Genetically-engineered Human Neural Stem Cells with Rabbit Carboxyl Esterase Can Target CNS Lymphoma

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Abstract. Background: Despite advances in its treatment, CNS lymphoma remains a devastating disease. Taking advantage of the tumour-tropic properties of neural stem cells (NSCs) is a novel therapeutic strategy. To apply this strategy to the treatment of CNS lymphoma, we investigated the role of NSCs expressing carboxyl esterase (HB1.F3.CE), which activates irinotecan. Materials and Methods: In order to find in vitro bystander effects of engineered NSCs, we performed cell viability assays. In vivo, the HB1.F3.CE cells were injected into the brain of mice with orthotopic CNS lymphoma. Mice were then treated with irinotecan by systemic administration. Results: The HB1.F3.CE cells significantly inhibited the growth of Raji cells with irinotecan treatment. In vivo, the HB1.F3.CE cells migrated into the tumour and significantly reduced tumour volume. In addition, survival of mice was prolonged by treatment with HB1.F3.CE and irinotecan. Conclusion: Transplantation of human NSCs encoding CE into brain, combined with irinotecan therapy, may be an effective treatment regimen for CNS lymphoma.

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Key Words: Irinotecan, carboxyl esterase (CE), CNS lymphoma, neural stem cells, rabbit.

The current primary therapeutic option for central nervous system (CNS) lymphoma is chemotherapy with intravenous high-dose methotrexate (1-3), and this treatment leads to durable remissions in some patients. However, tumour relapse occurs in most cases, and the overall and progression-free survival range from 14 to 55 months and 6 to 10 months, respectively (4). Although temozolomide, etoposide, ifosfamide, rituximab, and stem cell transplantation have been tried as salvage treatment, irinotecan is one of the most promising options since it has an excellent CNS penetration (5-7). Unfortunately, despite high response rates in phase II studies against a broad range of solid tumour types, irinotecan has not been used to treat malignant lymphoma, mainly due to its common severe side-effects of leucopenia and diarrhea even at normal treatment doses (8).

Carboxyl esterase (CE) is mainly expressed in the liver. It hydrolyzes and activates the pro-drug irinotecan to produce an anti-neoplastic topoisomerase-1 inhibitor, 7-ethyl-10-hydroxy-camptothecin (SN-38) (9, 10). Selective expression of CE in tumour could achieve the same therapeutic effects but at lower systemic concentrations of toxic SN-38, thus reducing the undesirable side-effects of irinotecan. The recent discovery of the inherent tumour-tropic properties of neural stem cells (NSCs) potentially provides a novel approach to target therapeutic gene products specifically to multiple tumour sites and invasive tumour cells in the CNS (11). In the present study, we established the proof-of-concept that CNS lymphoma can be effectively targeted by NSCs expressing CE (HB1.F3.CE cells) and demonstrated the therapeutic value of transplantation of HB1.F3.CE cells and systemic injection of irinotecan in a mouse model of CNS lymphoma.
Materials and Methods

Cell culture. HB1.F3, immortalized human fetal NSCs, and HB1.F3 cells producing rabbit CE (HB1.F3.CE) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen, Grand Island, NY, USA), which is hereafter referred to as 10% DMEM. Raji human Burkitt lymphoma cells (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS, 2 mmol/L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen).

Genetic engineering of the human NSC line HB1.F3.CE. HB1.F3 cell line was prepared as described previously (12, 13). The clonal HB1.F3.CE cell line was constructed from the parental HB1.F3 line as described previously (13-15). Briefly, an expression plasmid encoding rabbit CE was constructed and transduced into HB1.F3 cells using a retroviral system. Successful transduction of the HB1.F3.CE cells was confirmed by reverse transcription–PCR using the following primer pair: sense, 5’-TGCTGGGCTATCCACTCTCT-3’; antisense, 5’-CTTCAAGCTCCTCCTGTGTGA-3’ (15). To confirm the activity of CE in HB1.F3.CE cells, 1×10⁶ HB1.F3, HB1.F3.CE or Raji cells were plated in 96-well plates. Twenty-four hours after seeding, 0, 0.001, 0.01, 0.1, 1, 10, or 100 μg/ml irinotecan (Sigma, St. Louis, MO, USA) was applied for 48 h. The status of the cells was then analyzed using a microscope, and viability was determined with a colorimetric assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Rockville, MD, USA).

