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Successful lipofectin mediated transduction of a pIRES-fl-tk gene in human breast carcinoma: effective inducing the expansion as well as increasing the activity of dendritic cells

Research Article

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Abbreviations: dendritic cell, (DC); of antigen-presenting cell, (APC); granulocyte-macrophage colony-stimulating factor, (GM-CSF); stem cell factor, (SCF); FLT-3 ligand, (FL); bone marrow, (BM); cord blood, (CB); peripheral blood, (PB); herpes simplex virus thymidine kinase, (HSV-tk); ganciclovir, (GCV); counts per minute, (cpm); triplicate wells±standard deviation, (SD); internal ribosome site, (IRES); encephalomyocarditis virus, (ECMV)

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Summary

A novel approach to the treatment of cancer is based on combining the gene therapy and DC (dendritic cell) immunotherapy. In this study, DC were induced and expanded from cord blood and PBMC (peripheral blood mononuclear cell). The two kinds of DC exhibited the capacity to stimulate the proliferation of allogeneic T lymphocytes. We further constructed a recombinant vector carrying herpes simplex virus thymidine kinase gene (tk), internal ribosome entry site (IRES), Flt-3 ligand gene (fl) and transfected it into the human breast carcinoma cell line MCF-7. The supernatant of MCF-7/FL-IRES-TK cells could stimulate the proliferation of $CD34^+$ cells from cord blood and increase the rate of $CD1a^+$ cells (DC) in the presence of GM-CSF, TNF-·.In vitro experiment demonstrated dose-dependent cell killing by a transduction of the HSV-tk gene followed by GCV (ganciclovir) treatment. We found that GCV treatment of HSV-tk-fl expressing cell line induced apoptosis. DC contacting with apoptotic cells can further stimulate the proliferation of T cells. These experiments suggested HSV-tk/GCV system is not only a tumor vaccine, but also a method to enhance the DC function. Taken together, our findings therefore indicated that MCF-7/FL-IRES-TK could provide apoptotic bodies to DC as well as secrete FL to generation DC.

I. Introduction

Dendritic cells (DCs) were first described by Steinman and Cohn in 1973. DCs are one type of antigen-presenting cell (APC), processing antigens into peptide fragments that bind to products of the MHC. The peptide-MHC complexes are then recognized by the T cell receptor for antigen. DCs can be identified by their characteristic morphology, the lack of B, T, NK, and the monocyte markers, the expression of high levels of MHC class I and II molecules, co-stimulatory and adhesion molecules, as well as their high stimulatory capacity in MLR (Schuler and Steinman, 1997). One of the most important goals of DC research is the development of DC-based strategies for enhancing immune responses against tumors. Studies on DCs have been greatly hampered by difficulties in preparing the cells in sufficient numbers. The possibility of generating large numbers of autologous dendritic cells that can be used in the manipulation of the immune response against cancer and infections has tremendously boosted DC researches. Recently, the methods for generation large numbers of DCs are being developed (Bykovskaja et al, 1998). DC precursors have been isolated within the $CD34^+$ cell fraction in bone marrow (BM), cord blood (CB), and peripheral blood (PB). The in vitro development of fully functional mature DCs from $CD34^+$ cells is strictly dependent upon stimulation with cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), stem cell factor (SCF) and FLT-3 ligand (FL) (Michelle et al, 1998). After successfully inducing DC, modulation of DC function may

determine the efficacy of anti-tumor immunity, so exploring new methods to enhance the DC capacity is desirable (Paglia and Guzman, 1998).

An emerging strategy for cancer gene therapy involves the transfer of the herpes simplex virus thymidine kinase (HSV-tk) gene into tumor cells, rendering them susceptible to the cytotoxic effects of ganciclovir (GCV) (Eijun et al, 1998). Treatment with GCV also leads to the death of cells that do not express the HSV-tk gene that are in the immediate vicinity of HSV-tk-expressing cells. This has been called the "bystander effect". The bystander effect greatly enhance the efficacy of HSV-tk-mediated cytotoxicity (Denning, 1997). Apoptosis has been suggested as a mechanism by which bystander cell death is mediated (Hamel et al, 1996). Recent evidence indicate that DCs in contact with apoptosis cells can further increase the capabilities of presenting antigens or stimulating T cells (Albert et al, 1998). Therefore, development of novel approaches for DC are indispensable, combining with tk gene therapy may be a good candidate.

