Small Molecule Therapeutics

Molecular Cancer Therapeutics

Human Neural Stem Cells Overexpressing a Carboxylesterase Inhibit Bladder Tumor Growth

Sung S. Choi[1], Byung Hoon Chi[2], In Ho Chang[2], Kyung Do Kim[2], Sang-Rae Lee[3], Seung U. Kim[4], and Hong J. Lee[1]

Abstract

Bladder cancer is a significant clinical and economic problem. Despite intravesical chemotherapy and immunotherapy, up to 80% of patients with non-muscle–invasive bladder cancer develop recurrent tumors, of which 20% to 30% evolve into more aggressive, potentially lethal tumors. Recently, bladder cancer cells are considered to be mediators of resistance to current therapies and therefore represent strong candidates as biologic targets. No effective chemotherapy has yet been developed for advanced bladder cancer. It is desirable that a drug can be delivered directly and specifically to bladder cancer cells. Stem cells have selective migration ability toward cancer cells, and therapeutic genes can be easily transduced into stem cells. In suicide gene therapy for cancer, stem cells carry a gene encoding a carboxylesterase (CE) enzyme that transforms an inert CPT-11 prodrug into a toxic SN-38 product, a topoisomerase 1 inhibitor. In immunodeficient mice, systemically transplanted HB1.F3.CE stem cells migrated toward the tumor implanted by the TCCSUP bladder cancer cell line, and in combination with CPT-11, the volume of tumors were significantly reduced. These findings may contribute to the development of a new selective chemotherapeutic strategy against bladder cancer. *Mol Cancer Ther; 1–8. ©2016 AACR.*

Introduction

Urothelial carcinoma is the most common type of bladder cancer. Although more than 75% of all bladder cancer cases are non-muscle–invasive that can be treated by transurethral resection, 20% to 40% of patients who undergo complete transurethral resection for non-muscle–invasive bladder cancer present a more advanced disease or progression to muscle-invasive bladder cancer, within 5 years of follow-up (1–4). Systemic cisplatin-based combination chemotherapy regimens have become the gold standard in advanced bladder cancer. However, this standard chemotherapy is usually insufficient in the treatment of bladder cancer therapy because of occurrence of drug resistance as well as high systemic toxicity, including mucositis, neutropenia, infections, gastrointestinal complications, and toxic death rate (5).

Recently, the discovery of the inherent tumor-tropic properties of neural stem cells (NSC) has provided a novel approach to potentially overcome the primary challenge in developing chemotherapeutic regimens (6–8). Because NSCs act as delivery vehicles that can disseminate therapeutic gene products specifically to invasive tumor cells, the use of these therapeutic NSCs is highly attractive. Moreover, engineering NSCs to express a suicide gene/prodrug–activating enzyme system is a particularly safe way to administer gene therapy, because they can kill not only tumor cells by bystander effect but also NSCs themselves *in vivo* (8). We generated human NSCs overexpressing carboxylesterase (F3.CE) using F3 parental cell. The prodrug CPT-11 (irinotecan) is a chemotherapeutic agent that treats in metastatic colon cancer as first-line chemotherapy (9). CE hydrolyzes CPT-11 into cytotoxic SN-38, a topoisomerase I inhibitor (10). The conversion level of CPT-11 in human plasma is very low because of the lack of CE activity in human blood. As a result, rabbit liver CE (rCE), which is 100-fold more efficient than human CE, is preferentially used in combination with CPT-11 to improve therapeutic efficacy (11, 12).

In this study, we investigated the tumor-tropic properties and therapeutic potential of F3.CE-overexpressing rCE for advanced bladder cancer *in vitro* and *in vivo*.

Materials and Methods

Cell lines

To generate HB1.F3.CE (F3.CE) cells, immortalized HB1.F3 (F3) human NSCs were transfected with a retroviral vector containing the CE gene (Fig. 1A). F3.CE cells were prepared by transduction with a replication-deficient retrovirus harboring the pLPCX vector, which contains the rCE gene. Vectors were packaged by cotransduction of the pLPCX.rCE puromycin plasmid with the MV12 envelope–coding plasmid into 293T cells. The retroviral supernatant was used for multiple infections of F3 cells. Transduced F3.CE cells were selected with 3 mg/mL puromycin for 1 week. The TCCSUP line (HTB-5, ATCC) was isolated in 1974 from an anaplastic transitional cell carcinoma (TCC) in the neck of the urinary bladder of a 67-year-old female patient. The

[1]Biomedical Research Institute, Chung-Ang University Hospital, Seoul, Republic of Korea. [2]Department of Urology, Chung-Ang University College of Medicine, Seoul, Republic of Korea. [3]National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Chungbuk, Republic of Korea. [4]Division of Neurology, Department of Medicine, UBC Hospital, University of British Columbia, Vancouver, British Columbia, Canada.

Note: S.S. Choi and B.H. Chi contributed equally to this article.

