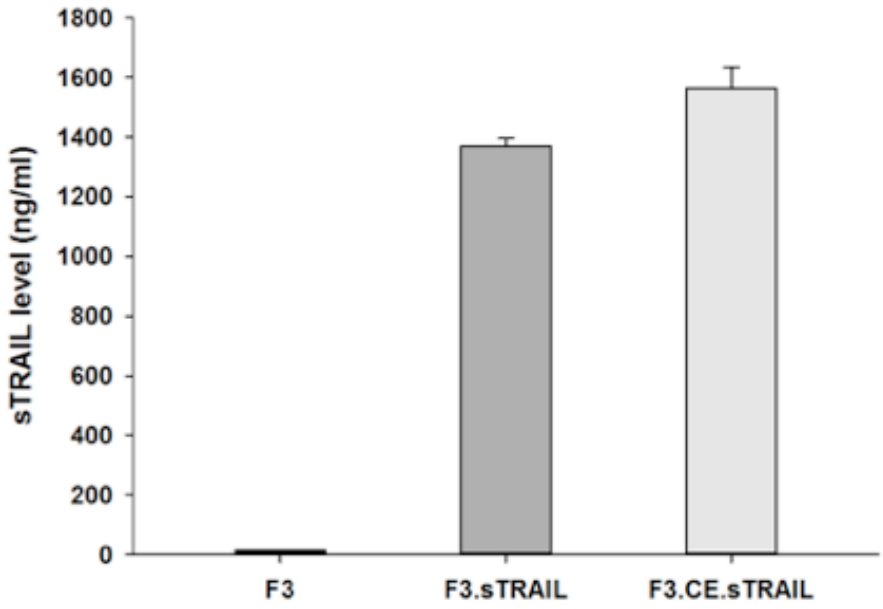

Figure 2. Secretion of sTRAIL from F3, F3.sTRAIL, and F3.CE.sTRAIL cells. The amount of sTRAIL secreted from F3.sTRAIL and F3.CE.sTRAIL cells was >1300 pg/ml when measured by ELISA, whereas the amount of sTRAIL from parental F3 cells was <50 ng/ml

Differential cytotoxic effects of CPT-11 on NSCs expressing CE and sTRAIL.

To confirm the activity of rCE and sTRAIL in F3.CE, F3.sTRAIL, and F3.CE.sTRAIL cells, the cytotoxic effect of CPT-11 on F3 or F3-based therapeutic cells was analyzed using a cell viability assay. F3 cells (the parental NSCs) showed approximately 30% cell death after 48 h exposure to 1 μM CPT-11, whereas more cells (>70%) were killed when F3.sTRAIL cells were treated with CPT-11 (Figure 3b), suggesting that the cytotoxicity of CPT-11 can be fortified by the presence of sTRAIL in the media. When F3.CE and F3.CE.sTRAIL cells were exposed to the same conditions, markedly increased cell death was noted (>90%), indicating that rCE enzyme in these cells efficiently converted the prodrug CPT-11 to active topoisomerase inhibitor SN-38, resulting in a strong cytotoxic effect (Figure 3c).

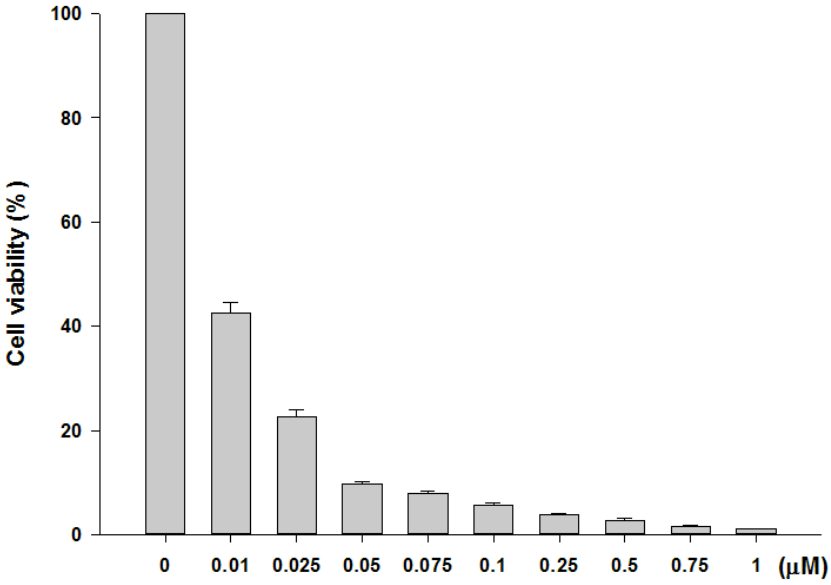

Figure 3a. Cytotoxic effect of CPT-11 on F3.CE cells. (a) F3.CE cells were incubated with varying concentrations of the prodrug CPT-11 for 48 h. CPT-11 at a concentration of 0.1 µM for 48 h killed >80% of F3.CE cells..

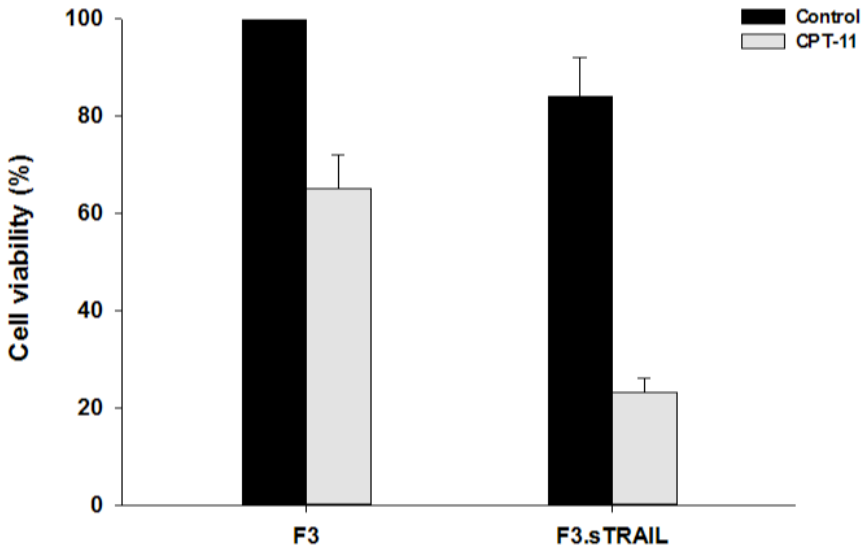

Figure 3b. Cytotoxic effect of CPT-11 on F3 and F3.sTRAIL cells. HB1.F3 and F3.sTRAIL cells were incubated with or without 1 μM CPT-11 for 48 h. Approximately 30% of F3 cells were killed by CPT-11, signifying a moderate cytotoxic effect of CPT-11. When F3.sTRAIL cells were exposed to the same amount of CPT-11, 70% of cells were killed, suggesting a possible additive cytotoxic effect.

