Abstract. Interest in gene therapy has recently increased; in particular, gene-directed enzyme/prodrug therapies have been found to be more advantageous compared to radiotherapy and/or chemotherapy. One of these, a cytosine deaminase (CD)/5-fluorocytosine (5-FC) system, is known to induce apoptosis by converting 5-FC, a prodrug, to its metabolically active form, 5-fluorouracil. The present study was designed to examine the migratory and therapeutic effects of engineered human stem cell lines against endometrial cancer using this strategy. The parental stem cells, HB1.F3, were modified to express Escherichia coli CD or human interferon-beta (IFN-β), thereby producing HB1.F3.CD and HB1.F3.CD.IFN-β cells, respectively. The parental and engineered stem cells (HB1.F3, HB1.F3.CD, and HB1.F3.CD.IFN-β) significantly migrated toward endometrial cancer cells (Ishikawa) more than primary bovine fibroblasts (bovine FB). In addition, important chemoattractant factors, including stem cell factor (SCF), vascular endothelial growth factor, vascular endothelial growth factor receptor 2, C-X-C chemokine receptor type 4, and c-KIT, involved in the tumor-tropic ability of stem cells were expressed in Ishikawa cells. In using a co-culture system and MTT assay, reduced viability of endometrial cancer cells was observed in the presence of HB1.F3.CD and HB1.F3.CD.IFN-β cells with prodrug 5-FC. Taken together, these results suggest that gene therapy employing genetically modified stem cells expressing CD and IFN-β may be effective for treating endometrial cancer.

Endometrial cancer, neoplastic growth of endometrial epithelial cells, has been recently reported as the most common malignancy in the female genital tract, accounting for approximately 50% of uterine cancer cases in the Western world (1-3). The mechanisms underlying the development of this type of cancer is unclear, but it mostly occurs in women who suffer from conditions which cause hormonal or endometrial cell cycle alteration, such as diabetes mellitus, menopause, hypertension, and obesity (4, 5). Endometrial adenocarcinoma can usually be classified into two categories: type 1 and type 2 (6, 7). Type 1 is known as a low-grade endometrioid adenocarcinoma mainly associated with obesity and high estrogen levels and emerging in both pre-/postmenopausal women (7, 8). Type 2, known as high-grade non-endometrioid carcinoma, is a more aggressive type of cancer that develops independently of estrogen levels and is commonly found in elderly women (6, 7).

Several types of cancer, including those in the colon, rectum, breast, and endometrial, are treated with chemotherapeutic agents such as cisplatin and 5-fluorouracil (5-FU) (9). 5-FU is one of the oldest antimetabolite drugs still used, despite its many side-effects, and it is commonly used as a potent antitumor agent (10). This agent inhibits cancer cell growth by interfering with transcription. It is converted to the cytosine analog drug 5-fluorocytosine (5-FC) by cytosine deaminase (CD) (11). CD is an enzyme that catalyzes the deamination of cytosine to uracil and is found in bacteria and yeast (12). Currently, this enzyme is being used in gene therapy for several tumor types and various stem cells have been genetically modified to express the Escherichia coli CD gene. Engineered gene/prodrug systems offer several advantages compared to radiotherapy and chemotherapy for cancer patients. In particular, the CD/5-FC

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Key Words: Endometrial cancer, engineered stem cells, cytosine deaminase, interferon-β.
system is a gene-directed enzyme/prodrug therapy (GEPT) that induces apoptosis following the administration of the prodrug 5-FC (13). Generally, GEPT involves the activation of a prodrug by an enzyme, and requires a vehicle for the enzyme gene delivery (14, 15).

For our study, we designed a ‘double-punch’ strategy that involves the expression of both the E. coli CD and human interferon-beta (IFN-β) in engineered stem cells. Interferons (IFNs) are cytokines which respond to viral infection and activate immune cells (16, 17). IFNs can be divided into two categories: type I and type II (18). Type I IFNs include IFN-alpha (IFN-α) and IFN-β, while IFN-ω and IFN-γ belong to the type II IFN family (19). Both type I and type II IFNs bind to specific type I (IFNAR) and type II cell surface receptors IFN receptors (IFNGR), respectively (20). In particular, IFN-β is a potent cytokine and has been used for the treatment for several diseases (17). However, the treatment is not extremely effective in vivo because IFN-β has a short half-life (21).