Corresponding Authors: Hong J. Lee, Chung-Ang University Hospital, Heukseok-dong, Dongjak-gu, Seoul 156-756, Republic of Korea (South). Phone: 8210-6276-2174; Fax: 8220-70462-37719; E-mail: leehj71@gmail.com; and Seung U. Kim, Division of Neurology, Department of Medicine, UBC Hospital, University of British Columbia, Vancouver, BC V6T 2B5, Canada. E-mail: 1428rol@gmail.com

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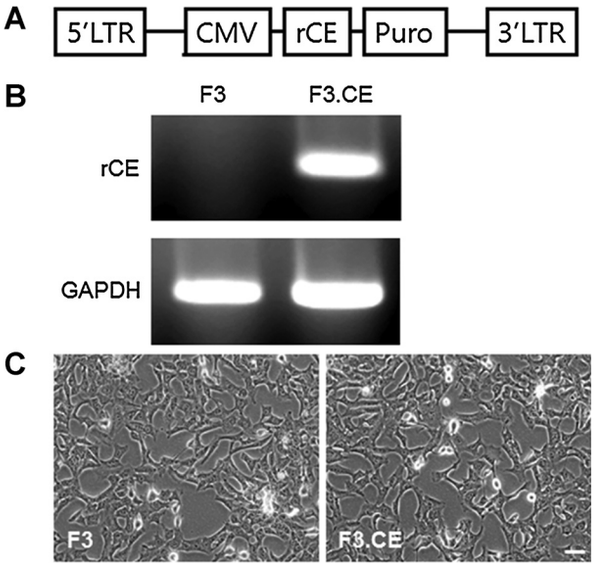

Figure 1.
Establishment of F3.CE cells. A, retroviral vector carrying the CE gene (pLPCX.CE). The rabbit CE gene is driven by the cytomegalovirus promoter (CMV). B, CE transcript was expressed in F3.CE, but not in F3 cells confirming by RT-PCR. C, cell morphology after CE gene transduction. LTR, long terminal repeat; Puro, puromycin.

established F3.CE cells and a human bladder cancer cell line, TCCSUP, were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

RT-PCR analysis

Total RNA from F3 and F3.CE cells was isolated in TRIzol reagent (Life Technologies), as described in the supplier instructions and stored at −80°C until further use. RT-PCR was performed with the RT DryMIX (Enzynomics) and PCR Premix Kits (Takara). For cDNA synthesis, 1 μg of total RNA and oligo dT primers (Promega) were reacted in RT DryMIX tubes at 50°C for 1 hour and at 95°C for 5 minutes. The resulting cDNAs were used for PCR with primers that are specific for the rCE gene (sense: 5′-ATGATGGCCTGGCTCTTTCT-3′; and antisense: 5′-TCTCGGAAAATTGCTCGATG-3′) and the GAPDH gene (sense: 5′-CGTGGAAGGACTCATGAC-3′; and antisense: 5′-CAAATTCGTTGTCATACCAG-3′). PCR cycling parameters consisted of 30 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. The PCR products were separated by electrophoresis on 1.5% agarose gels (Promega) and visualized using Davinch-Chemi System (Davinch-K). All experiments were performed three times, and the relative densities of each band were determined using the ImageJ program (NIH, Bethesda, MD).

Cell viability assay *in vitro*

To determine the appropriate concentration of CPT-11 for *in vitro* experiments, F3.CE cells were cultured under various concentration of CPT-11 (0.05–5 μmol/L). Cells (3×10^4) were incubated with CPT-11 for 48 hours in a 6-well plate, and cultured cells were harvested and analyzed using Muse Cell Analyzer (Millipore) following the manufacturer's instructions. Briefly, harvested cells were stained with Muse Cell Viability Kit (Millipore; 1:10 ratio) for 5 minutes.

To examine the therapeutic effect of F3.CE cells against bladder cancer cells, TCCSUP and F3.CE cells were seeded in a 6-well plate at a 1:1 ratio (3×10^4 cells each/well). After a 48-hour treatment with 1 μmol/L CPT-11, these cells were stained with Muse Cell Viability Kit as described above.

Apoptosis assay *in vitro*

To determine the cytotoxic effect of F3.CE in the presence of 1 μmol/L CPT-11, TCCSUP and F3.CE cells were cocultured at a 1:1 ratio as described above. Apoptosis was analyzed using Muse Annexin V and Dead Cell Assay Kit (Millipore) at the endpoint. These cells were incubated with Annexin V and Dead Cell Reagent (7-AAD) for 20 minutes, and the events for dead, late apoptotic, early apoptotic, and live cells were counted.

***In vitro* "bystander effect" experiments**

TCCSUP cells were plated in 6-well plates with F3 or F3.CE cells (total 6×10^4 cells/well, ratios of TCCSUP cells to F3 or F3.CE cells = 100:0, 75:25, 50:50, 25:75, or 0:100). Cell viability and cell death were analyzed using Muse Cell Analyzer at 48 hours after treatment with 1 μmol/L CPT-11 as described above.