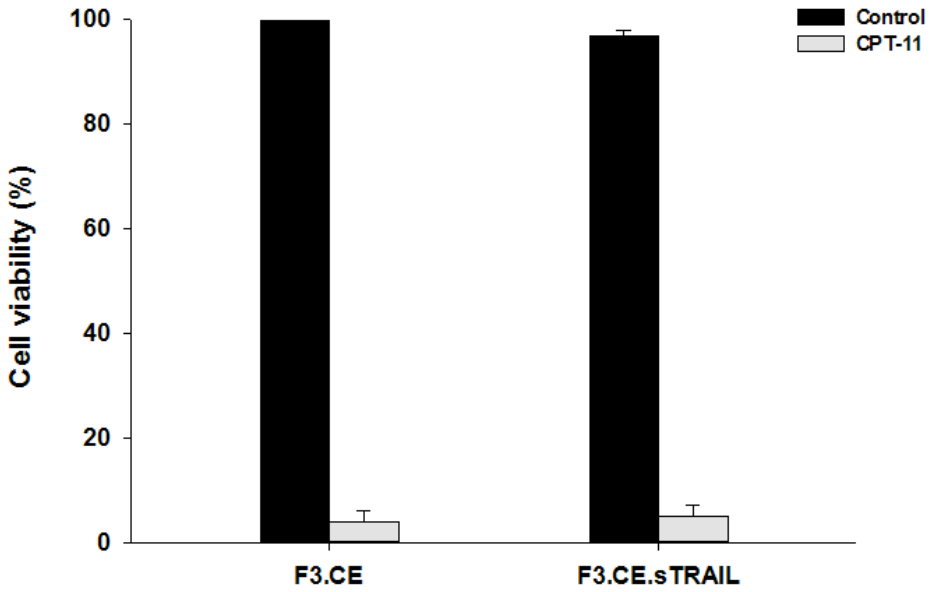

Figure 3c. Cytotoxic effect of CPT-11 on F3.CE and F3.CE.sTRAIL cells. F3.CE and F3.CE.sTRAIL cells were incubated with 1 μM CPT-11 for 48 h. More than 90% of both F3.CE and F3.CE.sTRAIL cells were killed, indicating a strong cytotoxic effect of SN-38 due to the conversion of the prodrug CPT-11 to SN-38 by the rCE enzyme expressed in the therapeutic cells.

In vitro bystander effects on lung cancer cells

The bystander effects of the therapeutic cells on A549 and H460 cells were determined using a co-culture system. Application of 1 μM CPT-11 to A549 or H460 cells had little effect on survival until 48 h after treatment in the absence of therapeutic cells or when cultured with parental F3 cells (Figure 4a). In contrast, the survival of A549 cells and H460 cells co-cultured with F3.CE cells (tumor cells:therapeutic cells = 100:0, 75:25, 50:50, or 25:75) was significantly reduced by 48 h exposure to 1 μM CPT-11. Cell survival after co-culture of A549 with F3.sTRAIL or F3.CE sTRAIL cells was also measured (Figure 4b). Interestingly, co-culture with F3.sTRAIL cells also resulted in increased cell death in the presence of CPT-11, and the ratio of apoptotic to live cells was comparable to the ratio seen after co-culture with F3.CE or F3.CE.sTRAIL cells. Thus, this bystander effect was compared among co-cultures of A549 cells with F3, F3.CE, F3.sTRAIL, or F3.CE.sTRAIL cells (tumor cells:each therapeutic cell type = 50:50) with addition of 0, 0.1, or 1 μM CPT-11. Cell survival was reduced in co-culture with F3.CE or F3.CE.sTRAIL cells after CPT-11 treatment and in co-culture with F3.sTRAIL cells (Figure 4c), as described above. The same results were obtained with H460 cells (Figure 4d). Without CPT-11, co-culture with therapeutic cells had no effect on the survival of either of the tumor cell types, except for co-culture with F3.sTRAIL or F3.CE.sTRAIL, which led

to mild cytotoxic effects (Figure 4c) that could be attributed to the influence of secreted sTRAIL from these cells.

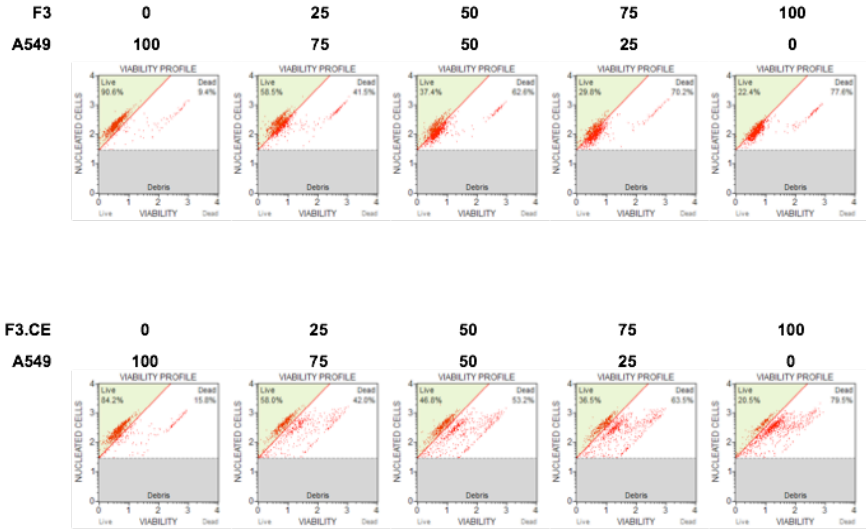

Figure 4a. A549 lung cancer cells were co-cultured with F3 or F3.CE cells in the presence of 1 μM CPT-11 for 48 h, and apoptotic cells and live cells were counted using Muse® Cell Analyzer (Millipore). When co-cultured with parental F3 NSCs, the apoptotic cell ratio was unaltered in any combination of cells. However, in A549 cells cultured with F3.CE cells in the presence of 1 μM CPT-11, the number of apoptotic cells dramatically increased after 48 h. In this condition, more cells were killed as the ratio of F3.CE cells to tumor cells increased.