Flt-3 ligand (FL) is a recently identified cytokine having a central role in the proliferation, survival and differentiation of early murine and human hematopoietic precursor/stem cells, including those of DC progenitors in the peripheral blood (Shurin et al, 1998). FL is a weak growth stimulator alone, but it acts synergistically in vitro with a number of other hematopoietic growth factors such as IL-3, IL-6, IL-11, IL-12, SCF and GM-CSF. Many studies suggested that FL significantly increased the yield of functionally DCs, and this may serve as a basis for the evaluation of FL as a useful cytokine to increase DC yield for DC-based immunotherapy for cancer (Peron et al, 1997). So, in our study, we transfered fl gene into tumor cells and researched its role on the generation of DC.

Taken together, an ideal antitumor strategy would contain a source of tumor antigen, activated professional antigen presenting cells (APC) and a immunopotentiating cytokine (Sharma et al, 1997). So the multi-component vaccine by using DC combining with tk and fl gene therapy would be a good anti-tumor approach. In this study we reported that: (1) The percentage of $CD1a^+$ cells depended on the cytokine combinations and the dosage. DC from cord blood $CD34^+$ cells and PBMC depleting T and B cells were similar in stimulating allogeneic T lymphocytes proliferation. (2) MCF-7/FL-IRES-TK cells-derived FL had bio-activity and could enhance the production of DC from $CD34^+$ cells, as well as apoptotic cells induced by suicide gene further increased the ability of DC in stimulating T lymphocytes. Therefore, we have developed a method to evaluate genetic modulation of the autologous tumor to serve as an antigen, cytokine sources and immune enhancer for DC. Utilization of DC combining with suicide gene therapy as multi-component vaccine would be worthy of researching.

II. Materials and methods

A. Cell culture

Human breast epithelial cell line, MCF-7 were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL company USA) containing 10% heat-inactivated fetal bovine serum, 2mML-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and kept at 37ÆC in a humidified atmosphere containing 5% CO_2.

B. Vector plasmid construct and transduction of tumor cell line

A vector plasmid carrying the HSV-tk gene, fl gene was constructed from a vector pIRES1*neo* and a plasmid of tgCMV/Hytk. The pIRES1*neo* contains the internal ribosome site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. fl gene was digested with EcoRV, XbaI and inserted into EcoRV, XbaI site of pIRES1*neo* plasmid. After construction of pIRES-fl vector, HSV-tk gene was separated by agarose gel electrophoresis from tgCMV/Hytk plasmid with BglII and NotI, and then inserted into the relevant site of pIRES-fl. The resulting vector (pIRES-fl-tk) was mixed with lipofectin (GIBCO BRL company USA) and transfected into MCF-7 cell line. After 12h, the medium was replaced with DMEM and the cells were incubated for additional 48h. The cells were then transfer to a 75-cm^2 flask and grown to confluence. The fl /HyHSVtk transduced cells were selected in 800 μg/ml of hygromycin for 10 –15 days.

C. Generation of DC from two sources and flow cytometric analysis

Suspension cultures of cord blood $CD34^+$ cells were initiated with IMDM supplemented with 12.5% fetal calf serum , 12.5% horse serum and antibiotics at an initial density of $5x10^3$ cells /ml. All cultures were maintained at 37ÆC in humidified 5% CO_2 atmosphere for 10-14 d in the presence of optimized concentrations of the following rh-cytokines: GM-CSF (Glaxo, 40ng/ml USA); TNF-α (Genzyme, 50U/ml USA); SCF (Sandos, 100ng/ml USA); FLT-3 ligand (Immunex, 40ng/ml USA). T and B cell-depleted PBMC cultured in the same medium excepting different cytokine combination GM-CSF (40ng/ml) and IL-4 (400-1000U/ml Promega, USA). At weekly intervals, half of the culture medium was replaced by fresh medium and growth factors and the generation of functionally active DCs was assessed by phase-contrast microscopy, immunophenotyping and mixed lymphocyte reactions (MLR). The purification process of $CD1a^+$ cells was incubating anti-DC monoclonal antibodies X-11 (COULTER-IMMUNOTECH USA) with magnetic-beads for 30 min at 4ÆC firstly, and then added it into the medium of $CD1a^+$ cells. After 30 minutes, the column was put into the magnetism for a while, then the adsorbed cells were eluted when far away from magnetism, they were identified by FACS.