To examine CE secretion and the direct apoptotic effect of the CE gene against tumor cells, tumor cells were plated and cultured in HB1.F3 and F3.CE-conditioned media in the presence of 1 μmol/L CPT-11 for 1 to 3 days. Untreated cells and only CPT-11–treated tumor cells were used as controls.

Bladder cancer animal model

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (IRB: 11-0086) with approval by the Institutional Animal Care and Use Committee of Chung-Ang University Hospital (Seoul, Republic of Korea). Male BALB/c nude mice (CAnN.Cg-Foxn1nu/CrljOri) 6 weeks old (SAERONBIO Inc) were housed in a temperature-controlled environment with a 12-hour day/night cycle. All mice were fed with regular chow and water *ad libitum*.

Mice were anesthetized by intraperitoneal injection with Zoletil (30 mg/kg) and Rompun (10 mg/kg). TCCSUP bladder cancer cells (1×10^6 cells in 100 μL of PBS) were injected into the subcutaneous dorsa of mice in the proximal midline.

Mice were divided randomly into six groups, 9 mice per group. All mice received TCCSUP cancer cells. The control group was not treated (control group, TCCSUP only). The second group received the CPT-11 prodrug (CPT-11 group). The third group was implanted with F3 cells (F3 group), whereas the fourth group received F3 cells and CPT-11 (F3/CPT-11 group). The fifth group was injected with F3.CE cells (F3.CE group), whereas the sixth group was injected with F3.CE cells and CPT-11 (F3.CE/CPT-11 group). F3 or F3.CE cells (1×10^6 cells in 100 μL PBS) were implanted around the tumor mass twice, at 2 and 4 weeks after TCCSUP injection. After the injection of F3.CE cells, animals were treated with CPT-11 (3.75 mg/kg in 100 μL normal saline) intraperitoneally for 5 days. They were then rested for 2 days and then injected with CPT-11 for 5 days. All animals were sacrificed, and tumor cell masses were collected 2 weeks after the last CPT-11 treatment (Fig. 6).