In this study, we used a human neural stem cell (NSC) line, HB1.F3, obtained from fetal telencephalon cells and immortalized by a retroviral vector, v-myc oncopGene (22, 23). These stem cells were modified to create genetically engineered stem cells which produced suicide genes, E. coli CD and/or human IFN-β to make the HB1.F3.CD and HB1.F3.CD.IFN-β cell lines, respectively. All cancer cells produce many growth factors, such as stem cell factor (SCF), vascular endothelial growth factor (VEGF),stromal cell-derived factor-1 (SDF-1), and c-KIT, for survival and proliferation (24, 25). Genetically engineered stem cells can migrate toward tumors to interact with several growth factors produced by the cancer cell. We predicted that simultaneous suicide gene and cytokine expression would have synergistic effects and tested our hypothesis by using tumor-tropic abilities of genetically engineered stem cells.

**Materials and Methods**

**Cell lines and culture.** Ishikawa cells are a human endometrial adenocarcinoma derived from a glandular epithelial cell line and express estrogen and progesterone receptors (26-28). Primary bovine fibroblast (FB) cells were used as a control for a migration assay because these cells may exclude the effects of chemo-attractant factors secreted by human cells. Ishikawa and bovine FB cells were obtained from the Veterinary Biochemistry Laboratory at Chungbuk National University (Cheong-ju, Korea). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc. Logan, UT, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc.), antibiotic agents 100 unit/ml penicillin and 100 µg/ml streptomycin (Cellgro Mediatech, Inc., Manassas, VA, USA), 1% (v/v) antifungal agents, HEPES (Invitrogen Life Technologies, Carlsbad, CA, USA), and anti-mycoplasmal agents (Invivogen, San Diego, CA, USA). Human NSCs, HB1.F3, HB1.F3.CD, and HB1.F3.CD.IFN-β (Chung-Ang University, Seoul, Korea) were cultured under the same conditions. Cells were grown at 37°C in 5% CO2 and 95% air in a humidified cell incubator and were passaged using 0.125% EDTA-trypsin (PA Laboratories GmbH, Linz, Austria).

**RNA extraction and reverse transcription-PCR.** Total RNA was extracted from stem cells and cancer cells using Trizol reagent (Invitrogen Life Technologies), and the concentration of the extracted total RNA was determined by a spectrophotometer (Optizien, Mecasyas, Dea-jon, Korea) at 260 nm/280 nm. For reverse transcription-PCR, 1 µg of total RNA was converted into complementary DNA (cDNA). The mixture used for cDNA synthesis contained murine leukemia virus reverse transcriptase (M-MLV RT; iNtRON Biotechnology, Sungnam, Kyeonggido, Korea), 200 pM nonamer random primer (iNtRON Biotechnology), 10 pM dNTPs (iNtRON Biotechnology), RNase inhibitor (iNtRON Biotechnology), and 5× RT buffer (iNtRON Biotechnology). The cDNA (1 µl) produced from the total stem cell RNA was amplified by PCR to confirm the expression of bacterial CD and human IFN-β. PCR was conducted using cDNA from the cancer cells to assess the expression of the chemo-attractant ligands and receptors including SCF, c-KIT, chemokine receptor 4 (CXCR4), VEGF, and VEGFR2 with primers based on the published sequences of the genes. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the PCR positive control. The PCR reaction mixture contained each 10 pM forward and reverse primer, 2 unit Taq polymerase (iNtRON Biotechnology), 10× PCR buffer (iNtRON Biotechnology), 5 pM dNTP mixture (iNtRON Biotechnology), and 1 µl cDNA template. PCR was performed for 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 58°C, and extension for 30 s at 72°C. The forward and reverse primers and the expected size of the RT-PCR products are presented in Table I. The PCR products (8 µl) were loaded on a 1.5% agarose gel and compared to 100-bp ladders (iNtRON Biotechnology). The gels were scanned by a Gel Doc 2000 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**In vitro migration assay.** A modified transwell migration assay was performed to determine the migration abilities of the stem cells. Endometrial cancer and bovine FB cells (1×105 cells/well) were seeded in 24-well plates and incubated at 37°C in a CO2 incubator for 6 h. The medium was changed to serum-free medium after washing the cells three times with phosphate-buffered saline (PBS), and the cells were then incubated for 24 h. Before incubation for 24 h, transwells (8 µm; BD Biosciences, Franklin lakes, NJ, USA) pre-coated with fibronectin (250 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS were inserted into the 24-well plates. The next day, 2 µM chloromethylbenzamidole-Dil (CM-Dil; Invitrogen Life Technologies) pre-labeled stem cells (1×105 cells/well) were plated in the upper transwell chamber. For pre-labeling of the stem cells, they were incubated with CM-Dil solution at 37°C for 5 min and then placed at 4°C for 15 min. These pre-labeled stem cells in a transwell upper chamber were incubated with endometrial cancer cells or bovine FB cells in the lower chamber at 37°C for 24 h. The endometrial cancer and bovine FB cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen Life Technologies). DAPI staining solution (200 ng/ml) was added to the lower chamber and the plates were incubated at 37°C for 10 min in the dark. After staining, the stem cells stained with CM-Dil and endometrial
cancer or bovine FB cells stained with DAPI were detected by fluorescence microscopy (IX71 inverted microscope; Olympus, Tokyo, Japan) following washing twice with PBS.