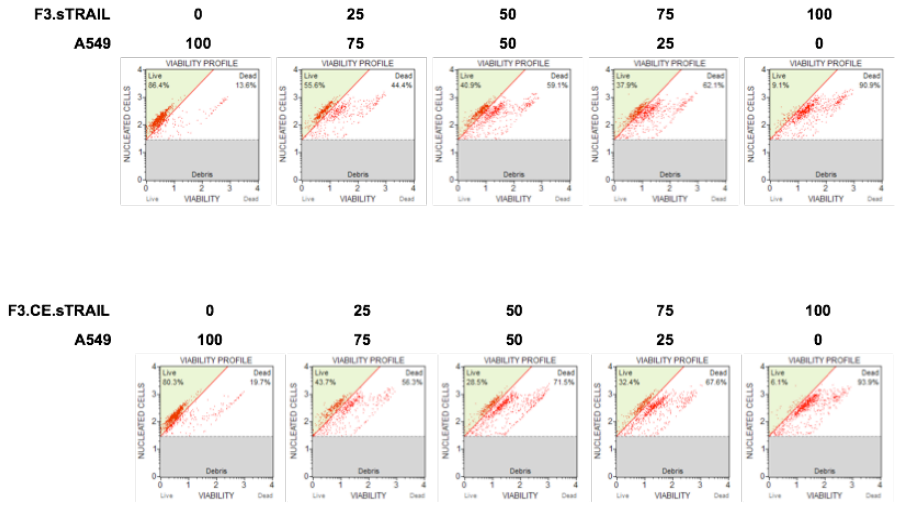

Figure 4b. A549 lung cancer cells were co-cultured with F3.sTRAIL or F3.CE.sTRAIL cells in the presence of 1 μM CPT-11 for 48 h, and apoptotic cells and live cells were counted. The apoptotic fraction of A549 cells increased when the cells were cultured with F3.sTRAIL or F3.CE.sTRIAL cells in the presence of 1 μM CPT-11 for 48 h.

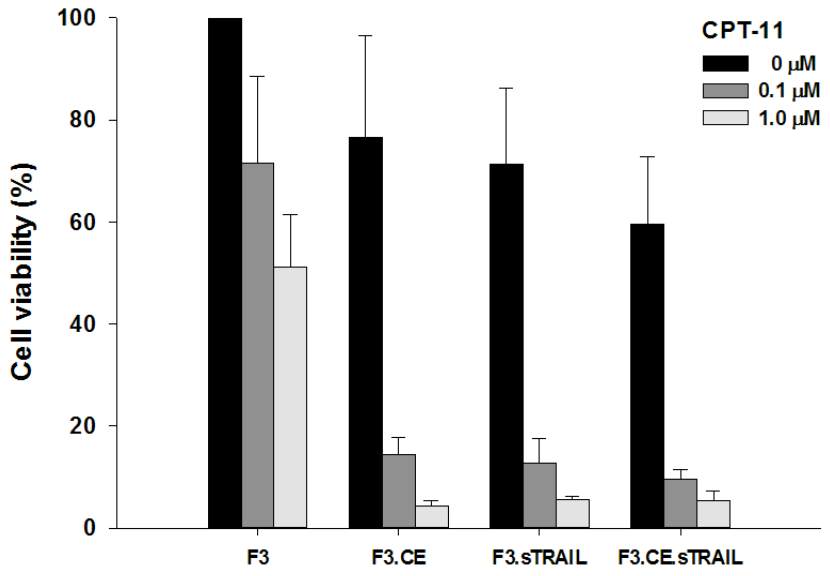

Figure 4c. Bystander killing effects of therapeutic cells were compared in a co-culture system with A549 cells. A549 cells with F3, F3.CE, F3.sTRAIL, or F3.CE.sTRAIL cells were seeded in 96-well plates (1×10^4 total cells per well, A549 cells:therapeutic cells = 50:50). After 24 h, cells were treated with 0.1 or 1 μM CPT-11 for 48 h and cell survival was determined (each group, n=3) ($P < 0.05$). Therapeutic cells expressing rCE, sTRAIL, or rCE plus sTRAIL exerted a cytotoxic effect on A549 cells in the presence of CPT-11, and more cells were damaged when a higher concentration of CPT-11 was added.

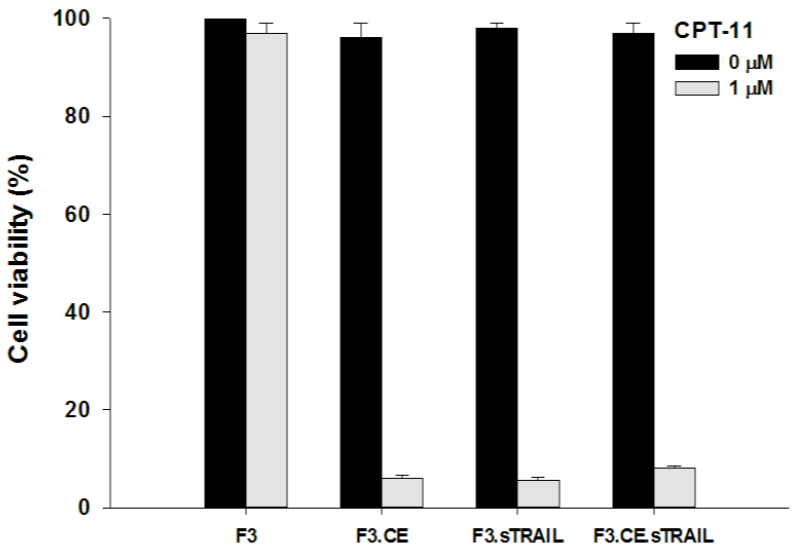

Figure 4d. Bystander killing effects of therapeutic cells were compared in a co-culture system with H460 cells. H460 cells were plated with F3, F3.CE, F3.sTRAIL, or F3.CE.sTRAIL cells in 96-well plates (1×10^4 total cells per well, H460 cells:therapeutic cells = 50:50). After 24 h, cells were treated with 1 μM CPT-11 for 48 h and cell survival was determined (each group, n=3; $P < 0.05$). Therapeutic cells expressing rCE, sTRAIL, and rCE plus sTRAIL exerted a cytotoxic effect on H460 cells when the cells were treated with CPT-11