In vitro experiments of the bystander effect. Raji and HB1.F3 or HB1.F3.CE cells in 10% DMEM were seeded in 96-well plates (total 1×10⁴ cells per well; Raji cells:HB1.F3 or HB1.F3.CE cells =75:25, 50:50, 25:75, or 0:100). Irinotecan (1 μg/ml) was added to the mixed cell cultures 24 h after seeding for 48 h, and cell viability was determined as described above. The relative survivals of Raji and HB1.F3.CE co-cultures were compared to the survival of Raji and HB1.F3 co-cultures (set to 100%) for each ratio. To rule out the direct effects of HB1.F3.CE and HB1.F3 cells on the survival of tumour cells, conditioned media were prepared (HB1.F3.CE and HB1.F3 conditioned media, respectively) as follows: 1×10⁶ HB1.F3 or HB1.F3.CE cells in 10% DMEM were seeded in a T-75 flask. On the next day, 1 μg/ml irinotecan was applied followed by incubation for 48 h. Conditioned media from HB1.F3 and HB1.F3.CE cells were then obtained by filtration (0.2 μm). Raji cells were treated with conditioned media (50%, 25%, 12.5%, and 0%) from HB1.F3 or HB1.F3.CE cells for 48 h. The relative survival of Raji cells supplemented with conditioned media (set at 100%) was compared at each ratio.

Animal model of CNS lymphoma. Animal experiments in this study were reviewed and approved (no: C-A7-210-3) by the Institutional Animal Care and Use Committee (IACUC) at Samsung Biomedical Research Institute (SBRI). Anesthetized BALB/c-nu mice (6- to 7-week-old males) were secured in a rodent stereotactic frame, and a hollow guide screw was implanted into a small drill hole made 2 mm left and 1 mm anterior to the bregma. Raji cells [5×10⁵ in 5 μl Hanks’ balanced salt solution (HBSS)] were injected through this guide screw into the white matter at a depth of 2 mm [anterior/posterior (AP) +1.0 mm, medial/lateral (ML) +1.7 mm, dorsal/ventral (DV) −3.2 mm].

In vivo analysis of tumour-tropism of human NSCs. HB1.F3.CE cells were labelled with PKH26, red fluorescent dye for staining of cell membranes, according to the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA). Seven days after tumour cell injection, 1×10⁶ PKH26-labelled HB1.F3.CE cells in 5 μl HBSS were stereotactically implanted into the contralateral hemisphere (AP +1.0 mm, ML -1.7 mm, DV −3.2 mm).

Forty-eight hours after the HB1.F3.CE cell injection, the animals were anaesthetized, and perfusion fixation was performed by transcardially perfusing ice-cold phosphate-buffered saline (PBS), followed by ice-cold 4% paraformaldehyde in PBS. The brains of the animals were immediately removed, post-fixed in the same fixative overnight, and processed for frozen embedding using standard experimental procedures. The frozen blocks were cut into 8-μm coronal sections. Nuclei were counterstained with 0.001% 4’,6-diamidino-2-phenylindole (DAPI) in PBS.

In vivo therapeutic efficacy of HB1.F3.CE cells. Seven days after Raji cell injection, animals were subjected to contralateral injection (AP +1.0 mm, ML −1.7 mm, DV −3.2 mm) of 5 μl HBSS (control and irinotecan, each group n=5) or 1×10⁵ HB1.F3.CE cells in 5 μl HBSS (each group n=5). Forty-eight hours after the NSC injection, the control and the HB1.F3.CE groups received intravenous injections of normal saline (100 μl), while the other two groups received irinotecan (3.75 mg/kg in 100 μl normal saline) every day for five days. Five days after the last irinotecan injection, brains were removed and cut into 4-6 mm thick coronal slices. The brain slices were fixed in 10% formalin/PBS, embedded in paraffin, sectioned into 4-μm sections, and stained with haematoxylin and eosin (H&E). Tumour volumes were measured in the sections with the largest width using the formula, largest width²×largest length×0.5. For survival analysis, seven mice of each group were treated as described above. Weight loss >25% of maximal body weight was regarded as mortality.

Statistical analysis. Statistical comparisons were performed using the Student’s t-test. Survival analysis was performed using the Kaplan–Meier and log-rank tests. p-Values less than 0.05 were considered statistically significant.

Results

HB1.F3.CE—a human NSC line expressing CE. HB1.F3 and HB1.F3.CE cells of the same passage and stock as in our previous research (13) were utilized in this study. We reconfirmed the detection of the CE transcript in HB1.F3.CE cells but not in HB1.F3 cells (data not shown). The sensitivity of HB1.F3.CE cells to irinotecan was compared to that of parental HB1.F3 and Raji human Burkitt lymphoma cells. While 1 μg/ml irinotecan had no significant effect on the survival of HB1.F3 or Raji cells (Figure 1A), the survival of HB1.F3.CE cells was dramatically reduced by 48-h exposure to irinotecan at a concentration <1 μg/ml.