In vitro cell viability assay. An MTT (3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed to confirm the viability of the endometrial cancer cells. Briefly, an initial experiment was performed to check for the effects of various prodrug 5-FC (Sigma-Aldrich Corp.) concentrations and a subsequent experiment was conducted to identify the effect of various stem cell numbers. After determining the number of viable cells with trypan blue staining, Ishikawa cells (4,000 cells/well) were seeded in 96-well plates for 24 h in an incubator at 37°C. The next day, HB1.F3, HB1.F3.CD, and HB1.F3.CD.IFN-β cells (8,000 cells/well) were co-cultured with the Ishikawa cells for 24 h. The wells were treated with 5-FC diluted to different concentrations in saline (100, 200, 300, 400, and 500 μg/ml) for 4 days. Subsequently, an MTT assay was performed to measure cell viability. A 5 mg/ml MTT stock solution (Sigma-Aldrich) was diluted in PBS, then 10 μl of the MTT stock solution was added to each well and the plates were incubated for 4 h at 37°C. The yellow colored formazan crystals that formed were dissolved in 100 μl dimethyl sulfoxide (DMSO; Junsei Chemical Co., Ltd., Tokyo, Japan). After shaking, the absorbance was measured at 540nm using an ELISA plate reader (VERSA man; Molecular Devices, CA, USA). A second experiment was performed using the same protocol. However, different numbers of stem cells (8,000, 16,000, and 24,000 cells/well) were added to the cultured plate on day 2, and 5-FC was added to the cells at a single concentration (500 μg/ml). Cell viability was measured using an MTT assay. Each experiment was conducted in duplicate (n=6).

Statistical analysis. To assess the statistical significance between each in vitro experimental group, statistical analyses were performed by one-way ANOVA followed by Tukey’s test using Prism Graph Pad (v4.0; Graph Pad Software Inc., San Diego, CA, USA). A p-value <0.05 was considered statistically significant. All experimental data are expressed as the mean±standard deviation (S.D).

Table 1. The sequences of the primers used in this study and predicted sizes of the PCR products.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5'-&gt;3')</th>
<th>Predicted size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>Forward: GCGCGAGTCACCGCCAGCCACACCACGC</td>
<td>559</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTTTGTATCGATGCTTCTGGCTGC</td>
<td></td>
</tr>
<tr>
<td>IFN-β</td>
<td>Forward: AAAGAAGCAGCAATTTTCCAGC</td>
<td>296</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTTCTCCAGTTTTTCTTCACA</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward: AAGGCTTCTCGTGCCCCCTGTAC</td>
<td>377</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTCTCTCTCTCTGCACCGCTCACAC</td>
<td></td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Forward: ACGCTGACATGTACGCTCATAT</td>
<td>438</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCCAAGCTTGTACCAGTGTAG</td>
<td></td>
</tr>
<tr>
<td>c-KIT</td>
<td>Forward: GCCCCAAATAGATTTTGGATATTT</td>
<td>570</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCATCTTTACAGCGACAGTC</td>
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</tr>
<tr>
<td>CXCR4</td>
<td>Forward: CTCTCAAAAGGAAGCCAGGTGGAACAT</td>
<td>558</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGACTGTACGTCAGGTCTGAAATCA</td>
<td></td>
</tr>
<tr>
<td>SCF</td>
<td>Forward: ACTTTGATCTTCACTTGCTATT</td>
<td>505</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCTCAAAAGGAAGCCAGGTGGAACAT</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: ATGTTCGTACATGGTCTGAAACCA</td>
<td>351</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGGCACAGTTTCCTCATACGGCGAC</td>
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</tr>
</tbody>
</table>