Changes in the expression of TRAIL receptors

FACS analysis of the TRAIL receptors DR4, DR5, DcR1, and DcR2 in lung cancer tumor cells and F3 NSCs revealed different rates of expression and response patterns to CPT-11 treatment. When DR4 expression was measured, >70% of H460 cells were found to have DR receptors on their surface, whereas the DR4 expression rates of A549 and HB1.F3 cells were less than 30% (Figure 5a), possibly explaining the relative higher sensitivity of H460 cells to TRAIL therapy that has been reported in the literature (24). After 0.1 or 1 μM CPT-11 was added to the cell culture, DR4 expression changed, with expression in A549 and F3 cells increasing by approximately 70%. The increased expression was noted with a low concentration (0.1 μM) of CPT-11 in A549 cells without changes in cell morphology related to cellular damage or survival (data not shown). In contrast, the expression rate of DR4 in H460 cells was not changed or possibly slightly decreased when the cells were exposed to CPT-11. When the expression of DR5 was analyzed, all cells examined showed a high rate of expression (>80%), and this expression was unaltered by low- and high-concentration CPT-11 treatment (Figure 5b).

The expression of DcR1 in tumor cells was low (<5%) in both A549 and H460 cells, and the rate was unaffected by exposure to CPT-11 (Figure 5c). In F3 cells, DcR1 receptor expression was slightly higher than that in tumor cells (13%) and increased after 1 μM CPT-11 addition by approximately 90%. DcR2 was strongly expressed in A549 cells (40%)

and in F3 NSCs (71%) but found to be low in H460 cells. The presence of CPT-11 in the cell culture did not change the expression of DcR1 in the cells (Figure 5d).

Thus, A549 cells showed less DR4 and more DcR2 receptors than H460 cells, and treatment with CPT-11 increased DR4 expression in A549 cells, which may be related to previous reports on the TRAIL resistance of A549 cells (24) and the synergistic activity of CPT-11 and TRAIL in apoptosis (39,40).

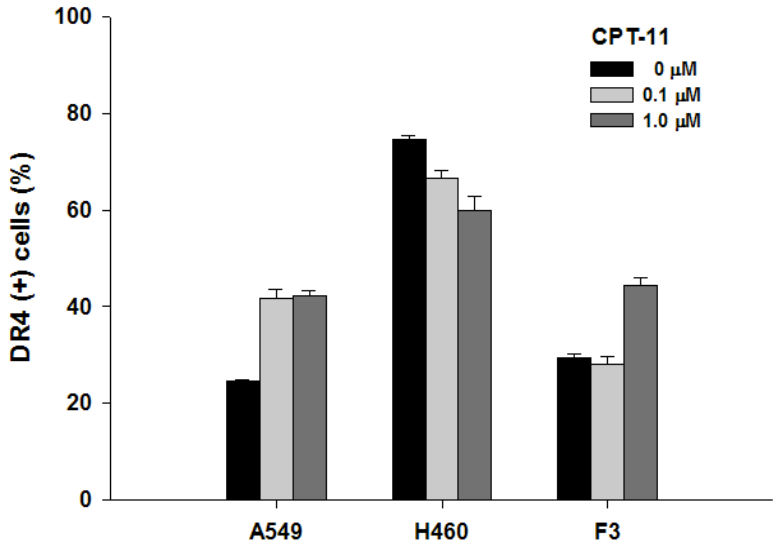

Figure 5a. The rate of DR4 receptor expression was high in H460 cells (>70%) but relatively low in A549 and F3 NSCs (<30%) when measured by FACS analysis. After 0.1 or 1 μM CPT-11 treatment for 24 h, the rate of DR4 expression of A549 and F3 NSCs was increased, whereas that of H460 cells was largely unaltered or slightly decreased.

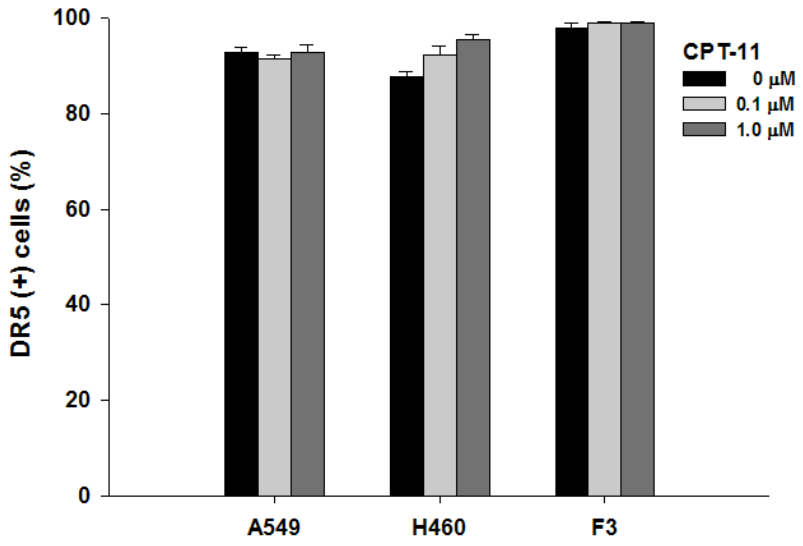

Figure 5b. The rate of DR5 receptor expression was similarly high (>80%) in both lung cancer cells and F3 NSCs. Changes after CPT-11 treatment were not evident in any cells.