In vitro bystander effects in HB1.F3.CE cells. In vitro bystander effects in HB1.F3.CE cells were determined using a co-culture system to compare between the conditioned medium from HB1.F3.CE cells and that from parental
Figure 1. Sensitivity of HB1.F3.CE cells to irinotecan. A: HB1.F3 and HB1.F3.CE cells (1×10^4) were cultured under different concentrations of irinotecan at 37°C for 48 h. Relative survival was determined and compared. *p<0.05 vs. Raji cells; #p<0.05 vs. HB1.F3 cells. B: The bystander effect of carboxyl esterase (CE) produced by HB1.F3.CE cells was confirmed using a co-culture system. Raji cells co-cultured with HB1.F3 or HB1.F3.CE cells were seeded in 96-well plates (total 1×10^4 cells per well; Raji cells:HB1.F3 or HB1.F3.CE cells = 75:25, 50:50, 25:75, or 0:100). After 48 h in co-culture, cells were treated with 1.0 μg/ml irinotecan for 48 h and cell survival was determined (each group, n=3). *p<0.05. C: Raji cells (1×10^4) were seeded in 96-well plates. After 24 h, 50%, 25%, 12.5%, or 0% of conditioned media from HB1.F3 and HB1.F3.CE cells were added for 48 h. The relative survival of Raji cells was determined (each group, n=3). *p<0.05.
HB1.F3 cells. No toxic effects of irinotecan (1 μg/ml) were observed when Raji cells were co-cultured with parental HB1.F3 cells (Figure 1B). In contrast, the survival of Raji cells co-cultured with HB1.F3.CE cells (Raji cells:HB1.F3.CE cells=75:25, 50:50, or 25:75) was significantly reduced by 48-h exposure to 1 μg/ml irinotecan (p<0.05, Figure 1B). In the absence of irinotecan, the survival of Raji cells co-cultured with HB1.F3 or HB1.F3.CE cells was not affected (data not shown).

To rule out the direct effects of HB1.F3.CE and HB1.F3 cells on the survival of Raji tumour cells, conditioned media were prepared by filtering media from HB1.F3.CE or HB1.F3 cells treated with 1 μg/ml irinotecan for 48 h. Raji cells were then treated with either 12.5%, 25%, or 50% of the conditioned medium for 48 h. The survival of Raji cells was significantly inhibited by HB1.F3.CE-conditioned media compared to HB1.F3-conditioned media (p<0.05, Figure 1C). These results indicate that HB1.F3.CE cells activated sufficient amounts of irinotecan to effectively kill Raji cells in vitro.

In vivo tumour-tropic property of HB1.F3.CE cells. We recently established an animal model of CNS lymphoma by stereotactically injecting Raji cells into the brains of immunocompromised mice, producing huge infiltrative intracerebral tumours several weeks after the injection. Specific pathological characteristics of CNS lymphoma, such as extensive leptomeningeal seeding, have been described (16). Seven days after stereotactic implantation of 5×10⁵ Raji cells into immune-deficient mouse brains, 1×10⁵ PKH26-labelled HB1.F3.CE cells were injected stereotactically into the contralateral brain hemispheres. The distribution of HB1.F3.CE cells was determined two days after HB1.F3.CE cell injection (Figure 2A). PKH26-positive HB1.F3.CE cells...
were identified in the tumour core, while few HB1.F3.CE cells were found in the injected hemisphere or in brain areas without tumour (Figure 2B), confirming the in vivo tumour-tropic activity of HB1.F3.CE cells.

In vivo therapeutic efficacy of HB1.F3.CE cells. The in vivo therapeutic efficacy of HB1.F3.CE cells against CNS lymphoma was independently assayed by tumour volume measurement and survival analysis. Seven days after stereotactic implantation of $5\times10^5$ Raji cells into the brains of immune-deficient mice, animals were subjected to contralateral injection of $5 \mu l$ HBSS or $1\times10^5$ HB1.F3.CE cells in $5 \mu l$ HBSS. Forty-eight hours after the NSC injection, the control and HB1.F3.CE groups received intravenous injections of normal saline, while the other two groups received intravenous injections of irinotecan ($3.75 \text{ mg/kg}$) every day for five days. When tumour volumes were determined in the brain tissue at five days after the last irinotecan injection (Figure 3A), tumour volumes in the brains of the HB1.F3.CE/irinotecan group were significantly reduced (Figure 3B and C) compared with those in the control ($p=0.008$), HB1.F3.CE ($p=0.03$) and irinotecan group ($p=0.01$). The tumour volumes in the HB1.F3.CE/irinotecan group were reduced by up to 60% compared to those in the control group.