Figure 1. Expression of Escherichia coli CD and human IFN-β in the three stem cell lines. After total RNA was extracted from the three stem cell lines, cDNA was synthesized by reverse-transcription. This cDNA was then used to perform PCR. The size of the PCR products was confirmed by 1.5% agarose gel electrophoresis. The sizes of CD and IFN-β were predicted to be 559 bp and 296 bp, respectively. GAPDH was used as the positive control and the negative control reaction was performed without template. Lane 1: 100-bp molecular weight marker, lane 2: negative control without template, lane 3: HB1.F3 cells cDNA, lane 4: HB1.F3.CD cells cDNA, lane 5: HB1.F3.CD.IFN-β cells cDNA.
Figure 2 Continued overleaf
Results

E. coli CD and human IFN-β gene expression. The gene expression of E. coli CD and human IFN-β was examined in the engineered stem cells using reverse transcriptase PCR (RT-PCR). The CD gene in HB1.CD and HB1.CD.IFN-β cells was detected at 559 bp, and human IFN-β gene at 296 bp was only detected in HB1.F3.CD.IFN-β cells. Additionally, human GAPDH was detected at 351 bp as a positive control, and its expression was identified in HB1.F3, HB1.F3.CD and HB1.F3.CD.IFN-β cells (Figure 1).

In vitro migration of the engineered stem cells. To assess the migration ability of the engineered stem cells, a modified transwell migration assay was performed. All of the stem cells, HB1.F3, HB1.F3.CD and HB1.F3.CD.IFN-β, migrated towards endometrial cancer cells more than primary bovine fibroblasts (bovine FB cells) as shown in Figure 2A and B. The number of stem cells that migrated was determined and this showed that the engineered stem cells migrated at a significantly higher rate toward the endometrial cancer cells compared to the bovine FB primary cells (Figure 2C).

In vitro therapeutic efficacy of CD and IFN-β expression. Using the co-culture system and MTT assay, the cytotoxic effects of 5-FC and 5-FU were measured in endometrial cancer cells in the presence of the engineered stem cells. When Ishikawa cells were treated with 5-FU, cell viability was reduced to about 80% as shown in Figure 3A. We also measured the cell viability of Ishikawa cells treated with different concentrations of the prodrug, 5-FC, in the presence of HB1.F3.CD and HB1.F3.CD.IFN-β cells. The cell viability of endometrial cancer cells treated with 5-FC was reduced in the presence of the HB1.F3.CD and HB1.F3.CD.IFN-β cells (Figure 3B). Cell viability of Ishikawa cells was reduced when cells were co-cultured with HB1.F3.CD.IFN-β rather than HB1.F3.CD cells, but this was not significant (Figure 3B). When the concentration of 5-FC was fixed at 500 μg/ml and the number of stem cells varied (8,000, 16,000 and 24,000 cells/well) in the co-culture system, endometrial cancer cell viability was not significantly affected by the number of stem cell present (Figure 3C). Overall, the growth of endometrial cancer cells was little inhibited when co-cultured with HB1.F3.CD.IFN-β cells than HB1.F3.CD cells.

Identification of chemoattractant factors in cancer cells. We confirmed the expression of chemoattractant ligands and receptors in the endometrial cancer cells with RT-PCR. The expression of chemoattractant factors including SCF, CXCR4, VEGF, VEGFR2, and c-KIT were assessed, assuming that
these factors may play a role in the tumor-tropic effect of the stem cells in vitro and in vivo. In the Ishikawa cells, all of these factors were clearly expressed although the expression VEGFR2 was relatively weak, as shown in Figure 4.

Discussion

It is estimated that approximately 40,000 new cases of endometrial cancer are diagnosed annually in the United States and about 7,000 women die every year from this disease, thus endometrial carcinoma is the fourth most common malignancy and the eighth leading cause of cancer-related death in women (29). Although extensive research on endometrial cancer is currently ongoing, the exact pathogenesis and biological aspects of this disease are not well elucidated. Nowadays, treatment for endometrial cancer includes several types of approaches such as surgery for removing the uterus, radiation therapy, hormone therapy, and chemotherapy (30, 31). Among these, chemotherapy and radiotherapy have been most commonly used, but the accompanying side-effects are serious and these modalities are associated with a high risk of disease recurrence (32, 33). Stem cell-based therapies are a potential alternative treatment for many types of human cancer.

Stem cells are characterized by both self-renewal and the ability to differentiate into various cell types (34). Recently, stem cell therapy using multi-pluripotent stem cells has been used for treating malignant cancer and promoting self-renewal in damaged tissues (35). However, the use of
embryonic stem cells taken from a developing human embryo has numerous ethical problems; therefore, the therapeutic use of these stem cells has been extremely restricted (36, 37). However, adult stem cells including, NSCs, mesenchymal stem cells (MSCs), and umbilical cord blood stem cells are easily obtained and have been successfully used in numerous patients (38, 39). We have employed human NSCs immortalized by a retroviral vector to elucidate their therapeutic potential for treating human cancer (40). Therapeutic efficacy of these stem cells for treating brain tumors such as brain stem glioma and medulloblastoma was demonstrated in vitro and in vivo by other previous studies (41-43).