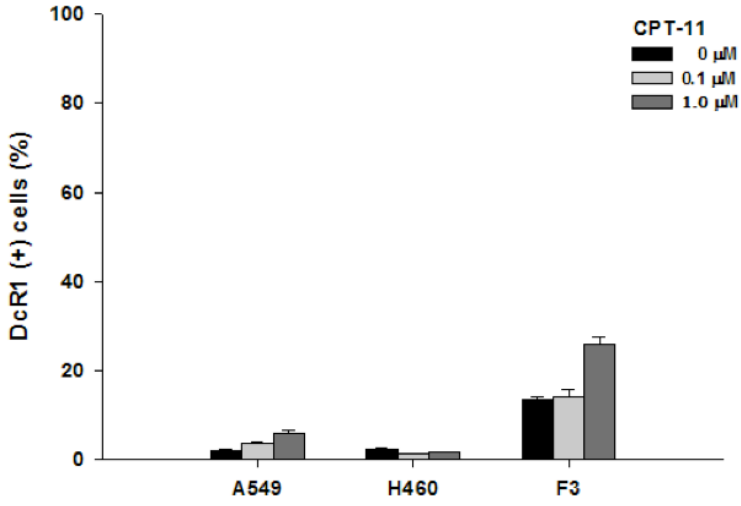

Figure 5c. The rate of DcR1 expression was relatively low in all cells examined, <5% in A549 and H460 tumor cells and approximately 13% in F3 NSCs. Exposure to CPT-11 did not affect the expression rate of DcR1 in the cells, except in F3 NSCs, in which DcR1 expression moderately increased with 1 μM CPT-11 treatment.

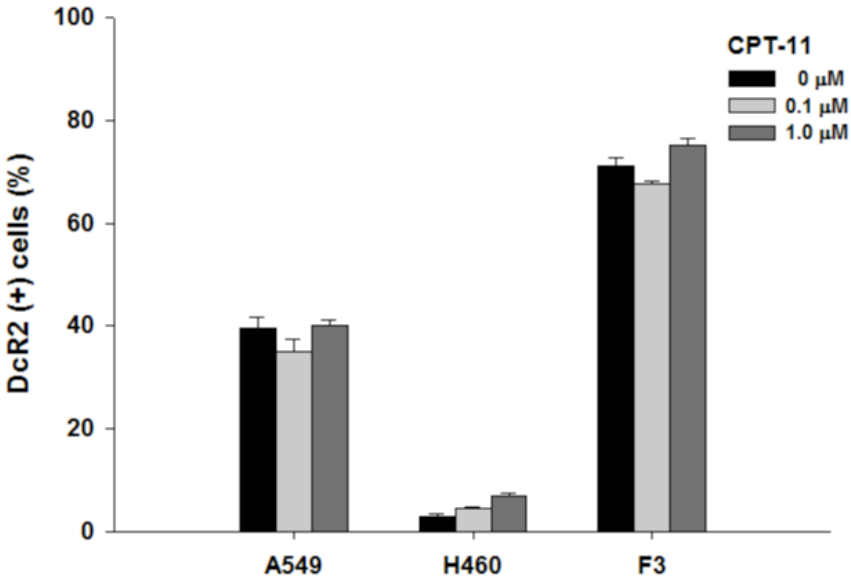

Figure 5d. The rate of DcR2 expression was 40% in A549 cells and 71% in F3 NSCs. In H460 cells, DcR1 expression was low (<5%). CPT-11 did not alter the expression of DcR1.

In vivo therapeutic efficacy

The timeline for the establishment of the lung cancer brain metastasis animal model and subsequent treatment using therapeutic cells and CPT-11 is shown in Figure 6.

Brain sections prepared from animals treated with F3.sTRAIL cells, F3.CE cells plus CPT-11, and F3.CE.sTRAIL cells plus CPT-11 showed a significant reduction in tumor volume (Figure 7). Large tumors were observed in brain sections of control animals and animals treated only with F3.CE cells without subsequent CPT-11.

The in vivo therapeutic efficacy of F3.CE cells against lung cancer brain metastasis was determined by tumor volume measurement and survival analysis (Figure 8). When tumor volumes were determined in brain tissue 2 days after the last CPT-11 injection, the brains of the mice of the F3.CE/CPT-11 group showed significantly smaller tumor volumes (median±s.d. = 8.43±1.37 mm^3) than the control (33.2±2.02 mm^3) and F3.CE (27.6±2.36 mm^3) groups. The mice of the CPT-11 alone group harbored similar sized tumors (28.7±3.13 mm^3) to the control and F3.CE group. The tumor volume of the F3.sTRAIL group was also significantly smaller (14.5±1.76 mm^3) than that of the control, F3.CE, and CPT-11 alone groups. The tumor volume of F3.CE.sTRAIL/CPT-11 group was significantly smaller (5.27±1.23 mm^3) than that of the control and was also significantly different from those of the F3.CE/CPT-11 and

F3.sTRAIL groups. There was an 84.1% reduction in tumor volume in the F3.CE.sTRAIL/CPT-11 group compared with the control group.

When the survival of treated mice was analyzed (Figure 9), the F3.CE/CPT-11 and F3.sTRAIL groups showed significantly prolonged survival periods (median±s.d. = 58.6±2.22 days and 55.0±2.08 days, respectively) compared with the control (40±1.29 days), F3.CE (42.5±1.44 days), and CPT-11 alone (38.5±1.19 days) groups. The survival of the F3.CE.sTRAIL/CPT-11 group was the longest (62.6±2.37 days) among all of the treatment groups, and the difference was significant when compared with the control, F3.CE, CPT-11 alone, and F3.sTRAIL groups, but not when compared with the F3.CE/CPT-11 group ($P = 0.151$)