In the immunophenotype studies, dual-colour immunofluorescence was performed using the following panel of monoclonal antibodies (MoAbs): phycoerythrin (PE)-conjugated anti-human CD1a (Pharmingen, San Diego, Calif USA); fluorescein isothiocyanate (FITC)-conjugated anti-human HLA-DR (Pharmingen, USA). Negative controls were isotype-matched irrelevant MoAbs. Cells were incubated in the dark for 30 min at 4ÆC in PBS-1% bovine serum albumin (BSA). After washing, cells were resuspended in PBS and analysed as reported above.

The function of two sources-derived DC was detected in T lymphocytes proliferation (the detailed method has been described in literature).

D. The activity of MCF-7/FL-IRES-TK supernatant Fl in $CD34^+$ cells proliferation

$CD34^+$ cells were isolated from cord blood by Magnetic Beads and seeded at density of $1x10^3$/ml into 96 wells plate. Six groups were designed according to the different cytokines combinations. Culturing for 10 days, cytokines were added every 48 hours, and then $CD34^+$ cells proliferation was detected. Experiments were performed in triplicate, and the S.D did not exceed 10%.

E. The role of FL in the expansion of DC

$CD34^+$ cells were isolated from cord blood and seeded at density of $5x10^3$/ml into 12 wells plate. Cells cultured in the same medium as described previously, and then cytokines were added according to the following cytokine combination: (1)GM-CSF+TNF-α, (2)GM-CSF+TNF-α+SCF, (3)GM-CSF+TNF-α+FL, (4)GM-CSF+TNF-α+SCF+FL and (5)GM-CSF+TNF-α+supernatant. At weekly intervals, medium and cytokines were replaced and cultured at 37ÆC in a humidified atmosphere containing 5% CO_2 till 8-12 day. Harvesting cells were incubated for 30 min at 4ÆC in PBS, 2% FCS, with the PE-conjugated CD1a mAb (1:100 final) or with control irrelevant isotype-matched mAb. After washing, cells were resuspended in PBS and analyzed with a FACScan.

F. In vitro cytotoxic assays of MCF-7/FL-IRES-TK cells to GCV (Nishihara et al, 1997)

The MCF-7/FL-IRES-TK cells were seeded at density of $1x10^3$ cells/well in 96-well microtiter plates. One day later, the cells were treated with various concentrations of GCV(10^{-4}-10^4 μg/ml) in 200 μl fresh medium. Medium was replaced with the same medium on the next day. The cell survival was detected by MTT method. MTT 20 μl (5mg/ml) was added into the medium of every well on day 3, after incubated at 37ÆC for 4h, the plates were centrifuged for 10 min at 1000 μg and the medium was discarded. To dissolve the crystal, 200 μl of DMSO and 25 μl of the glycin buffer were added to the wells. The OD was read at 570 nm. And then survival ratios were expressed as percentages relative to untreated control values.

G. Detection of radiosensitization and apoptotic phenomena of MCF-7/FL-IRES-TK cells

The MCF-7/FL-IRES-TK cells were divided into two groups: GCV (+) and GCV(-).Cells were seeded into a 10cm culture plate at low density (1000 cells), GCV (0.1μg/ml) was added into the GCV(+) groups for 48 hours. Every group was irradiated by $^{60}C_O$ under 5Gy, 10Gy, 15Gy and 20Gy respectively. Two weeks later, the survival cell clones were counted (larger than 5 mm) and the survival rate-effect curve was drawn according to the cell survival fraction, from which the SER (sensitizer enhancement ratio) was derived.

The MCF-7/FL-IRES-TK cells were plated at a density of $3x10^4$ cells/well in six-well culture dishes and were incubated in the absence or presence of the IC_{50} of GCV, and one of the GCV treating groups assisting with $^{60}C_O$ radiation (20Gy),one group only with $^{60}C_O$ radiation (20Gy) without GCV. Four days later, apoptosis phenomena in the cells was detected . DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (FACS 400 BDIS USA). Numbers of cells with sub-G_1 DNA content were determined with a program. Morphological changes of the cells were observed under the electron- microscope.