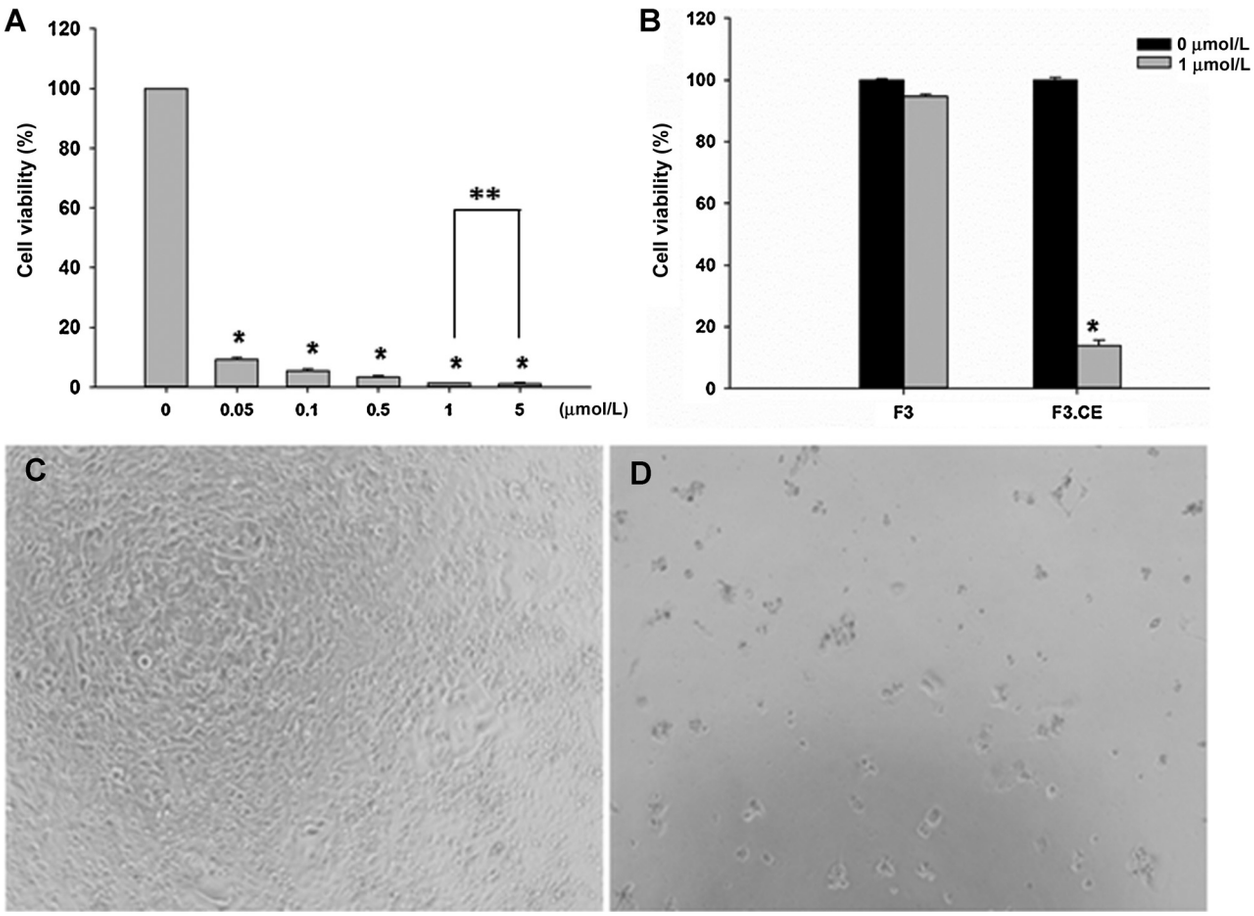

Figure 2.
Cytotoxicity of the F3.CE/CPT-11 system. A, F3.CE cells were cultured with various concentrations of CPT-11 for 48 hours. Survival of F3.CE cells was lower than 20% compared with control. B, therapeutic effect of F3.CE against human bladder cancer cells. The survival of TCCSUP cells was significantly reduced in the presence of 1 μmol/L CPT-11 and F3.CE cells. C and D, microscopic images in F3 (C) and F3.CE (D) groups after CPT-11 treatment. *, $P < 0.05$; **, no significance.

Measurement of tumor size

The tumor mass sizes were measured by caliper, and volumes were calculated according to the formula: volume = largest width^2 × largest length × 0.5. Results were expressed as the mean of tumor volume ± SE. Collected tumor cells masses were fixed in 4% paraformaldehyde and cryosectioned coronally at 10-μm thickness and stained with hematoxylin–eosin (H&E) staining. Stained tumor sizes were measured following the same procedure.

Statistical analysis

Two-way ANOVA and *post hoc* Tukey tests were used to evaluate differences in cell viability and tumor volume between groups at the significance level of 5%. Data are presented as means ± SE.

Results

Establishment of F3.CE cells

Retroviral vectors containing the rCE gene were transduced into HB1.F3 human NSCs to generate F3.CE cells overexpressing the CE gene. Expression of the CE gene was examined by RT-PCR. CE transcripts were expressed in F3.CE cells, but not in parental HB1.F3 (F3) cells (Fig. 1B). There was no change and no disruption in the cell morphology (Fig. 1C).

Cytotoxic effect of CE and CPT-11 on bladder cancer cells

To determine the concentration of CPT-11, F3.CE cells were incubated in various concentrations of CPT-11 (0.05–5 μmol/L). The optimal CPT-11 concentration for the *in vitro* experiments was found to be 1 μmol/L. Therefore, 1 μmol/L CPT-11 was used in all experiments (Fig. 2A). When F3.CE cells were cocultured with TCCSUP bladder cancer cells (1:1 ratio) for 48 hours with 1 μmol/L CPT-11, the survival of bladder cancer cells was significantly reduced in F3.CE (13.8 ± 1.8%), but not in the F3 group (94.7 ± 0.7%), under CPT-11 exposure compared with that in the absence of CPT-11 (Fig. 2B). The bladder cancer cell number also appeared reduced in the F3.CE group (Fig. 2D), but not in the F3 group (Fig. 2C), under the microscope.

Apoptotic cells were counted only as Annexin V–positive cells (early and late apoptotic cells) without necrotic cells. Annexin V–positive apoptotic bladder cancer cells significantly increased in the presence of F3.CE and 1 μmol/L CPT-11 (51.1 ± 1.3%) compared with F3 only (2.1 ± 0.2%), F3 and CPT-11 (2.3 ± 0.1%), and F3.CE (11.9 ± 1.4%) groups (Fig. 3A and B).