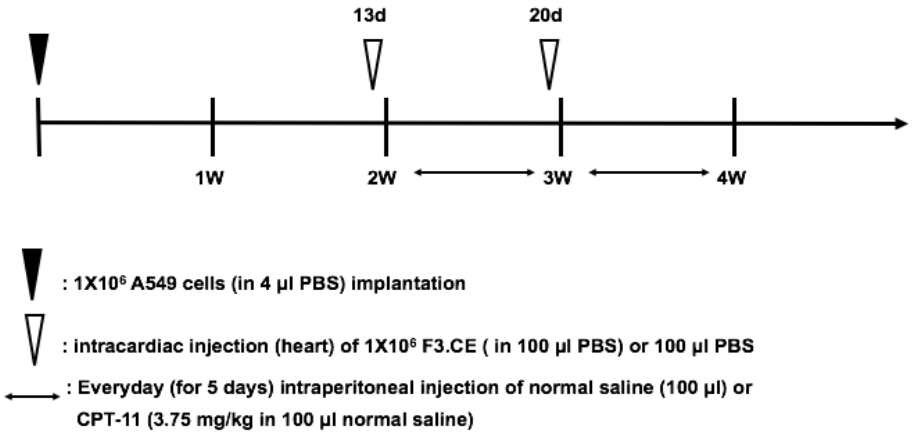

Figure 6. Timeline for the establishment of the lung cancer brain metastasis animal model and subsequent treatment with therapeutic cells and CPT-11. A549 human non-small cell lung adenocarcinoma cells (1×10^6 cells in 4 μl PBS) were implanted into the brains of 7-week-old SCID mice (n=9). Thirteen and twenty days after tumor cell implantation, therapeutic cells (1×10^6 cells in 100 μl PBS) were intracardially injected and CPT-11 (10 μM, 3.75 mg/kg) was intraperitoneally injected for a further 5 days (for a total of 10 days). The mice were divided into six groups as follows: in group 1 (control group), the mice were treated with 100 μl PBS without CPT-11 as a negative control; in group 2 (F3.CE group), the mice were treated with F3.CE cells in 100 μl PBS (intracardiac injection) without CPT-11; in group 3 (CPT-11 group), the mice were treated with CPT-11 alone; in group 4 (F3.sTRAIL group), the mice were treated with F3.sTRAIL; in group 5 (F3.CE/CPT-11 group), the mice were treated with F3.CE cells and CPT-11; and in group 6 (F3.CE.sTRAIL/CPT-11 group), the mice were treated with

F3.CE.sTRAIL cells and CPT-11. Two days following the last CPT-11 injection, all mice were euthanized and the brains were processed for histology.

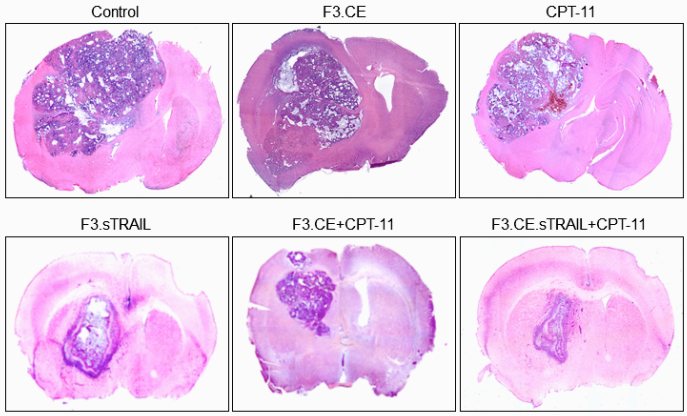

Figure 7. Representative images of brain slices from each group. Tumor-bearing mice treated with F3.CE cells plus CPT-11, F3.sTRAIL cells, and F3.CE.sTRAIL cells plus CPT-11 showed significantly smaller tumor volumes than other groups including the sham control group, tumor-bearing animals transplanted with F3.CE cells but without CPT-11 treatment, and animals injected with CPT-11 but without therapeutic cell transplantation

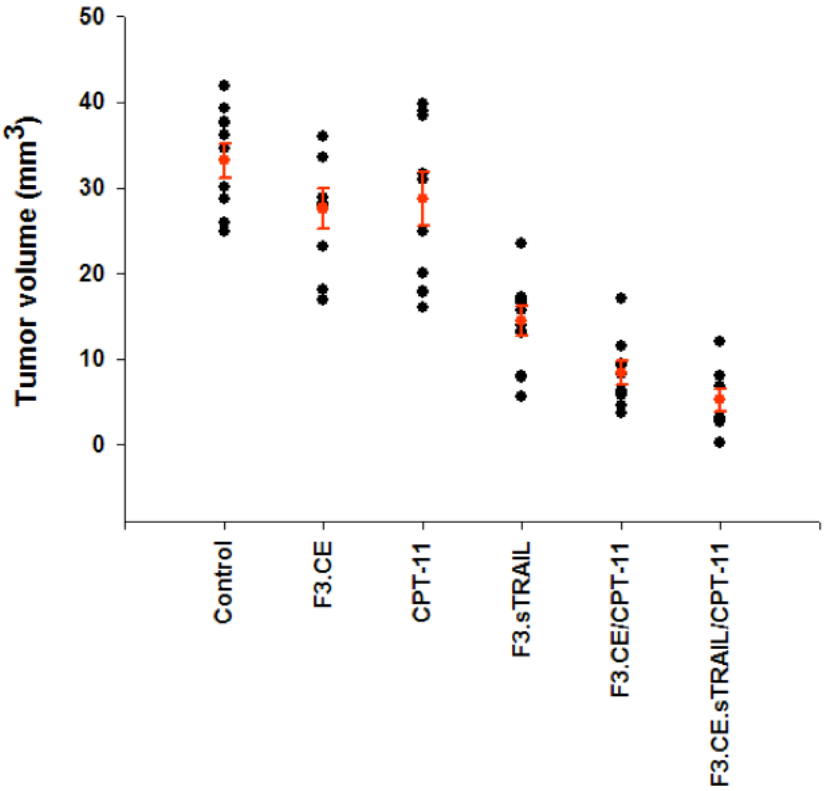

Figure 8. Treatment with therapeutic cells and CPT-11 has a significant therapeutic effect in vivo. Tumor-bearing mice treated with F3.sTRAIL cells, F3.CE cells plus CPT-11, and F3.CE.sTRAIL cells plus CPT-11 showed significantly smaller tumor volumes than other groups including the sham control group, tumor-bearing animals transplanted with F3.CE cell but without CPT-11 treatment, and animals injected with CPT-11 but without therapeutic cell transplantation ($P < 0.05$). The tumor volume of the F3.CE.sTRAIL/CPT-11 group was significantly smaller than that of the F3.sTRAIL group or F3.CE.sTRAIL group ($P < 0.05$).