H. Activation of allogeneic T-cell proliferation

The responder cell fraction (T cells) were isolated from peripheral blood monocyte by using CD2 magnetic-beads monoclonal antibodies. The stimulator cell fractions (DCs) primarily were cocultured with tumor cells from different managing groups (described in apoptosis detection) for 8h, and then DCs were irradiated (3000cGy). 10^5 T lymphocyte were cultured at 37ÆC in 5%CO_2 in 96-well plates with triplicate graduated numbers of sorted DCs. Wells were pulsed for 16 hours with 0.5 μCi tritiated thymidine immediately before harvest at 4 days. Cells were harvested onto filter paper and thymidine incorporation was measured with a liquid scintillation counter. Data are expressed as mean counts per minute (cpm) of triplicate wells±standard deviation (SD).

III. Results

A. Functional in vitro evaluation of transduced genes expressed by the pIRES1neo vector in MCF-7 cells

pIRES1neo vector has the advantage of coordinate expression of two genes (**Figure 1**). To determine if both the cytokine and drug sensitivity genes were functional in transduced cells, we assessed the bio-activity of tumor-derived FL and GCV sensitivity of tumor cells *in vitro*. The results demonstrated that fl and tk genes have been successful transduced in MCF-7 cells by PCR and Southern blot (data not shown), and FL secreted in the medium was determined by human FL-specific ELISA and Western blot. To determine if tumor-derived FL was

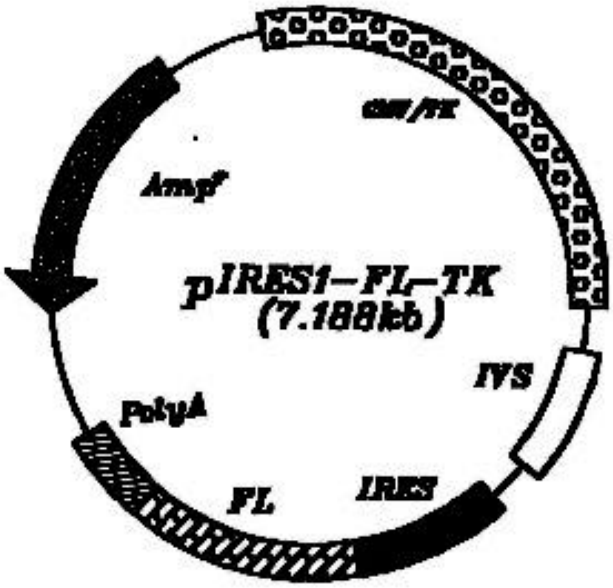

Figure 1. Diagram of the human fl/Hytk vector. The IRES sequence is from the encephalomyocarditis virus (EMCV), which permits the translation of two open reading frames from one messenger RNA. Hytk is a fusion gene which hygromycin phosphotransferase gene fusing in-frame to the herpes simplex thymidine kinase gene. The fl-IRES-tk vector was selected in hygromycin medium.

bio-active, allogeneic $CD34^+$ cells were cultured in cell free supernatants from fl/HyHSVtk transduced tumor cells. Compared to supernatants from parental tumor cells, fl/HyHSVtk transduced tumor cell supernatants significantly enhanced $CD34^+$ cells proliferation combining with GM-CSF. The vector used in this study contains the HSVtk gene, it was a fusion gene and could been selected by hygromycin. Cells expressing HSVtk are sensitized to the toxic effects of GCV. Low concentration of GCV (0.1ug/ml), which alone showed little cytotoxicity, could enhance radiation-induced cytotoxicity (radiosensitization) and increased the rate of apoptosis.