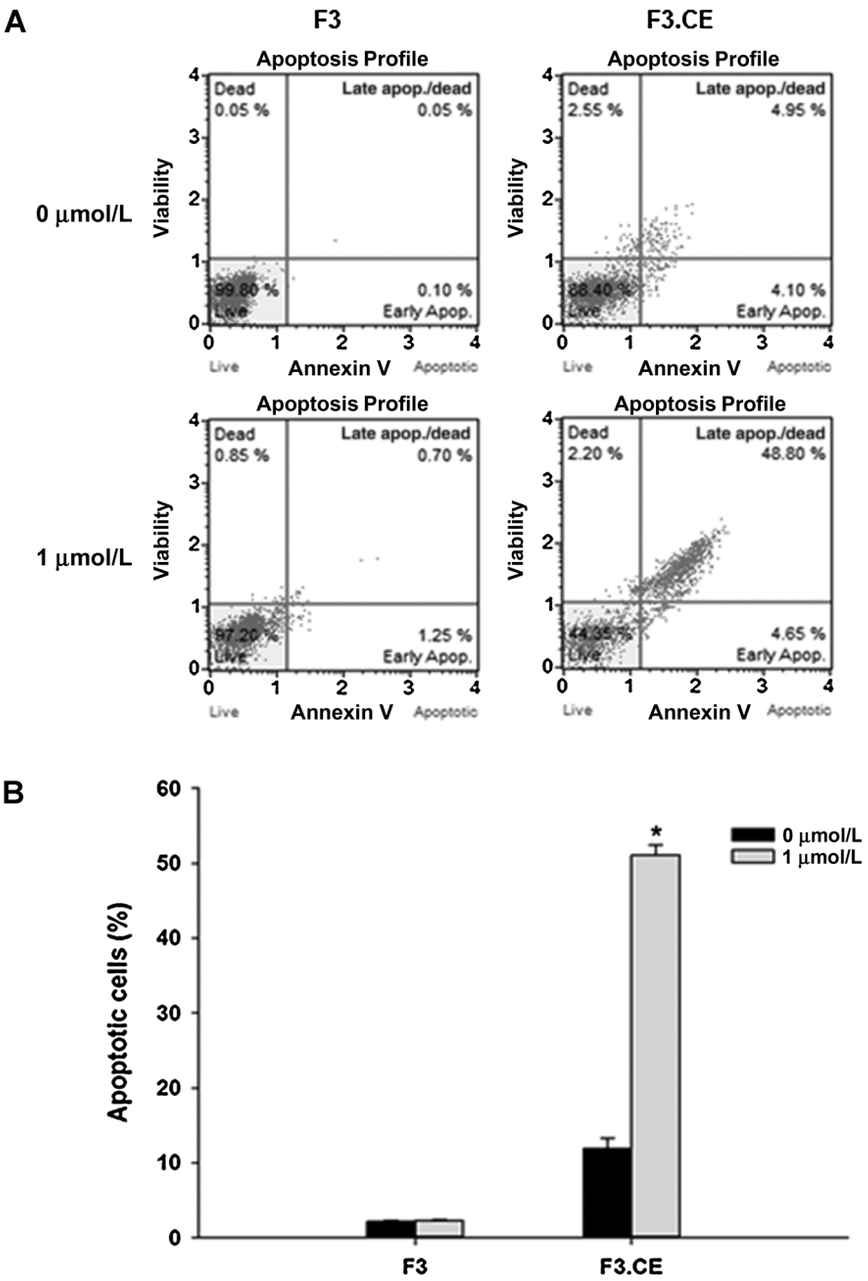

Figure 3.
Analysis of apoptosis in mixed culture with cancer and stem cells. A, Annexin V staining of early and late apoptotic (Apop.) bladder cancer cells cultured in the presence of F3 (0 μmol/L), F3.CE (0 μmol/L), F3 (1 μmol/L), and F3.CE (1 μmol/L CPT-11). B, data, mean ± SE; *, $P < 0.05$.

***In vitro* bystander effects of F3.CE**

The *in vitro* bystander effects of F3.CE were determined in a coculture system, with TCCSUP bladder cancer cells cocultured with F3 or F3.CE cells at various concentrations (ratios of TCCSUP cells to F3 or F3.CE cells = 100:0, 75:25, 50:50, 25:75, or 0:100). In the coculture experiment, the parental F3 cells did not induce any cytotoxic effect on TCCSUP cells after CPT-11 treatment; however, serial mixture of F3.CE and TCCSUP cells was shown to significantly reduce their viability under the exposure of 1 μmol/L CPT-11 to TCCSUP human bladder cancer cells ($P < 0.05$; Fig. 4). These results indicate that a small number of F3.CE cells overexpressing CE can activate sufficient amounts of CPT-11 to kill TCCSUP cells *in vitro*.

To confirm whether cell death was induced by the cytotoxic effects of F3.CE secreted in response to CPT-11, the media were conditioned with F3 and F3.CE for 2 days. TCCSUP cells were cultured in normal growth media, 1 mmol/L CPT-11 only media, or the F3 or F3_CE–conditioned media in the presence of 1 mmol/L CPT-11 for 1 to 3 days. The viability of TCCSUP cells in CPT-11 and F3-conditioned media was maintained at similar levels to that of controls, whereas the F3_CE–conditioned media decreased the viability and induced apoptosis in a time-