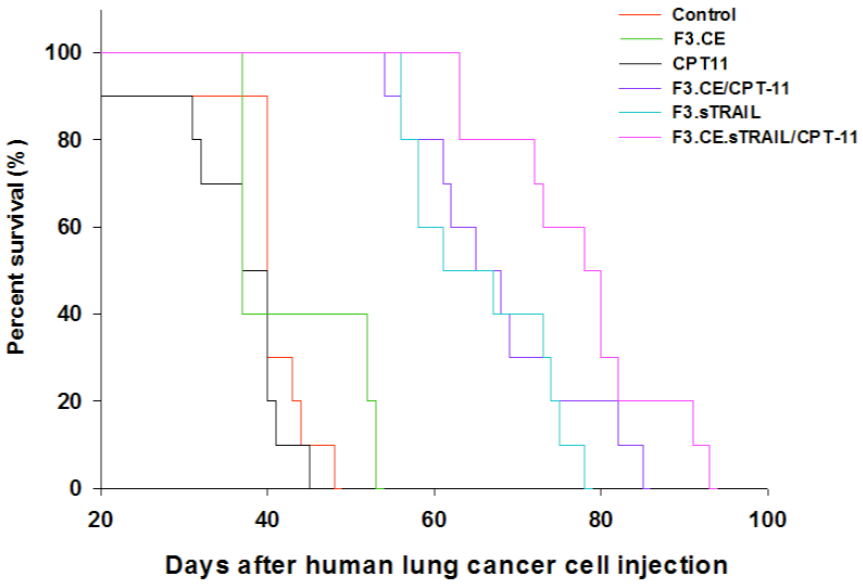

Figure 9. Survival analysis of mice treated with the therapeutic cells and CPT-11. The Kaplan-Meier graph shows significantly longer survival periods of the mice administered the F3.sTRAIL, F3.CE/CPT-11, and F3.CE.sTRAIL/CPT-11 cells than those of the control, F3.CE group, or CPT-11 group ($P < 0.05$). The survival of the F3.CE.sTRAIL/CPT-11 group was the longest, significantly longer than that of F3.sTRAIL, but the difference from that of the F3.CE/CPT-11 group was not statistically significant ($P = 0.151$).

Discussion

In our present study, we showed that NSCs expressing rCE or sTRAIL therapeutic genes can exert an anti-cancer action on metastatic brain tumors from lung cancer and that the anti-cancer effects are synergistic or additive when rCE/CPT-11 and sTRAIL therapy are combined. This type of stem cell-based study could be readily translatable to clinical studies because a similar method is currently being used in an investigational phase I clinical trial in adult patients with recurrent glioblastoma (clinical trial ID NCT01172964; http://clinicaltrials.gov/ct2/show/NCT01172964). NSCs encoding sTRAIL may exert cytotoxic activity in tumor cells by binding cell surface TRAIL receptors. TRAIL-targeted therapy has been suggested to be a useful anti-cancer treatment because TRAIL selectively affects tumor cells without interfering with normal tissue (41). Tumor cells abundantly express TRAIL receptors containing death domains, such as DR4 and DR5, whereas non-neoplastic cells have a decoy receptor that lacks the structures required for the transmission of death signals to the downstream cascade (23). However, a number of tumor cells show insufficient TRAIL sensitivity and may be resistant to TRAIL therapy, which may be partly attributed to differences in receptor expression among various cancer cells. A549 and H460 cells, which were used in the present study for both in vitro and in vivo experiments, have a different susceptibility to TRAIL apoptosis (24). We also confirmed that DR4 expression was strong in H460 cells, but not in A549 or NSCs. In TRAIL-

resistant cancer cells such as A549, combination chemotherapy could be a useful method, as exposure to a chemotherapeutic agent might render cancer cells more susceptible to TRAIL apoptosis (40). In the present study, when receptor expression was examined after CPT-11 treatment for 24 h, DR4 expression in A549 cells increased by 70% and decoy receptor DcR1 in NSCs increased by 90%, which might partially explain the increased anti-tumor effects of CPT-11 in the *in vitro* and *in vivo* experiments.

F3.sTRAIL cells showed increased cytotoxicity to A549 cells when combined with CPT-11 treatment both *in vitro* and *in vivo* in this study. Synergistic or additive effect of CPT-11 was evident when treated without the presence of rCE *in vitro*, because F3.sTRAIL cells demonstrated comparable cytotoxicity to F3.CE.sTRAIL cells in the presence of CPT-11 (Figure 4c). From this result, it could be suggested that the dose of CPT-11 for pre-conditioning of the cell to TRAIL susceptibility could be lower that the dose for cell death. The observation that treatment of low dose of CPT-11 (0.1 μM) in A549 cells resulted in DR4 and DcR1 expression (Figure 5a and 5c) without the change of cell morphology related to cellular damage may also support such difference between the doses for cell death and pre-conditioning for TRAIL susceptibility.

F3.sTRAIL cells alone showed significant tumor reduction and survival prolongation *in vivo*, whereas they had mild cytotoxicity *in vitro* when used alone without CPT-11. One of the reason for this discrepancy could be from the difference in the duration of exposure to sTRAIL, as we

employed 48-h time setting for in vitro experiment, but tumor-bearing animals has been exposed to TRAIL for a prolonged period, at least 10 days for histology analysis.

Combination of rCE/CPT-11 therapy with a TRAIL strategy may also provide a safety benefit as well as improved treatment efficiency (42). There have been concerns that long-term habitation of foreign cells inside the brain, where immune rejection is relatively restricted, might result in hazardous events, such as secondary malignancy or overproduction or unwanted production of biologically active materials. NSCs that can produce sTRAIL alone could not eliminate themselves since non-neoplastic cells are resistant to TRAIL apoptosis. However, cell death could be readily induced when NSCs additionally express rCE, or another type of suicide gene, by prescribing a prodrug that can be converted to a highly cytotoxic agent (43). This property may allow efficient and safer cell-based treatments.

The use of NSCs as drug delivery vehicles may offer a novel solution for overcoming the shortcomings of previous conventional treatment, by directing high concentrations of a chemotherapeutic agent selectively to the lesion, thus alleviating the limitations of current chemotherapy, which are a low efficacy and severe systemic toxicity. Recent studies have found that genetically engineered NSCs have advantages that could be useful for gene therapy treatment of brain tumors (7,13). Use of HB1.F3 immortalized human fetal NSCs in intracranial primary or metastatic brain tumors has been reported in multiple studies. F3 human NSCs engineered

to express Escherichia coli cytosine deaminase, which can convert the prodrug 5-fluorocytosine (5-FC) to active 5-fluorouracil (5-FU), reduced brain tumor growth in vitro and in vivo (11,12,36,44-48).