B. Generation of DC from different sources and comparison of their ability to stimulate T lymphocytes

As reported previously and confirmed by us, cord blood and peripheral blood can both effectively give rise to phenotypic DC when cultured in different cytokines combination (Thomas and Lipsky, 1996). Cultures of $CD34^+$cells with the combination of GM-CSF+TNF-α+SCF+FL yielded 27.18%±1.56% $CD1a^+$cells, much higher than that in the group of GM-CSF alone (0.65%±0.38%). FL was important for DC expansion from $CD34^+$cells, the percentage of $CD1a^+$ cells increased to 27.18%±1.56% from 12.3%±0.33% ($p<0.01$) when FL was added into the medium of GM-CSF+TNF-α+SCF group. After purification, the rate of $CD1a^+$ cells could reach to 67.6%±2.1% (the purification process was detailed described in Materials and Methods). The combination of GM-CSF+ high dose IL-4 (1000U/ml) was the most potential for expanding $CD1a^+$cells (21.8%±0.32%) from peripheral blood, the control experiment showed that IL-4(400U/ml) combined with GM-CSF only give birth to 3.30%±0.16% $CD1a^+$ cells **.** In the experiment we found that IL-4 500-1000U/ml was essential for blocking macrophage colony formation. Two kinds of DC expressed high level HLA-DR (93.7%±1.0%、94.7%±1.6%) and were similar in cell morphology , and also exhibited the capacity to stimulate the proliferation of allogeneic T lymphocytes. So, both of the cord blood and peripheral blood were useful sources for the generation of large numbers of plentiful typical DC, and could be adopted for the application according to different purposes.

C. MCF-7/FL-IRES-TK cells derived-FL enhances the $CD34^+$ proliferation and DCs production

Following lipofectin-mediated fl/HyHSVtk transduction of MCF-7 breast carcinoma cells and selection in hygromycin, FL secreted in the medium was determined by human FL-specific ELISA and Western blot. MCF-7/FL-IRES-TK clones produced 15.7ng of FL/ml/10^5cells per 4 d.

To evaluate the bio-activity of tumor-derived FL, $CD34^+$cells isolated from cord blood were cultured in cell free supernatants from transduced tumor cells, and low concentration of GM-CSF was added into the medium at the same time. Six groups were designed as follows: (1)GM-CSF(40ng/ml), (2)GM-CSF+standard FL, (3)GM-CSF+MCF-7/FL-IRES-TK cell supernatants, (4)GM-CSF+MCF-7/TK cell supernatants, (5)GM-CSF+MCF-7/FL cell supernatants and (6)GM-CSF+MCF-7 cell supernatants. Compared to supernatants from parental tumor cells, fl-HyHSVtk or fl gene transduced tumor cell supernatants showed higher biological activity than tk gene transduced tumor cell (**Figure 2**).