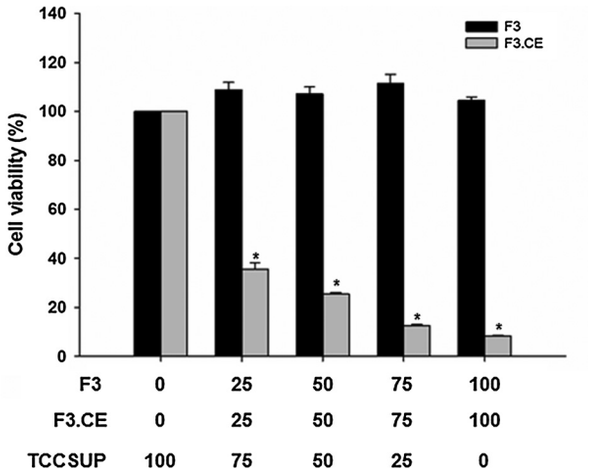

Figure 4.
Bystander cell killing effect of CE. The bystander effect of CE was examined in a coculture system of F3 or F3.CE cells and TCCSUP cells at various ratios (TCCSUP cells to F3 or F3.CE cells = 100:0, 75:25, 50:50, 25:75, or 0:100). Survival of human bladder cancer cells was significantly reduced under the exposure of 1 μmol/L CPT-11 and F3.CE cells for 48 hours ($n = 3$/group; *, $P < 0.05$), but not F3 cells.

dependent manner (Fig. 5). The number of apoptotic cells in TCCSUP cells treated with control, CPT-11 only, and F3-conditioned medium was up to 15%, whereas apoptotic cells in F3.CE conditioned media reached 64.35% (Fig. 5B). These results suggested that F3.CE cells secreted CE proteins into the medium, and the secreted CE proteins efficiently converted CPT11 into active SN-38 to induce death of TCCSUP.

In vivo therapeutic efficacy of F3.CE cells toward bladder cancer cells

The *in vivo* therapeutic efficacy of F3.CE cells against bladder cancer cells was determined by tumor volume measurement. Slices of tumor mass were stained with H&E and tumor areas measured (Fig. 7A). The tumor volumes in the F3.CE/CPT-11 group (11.1 ± 1.2 mm^3) were significantly reduced compared with control (TCCSUP only, 65.9 ± 5.1 mm^3), F3 (62.4 ± 3.6 mm^3), F3.CE (62.8 ± 4.8 mm^3), TCCSUP + CPT11 (62.4 ± 3.6 mm^3), and F3 + CPT11 (64.7 ± 6.6 mm^3; Fig. 7B). The tumor volumes in the F3.CE/CPT-11 group were reduced by 83% compared with the control group. These results suggest that F3.CE cells expressed CE, which in turn converted CPT-11 into SN-38, a potent toxic metabolite showing tumor-killing activity and acting as anticancer therapeutic. In addition, we did not find any cell mass near the implanted cancer in F3.CE/CPT-11 group at the endpoint. This suggested that F3.CE cells may be removed after CPT-11 treatment *in vivo*.

Discussion

The major advantage of using stem cells for treating cancer is their unique migration and infiltration ability into tumor bulks. This tumor-specific tropism can be utilized by equipping stem cells with products that have antitumor effects. In additon, the ability of stem cells to infiltrate throughout tumor masses is assumed to produce a more potent antitumor effect than other tools, such as modified liposomes, antibody–drug conjugates, and nanoparticles. Moreover, this tumor-specific tropism could be exploited to target minute distant metastases and infiltrate malignant satellites after complete resection of the main tumor. This tropism is due to interactions with protein receptors on tumor cells as well as important physiologic processes that influence the migratory behavior of transplanted NSCs, including inflammation, reactive astrocytosis, and angiogenesis (13–15). F3.CE cells were used to treat various cancer cell types (subdural medulloblastomas, breast cancer, and lung cancer). These previous studies also reported the tropism of stem cells to cancer cells (16–18).

To avoid tumor formation, F3.CE cells were transduced with the suicide gene encoding CE, which convert the nontoxic CPT-11 prodrug into the highly toxic SN-38 drug, showing significant therapeutic effects on some tumor cells, such as