Engineered MSCs obtained from bone marrow or umbilical cord have been recently used for many tumor-targeting studies (44). These cells are also capable of self-renewal and tissue reconstruction (45, 46), and were genetically engineered to express either CD or IFN-β for use as a delivery vehicle. They have been shown to possess tumor-tropic abilities that are attributed to the expression of growth factors, their receptors, and chemokines (47-50). The proliferation of several cancer cell lines including those derived from breast, lung, and brain was inhibited when treated with CD and 5-FC (51, 52). In particular, the therapeutic efficacy of engineered stem cells expressing IFN-β was increased when administered along with 5-FU (51). Taken together, these studies indicate that a specific and selective mediator or delivery system is crucial for enhancing the selective effectiveness of these prodrugs against de novo tumors. HB1.F3.CD and HB1.F3.CD.IFN-β cells, by virtue of their inherent migratory and tumor-tropic properties, represent a novel and potentially powerful approach for treating invasive tumors. Since no effective treatments are available for most metastatic tumors, these stem/progenitor cells expressing a suicide gene and cytokine, CD and IFN-β, have the potential to improve the prognosis of patients with metastatic cancer.

In this study, we tested the hypothesis that these engineered stem cells are highly effective for the genetic manipulation of endometrial cancer cells via gene transfer. Although an accurate molecular mechanism underlying the tumor-tropism of these stem cells has not been clearly elucidated, the tumor-tropic activities of HB1.F3.CD and HB1.F3.CD.IFN-β cells can provide an excellent method for specifically and selectively targeting human cancer cells. The tumor cell expression of factors such as SDF-1, SCF, VEGF, VEGFR2, and c-KIT may serve as chemoattractants for promoting an immune response, would have a synergistic effect. Furthermore, the ability of the engineered stem cells to migrate towards the endometrial cancer cells was determined through a modified migration assay. We found that the engineered stem cells migrated more towards the endometrial cancer cells rather than primary bovine FB cells. Expression of chemoattractant receptors and ligands which are important for in vivo cell migration were confirmed by RT-PCR. In the endometrial cancer cells, the expression of several chemoattractant factors, including SCF, CXCR4, VEGF/VEGFR2, and c-KIT, was observed, although VEGFR2 was more weakly expressed than the other factors. These chemoattractant molecules and their respective receptors may play a role in the tumor-tropic effects that enable the genetically engineered stem cells to selectively deliver a suicide enzyme to endometrial tumors. Further study is required to confirm the role of these factors in tumor cell recognition and/or tumor tropism of the engineered stem cells (42).

In the present study, HB1.F3.CD.IFN-β and HB1.F3.CD cells showed a high rate of therapeutic efficacy and treatment when administered with a prodrug, and induced the inhibition of endometrial cancer cell growth. However endometrial cancer cell therapy in our study, a greater synergistic effect of HB1.F3.CD.IFN-β cells used with the 5-FC/CD system was not observed compared to that with HB1.F3.CD and 5-FC. Furthermore, this finding indicates that a synergistic effect of IFN-β with a CD/5-FC system may not be derived from a ‘double-punch’ strategy of chemotherapy and immunotherapy for potential therapeutic applications for endometrial cancer patients. The secretion of IFN-β into culture media from HB1.F3.CD.IFN-β cells needs to be confirmed in future experiments. IFN-β is a potent cytokine but has a low clinical impact due to its short half-life (55).

Additionally, cell viability of endometrial cancer cells appeared not to be dose-dependent state of 5-FC. To find underlying mechanisms for this effect, the conversion rate of 5-FC to 5-FU via HB1.F3.CD/HB1.F3.CD.IFN-β cells in this type of tumor needs to be established. The doubling time of the endometrial cancer cells is very short when cultured in vitro. If 5-FC is not converted into 5-FU at a high enough rate, the inhibition of endometrial cancer cell growth would not be sufficient. Furthermore, the effect of IFN-β on cancer cell growth inhibition may be very weak because of the short half-life of secreted IFN-β. In conclusion, the results from the present study suggest that genetically modified stem cells expressing CD and IFN-β can be used as a gene-based therapy for treating endometrial cancer via their tumor-tropic capacity.

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF; No.2010-0003093) grant funded by the Korean government (MEST).
References