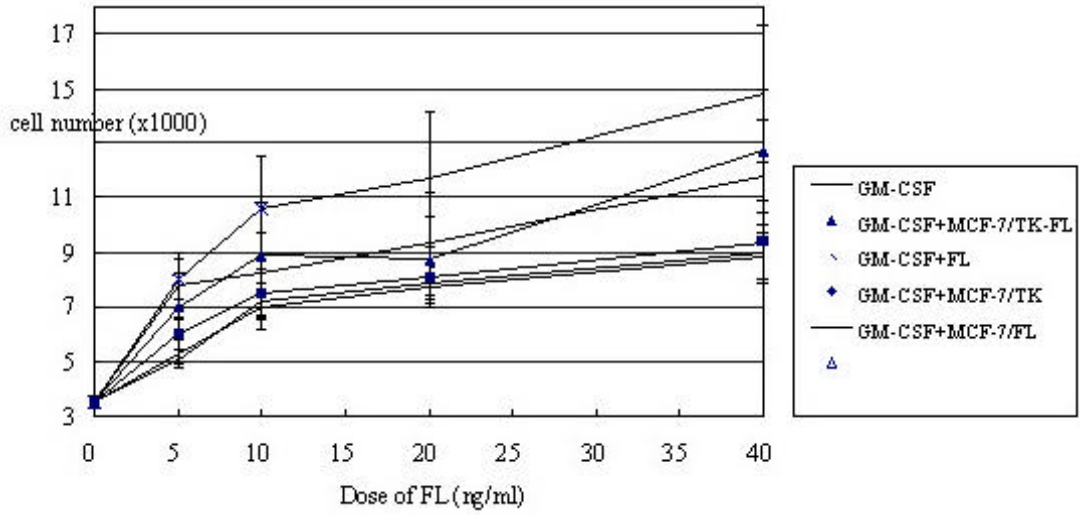

Figure 2. The activity of FL in the supernatant was determined by proliferation of $CD34^+$ cells. GM-CSF 40ng/ml was added into the culture medium. Cytokines were added every 48 hours, six groups were designed as follows: (1)GM-CSF, (2)GM-CSF+standard FL, (3)GM-CSF+MCF-7/FL-IRES-TK cell supernatants, (4)GM-CSF+MCF-7/TK cell supernatants, (5)GM-CSF+MCF-7/FL cell supernatants, and (6)GM-CSF+MCF-7 cell supernatants. Culturing for 10 days, then $CD34^+$ cells proliferation was detected. One-way ANOVA analysis was performed in this experiment. All results represent the means of triplicates, with standard deviations shown by error bars. Similar results were obtained from at least two independent experiments.

To further quest for the role of tumor-derived FL in generation of DC, we replaced standard FL with MCF-7/FL-IRES-TK cell supernatants in cytokine combinations and found that the rate of $CD1a^+$cells decreased to 8.17%±0.32% from 18.0%±0.11%, but it was higher than that in the control group of GM-CSF+TNF-α (4.12%±0.23%, $p<0.01$) (**Figure 3**). The result suggested that MCF-7FL-IRES-TK tumor-derived FL could enhance the DCs production.

D. The sensitivity of MCF-7/FL-IRES-TK cells to GCV

The GCV sensitivity of the genetically modified cells was assessed by the MTT assay. The cells expressing HSV-tk were first assayed for sensitivity to GCV (concentration from 10^{-4}-10^4 μg/ml) by determining the IC_{50} (**Figure 4**). There was no significant difference in cell growth between the parental and transduced cells. In dose-dependent experiment, the IC_{50} shifted from 500 μg/ml in MCF-7 cells to 0.5 μg/ml in MCF-7/FL-IRES-TK cells or 0.46 μg/ml in MCF-7/TK cells. The result showed that MCF-7/FL-IRES-TK cells and MCF-7/TK cells have successfully expressed the quality of tk and could be used in the following studies.

E. Detection of the radiosensitization and apoptotic phenomenon of MCF-7/FL-IRES-TK cells

GCV (0.1 μg/ml) was added into the culture plate for 48 hours, then cells were irradiated by different dosage. The results showed that, GCV (+) group was quite different from GCV (-) group after irradiation. SER (sensitizer enhancement ratio) calculated from the survival rate-effect curve was 3.48, which demonstrated that pIRES-tk-fl transduced MCF-7 could increased the radiosensitization of tumor cells (**Figure 5**).

We then evaluated whether apoptosis could be induced by GCV.

MCF-7/FL-IRES-TK cells were divided into four groups: (1) cells were incubated without any treatment, (2) cells were incubated in the presence of the IC_{50} of GCV, (3) the second group assisting with ^{60}CO(20Gy) radiation and (4) cells with ^{60}CO(20Gy) radiation without GCV. As shown in data, GCV treatment significantly increased the percentage of apoptotic cells in MCF-7/FL-IRES-TK (3.3%±0.6% to 19.3±1.0%; $P<0.01$), suggesting that the cytotoxicity induced by HSV-tk/GCV is in part mediated by an apoptotic mechanism. ^{60}CO(20Gy) radiation further increased the rate of apoptosis in GCV treatment (19.3±1.0% to 31.3±1.2%; $P<0.01$), the group only with ^{60}CO(20Gy) radiation without GCV was 6.6%±1.2% (**Figure 6**), reminding us that radiation combining with HSV-tk/GCV system would be a better candidate to induce apoptosis.