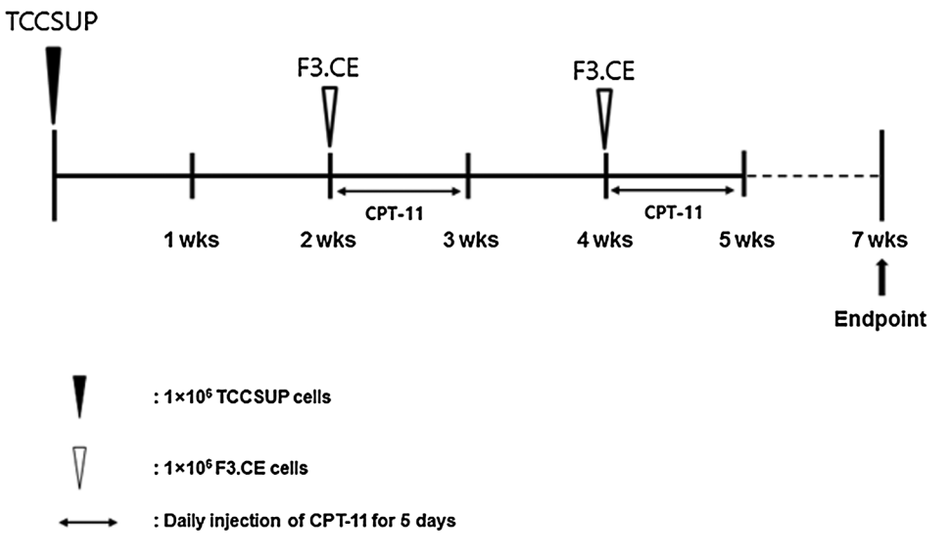

Figure 5.
Cytotoxic effects of F3.CE in the presence of CPT-11. To confirm the cytotoxic effect of F3.CE cells, the conditioned medium from F3 and F3.CE cell cultures was collected and treated with TCCSUP cells for 3 days. A, the viability of TCCSUP cells decreased in F3.CE-conditioned medium. B, F3.CE-conditioned medium induced the apoptosis of TCCSUP, whereas CPT-11 and F3–conditioned media did not have any cytotoxic effect on TCCSUP.

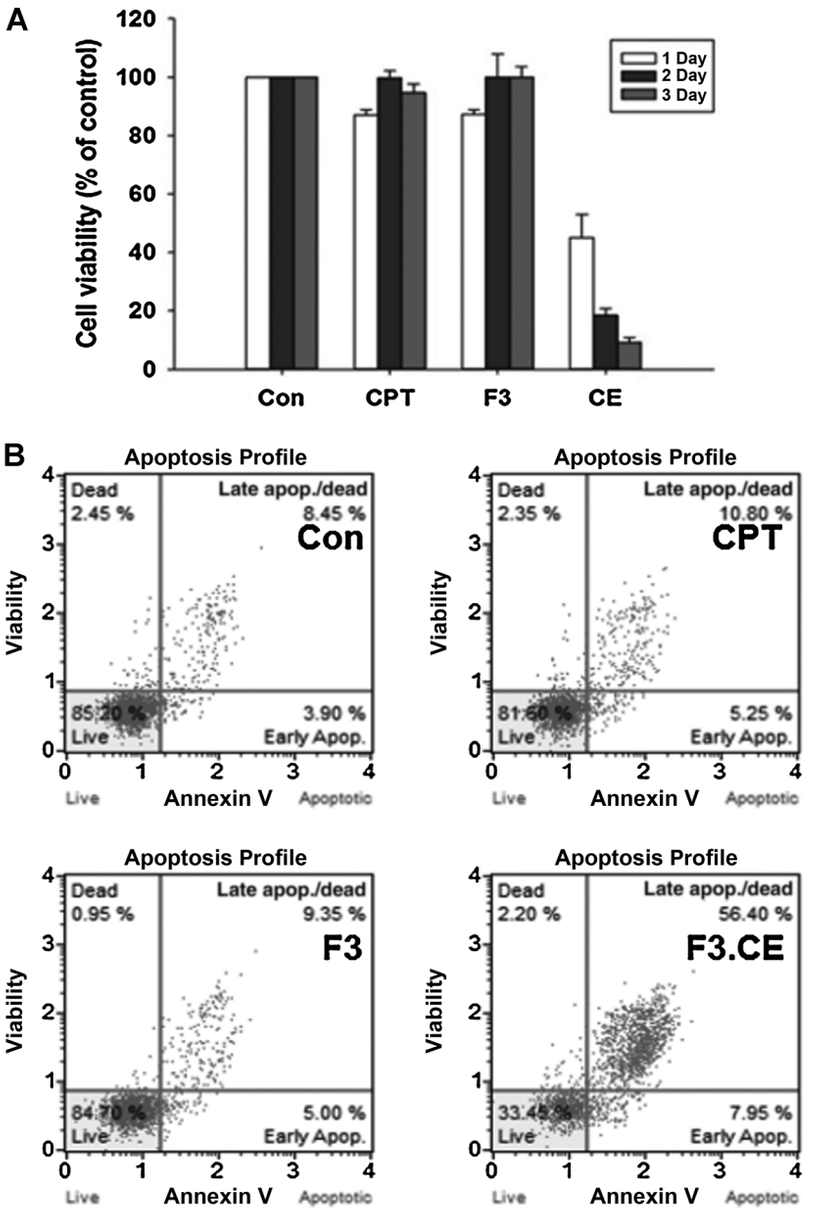

Figure 6.
Schematic timeline of *in vivo* experiments. F3 or F3.CE cells were implanted around the tumor mass twice, at 2 and 4 weeks after TCCSUP injection. After the injection of F3.CE cells, animals were treated with CPT-11 for 5 consecutive days with a break of 2 days. All animals were sacrificed, and tumor cell masses were collected 2 weeks after the endpoint of CPT-11 treatment. Con, control; Apop., apoptotic.

melanoma, ovarian cancer cell, and neuroblastoma (19–21). The toxic drug can remove the tumor mass as well as the stem cells themselves to avoid additional tumor formation from implanted stem cells. Safety issues as well as immune rejection of stem cells need to be solved before clinical application. However, stem cells from the central nervous system have the advantage to give rise to limited immune response because they do not express MHC class I or II antigens in their undifferentiated state (22). In a previous study, Lee and colleagues reported that F3.CE could avoid immune rejection by CD70–CD27 ligation between NSCs and T cells (23). Moreover, F3 cells were used as carriers of suicide genes for brain tumors and brain metastases without any additional tumor formation (21, 24).