The therapeutic effects of HB1.F3.CE cells against CNS lymphoma were confirmed by survival analysis (Figure 4A), using the same treatment schedule and groups as those utilized for the tumour volume analysis (n=7 per each group). The HB1.F3.CE/irinotecan group had a significantly prolonged survival period (Figure 4B, mean±SD=21.1±1.3 days, $p=0.0001$) compared with the other groups (control=16.2±2.7 days, HB1.F3.CE group=16.6±2.3 days, irinotecan group=17.6±0.5 days), confirming the in vivo therapeutic efficacy of HB1.F3.CE cells against CNS lymphoma.
Discussion

CNS lymphoma is an aggressive malignancy arising exclusively in the CNS. This type of lymphoma represents 4% of intracranial neoplasms and 4-6% of all extranodal lymphomas (17). Some registry studies suggest that the incidence of CNS lymphoma in immunocompetent patients is progressively increasing (18). Despite advances in chemotherapeutic modalities, a high relapse rate (approximately 70%) and leptomeningeal seeding (7-42%) after standard chemotherapies, including intrathecal methotrexate, are major concerns for this disease (19, 20). The lack of benefit of cyclophosphamide, doxorubicin, vincristine, prednisone/prednisolone (CHOP) therapy, which is a traditional treatment regimen for lymphomas, has also been reported (21). It has been suggested that relapse of CNS lymphoma even after high-dose methotrexate in addition to standard chemotherapy is mainly due to restoration of the blood–brain barrier, which impairs the penetration of chemotherapeutic agents into the CNS. Even though other drugs, such as cytarabine and rituximab, have been suggested for primary or salvage therapy, the treatment efficacy and side-effects need to be further elucidated (22). A more tumour-specific targeted approach in CNS lymphoma may, therefore, provide a much needed advantage over the standard systemic drugs.

Recent studies have found that immortalized and genetically-engineered NSCs display tumor-tropic activities that can be exploited for tumour-specific gene therapy for the treatment of brain tumours (23, 24). CE-producing NSCs (HB1.F3.CE), which selectively convert the pro-drug irinotecan to SN-38, have been shown to migrate towards tumour cells and have therapeutic effects on the tumours (9,

Figure 4. Survival analysis to investigate the effects of HB1.F3.CE and irinotecan. A: Timeline for the survival analysis, i.c., Intracranial; i.v., intravenous; HBSS, Hank's balanced salt solution. B: The Kaplan–Meier graph shows a significantly prolonged survival period in the HB1.F3.CE/irinotecan (IRI) group compared to the other groups (n=7 per each group), *p<0.05.
10, 13, 25, 26). Irinotecan itself has excellent CNS penetration. However, in CNS lymphoma, a much higher systemic dose is required to achieve a therapeutic concentration in the brain, leading to a concomitant increase in undesirable side-effects, such as severe diarrhea, neutropenia, and hepatotoxicity (27-29). Selective targeting by NSCs is therefore a promising approach to overcome these limitations, since increases in the intracranial or intratumoural concentration of irinotecan and SN-38 for treatment of CNS lymphoma can be achieved even using a relatively lower systemic dose of irinotecan.

Our data demonstrate that the intra-cerebral implantation of Raji cells into immune- compromised mouse brains provides an animal model that closely mimics the pathological characteristics of human CNS lymphoma (30). In this study, we tested the hypothesis that NSCs carrying a suicide gene have therapeutic effects in CNS lymphoma. The NSCs carrying the suicide gene (HB1.F3.CE) migrated to the contralateral tumour site, and a significant reduction in the tumour volume and increase in survival outcome was observed in the xenograft animal model. However, it is important to point out that our xenograft model using immune-deficient mice has limitations in representing real CNS lymphoma. For example, it limits the opportunity to examine the key role of the immune system. In addition, clinical application of this NSC therapy will require further testing in terms of safety for use of NSCs, the route of administration, the required cell number, and possible immune-rejection (31, 32). The human NSC line used in this study was immortalized by v-myc (33, 34) and the possibility of tumour formation by the v-myc oncogene in the HB1.F3 human NSCs cannot be excluded.

To the best of our knowledge, no studies regarding CNS lymphoma-targeting therapies using an NSC-based approach have been published thus far. Our results demonstrate for the first time that brain transplantation of human NSCs encoding the suicide enzyme CE, combined with systemic administration of irinotecan, may be an effective treatment regimen for CNS lymphoma.

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References