F3 NSCs encoding the rCE gene also demonstrated migratory potential to brain tumor sites and anti-tumor effects on subdural medulloblastoma (8) and metastatic tumors from breast cancer (9,10) after implantation. Carboxylesterase activates the prodrug CPT-11 (irinotecan) to the active metabolite SN-38, a 1000-fold more potent topoisomerase I inhibitor. In the current study, a gene for rCE was used because the activity of human carboxylase for converting the prodrug CPT-11 is lower and rCE is more efficient (36,49).

CPT-11 is a chemotherapeutic agent that specifically kills dividing cells. CPT-11 is approved for numerous cancers, including malignant gliomas, as it can penetrate the blood-brain barrier with relatively high efficiency. Promising results have been demonstrated in preclinical studies (8-10,13) and a clinical phase II study is also underway in patients with malignant brain tumors (50,51). However, the main obstacle that limits CPT-11 from more active clinical application in primary and metastatic brain tumors is systemic toxicity, which includes severe gastrointestinal disturbance, interference in immune function and leukopenia, and hepatotoxicity (51-53). Given that higher dosages of drugs are prescribed in patients with intracranial lesions, a balance between efficacy and adverse toxicity is crucial to achieving the optimal treatment result. Attempts have been made to overcome this problem by the use of additional chemotherapeutic

agents, such as bevacizumab, that can synergistically combine anti-tumor activity with interruption of tumor growth through a different biological mechanism (52). Another effective method is to localize drugs in the target region without raising systemic concentration, such as the use of biological vectors containing therapeutic material.

The present study demonstrates that brain transplantation of human NSCs encoded with the genes for the suicide enzyme rCE and sTRAIL combined with systemic administration of CPT-11 could be considered an effective treatment regimen for metastatic brain tumors from lung cancer. This strategy may be readily applicable in patients with brain metastases and could guide the development of future metastasis therapeutics.

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국문 초록

서론: 전이성 뇌종양은 임상적으로 성인에서 가장 흔한 중추신경계의 악성종양이며, 폐암환자의 주된 사망원인의 하나이다. 전이성 뇌종양의 치료로 수술적 제거, 방사선수술, 방사선치료, 항암화학치료를 시행하고 있으나, 만족할만한 치료효과를 충분히 얻지 못하고 있다. 본 연구에서는 신경줄기세포주의 암세포에 대한 표적이동성을 이용하여 항암화학요법의 효과를 증대시킬 수 있는 치료방법을 동물모델에서 검증하고자 하였다.

방법: 불멸화 인간태아유래 신경세포주인 HB1.F3 세포주에 토끼유래 카르복실에스터라제(rCE) 유전자와 secretable form of tumor necrosis factor-related apoptosis-induced ligand(sTRAIL) 유전자를 재조합하여, 카르복실에스터라제를 발현하는 세포주(F3.CE), sTRAIL 유전자를 발현하는 세포주(F3.sTRAIL), 그리고 두 유전자를 모두 발현하는 세포주(F3.CE.sTRAIL)를 확립하였다. 확립된 세포주를 CPT-11 이 함유된 배약액에서 폐암 세포주 A549와 H460과 공배양하여, 각각의 세포독정을 측정하였다. CPT-11 처리 후 sTRAIL의 리간드인 DR4, DR5, DcR1,

DcR2 발현의 변화를 ELISA 방법을 이용해 비교하였다. SCID 마우스의 대뇌에 A549 세포주를 이식하여 폐암 뇌전이 동물모델을 제작하고, F3.CE, F3.sTRAIL, 또는 F3.CE.sTRAIL 세포주를 체순환계에 주입한 후 CPT-11을 주입하였다. 각 치료군에서 치료효과의 차이를 조직학적 검사와 생존률 분석을 이용해 측정하였다.

결과: HB1.F3 신경줄기세포주에 rCE 유전자와 sTRAIL 유전자를 함입한 F3.CE, F3.sTRAIL, F3.CE.sTRAIL 세포주에서 각 유전자의 발현을 확인할 수 있었다. 각각의 치료유전자를 함입한 줄기세포주는 폐암 세포주와의 공배양에서 단독사용하는 경우에 비해 병합사용한 경우 더 큰 세포사멸효과를 보였다. TRAIL 리간드 중 DR4는 H460 폐암세포의 70%이상이 발현하고 있었으나 A549세포와 F3 신경줄기세포에서는 발현이 낮았다(<30%). DcR2 수용체는 H460 세포의 5%이하에서만 발현되었으나, A549 세포와 신경줄기세포에서는 각각 40%와 71%의 세포에서 발현되고 있었다. CPT-11 처리 후 A549 세포에서는 DR4의 발현이 70%, 신경줄기세포에서는 DcR1의 발현이 90% 증가하였다. 폐암 뇌전이 동물에 F3.CE, F3.CE.sTRAIL 치료세로를 단독 또는 병합하여 이식하고 CPT-11을 투여하였을 때, 대조군, 치료세포 없이 CPT-11만 투여한 군, 그리고 CPT-11 투여 없이 rCE 유전자를 가진 세포를 주입한

군에서는 큰 종양을 관찰할 수 있었으나, rCE 유전자를 가진 세포를 주입하고 CPT-11을 투여한 군과 sTRAIL 유전자를 가진 세포를 주입한 군에서는 종양의 크기가 유의하게 감소하였고, 생존기간 또한 증가하였다. rCE와 sTRAIL유전자를 모두 가진 세포를 주입한 후 CPT-11을 투여한 경우 단독으로 치료한 군이나 대조군에 비해 의미있게 가장 작은 종양 크기를 보였고, 생존기간도 증가하였다.

결론: 본 연구를 통해 폐암 뇌전이 동물모델에서 rCE 유전자와 sTRAIL 유전자를 동시발현하는 인간유래 신경줄기세포주의 항암치료효과와 두가지 치료유전자를 병행사용하였을 때의 상승 또는 부가효과를 확인하였다. 이러한 치료방법은 폐암 뇌전이 치료에서 기존의 항암화학치료의 단점을 보완하고 치료효율을 높일 수 있을 것으로 기대된다.

주요어: 전이성 뇌종양, 세포치료, 신경줄기세포,

카르복실에스터라제, CPT-11(irinotecan), sTRAIL,

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