In this study, we tested the hypothesis that NSCs carrying a suicide gene exert a bystander therapeutic effect on bladder cancer cells. We used HB1.F3 NSCs, a well-characterized human NSC line (22). The effect of NSCs showed an 83% reduction of tumor volume in a mouse model. F3.CE cells migrated to the implanted tumor sites and reduced the tumor volume in mice receiving administration of CPT-11. Therefore, our study suggests the potential of human NSCs as an effective delivery system to target and deliver CPT-11 to bladder cancer cells.

In conclusion, human NSCs transduced with CE were shown to exert a cytotoxic effect on implanted human bladder tumor cells in the presence of the CPT-11 prodrug *in vitro* and *in vivo*. These results further support the use of human NSCs expressing CE in the treatment of advanced bladder cancer in clinical trials.

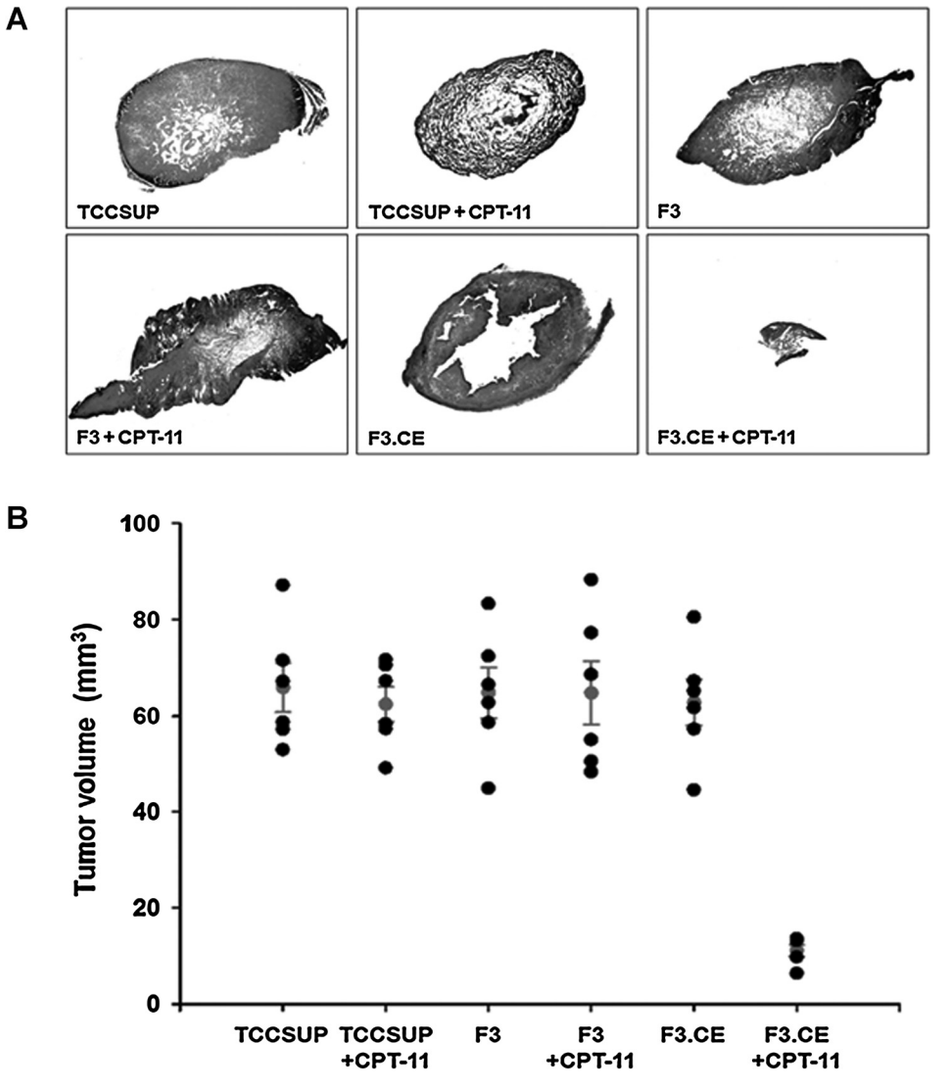

Figure 7.
Implantation of F3.CE has a therapeutic effect against TCCSUP cells in CPT-11 exposure. A, collected tumor masses were cryosectioned, stained with H/E, and measured. B, data, mean ± SE, $P < 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.S. Choi, I.H. Chang
Development of methodology: K.D. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-R. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.S. Choi
Writing, review, and/or revision of the manuscript: S.S. Choi, B.H. Chi
Study supervision: S.U. Kim, H.J. Lee

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