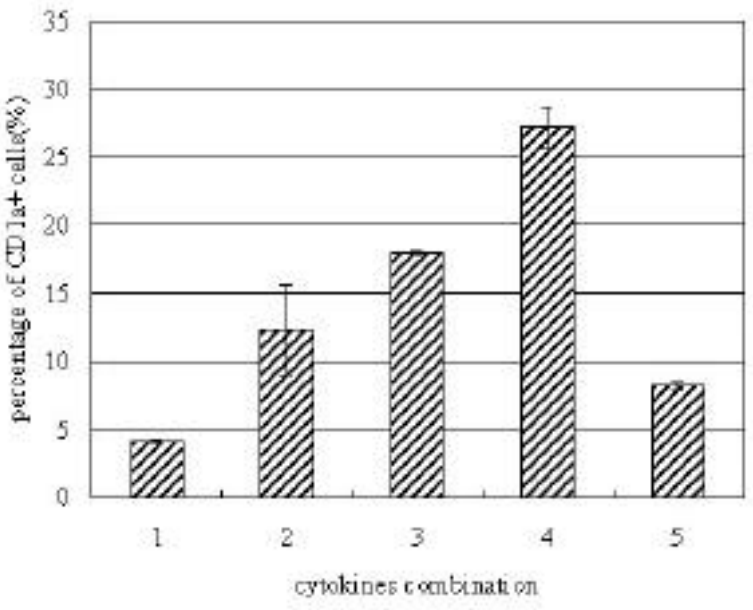

Figure 3. The percentage of $CD1a^+$ cells inducing from cord blood $CD34^+$ cells by different combination of cytokines. $CD34^+$ cells were isolated from cord blood and seeded at density of $5μ10^3$/ml into 12 wells plate. Cytokines were added according to the following combination: (1) GM-CSF+TNF-α, (2) GM-CSF+TNF-α+SCF, (3) GM-CSF+TNF-α+FL, (4)GM-CSF+TNF-α+SCF+FL,and (5) GM-CSF+TNF-α +supernatant. Expansion cells were cultured in medium with cytokines for 8-12d and then harvested to perform flow cytometry analysis. GM-CSF: 40ng/ml; TNF-α: 50U/ml; SCF: 100ng/ml; FL: 40ng/ml. Experiments were performed in triplicate , with standard deviations shown by error bars.

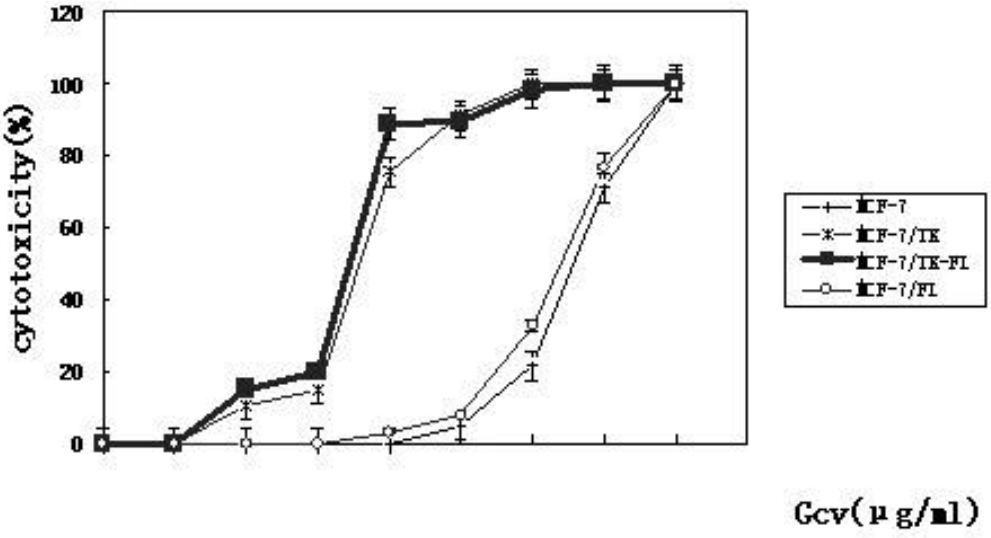

Figure 4. Dose-dependence of in vitro cytotoxicity of GCV in MCF-7/FL-IRES-TK cells. The four groups cells, (1)MCF-7/FL-IRES-TK, (2)MCF-7/TK, (3)MCF-7/FL and (4)MCF-7 cells, were seeded at density of $1\mu10^3$ cells/well in 96-well microtiter plates. One day later, the cells were treated with various concentrations of GCV(10^{-4}-10^4Ìg/ml) in 200Ìl fresh medium. Medium was replaced with the same medium on the next day. The cells were incubated with various doses of GCV for the indicated periods (4 days), followed by cell survival quantitation as described in Materials and Methods. All results represent the means of triplicates, with standard deviations shown by error bars and is expressed as a percentage relative to the value in untreated cells. Similar results were obtained from at least two independent experiments.

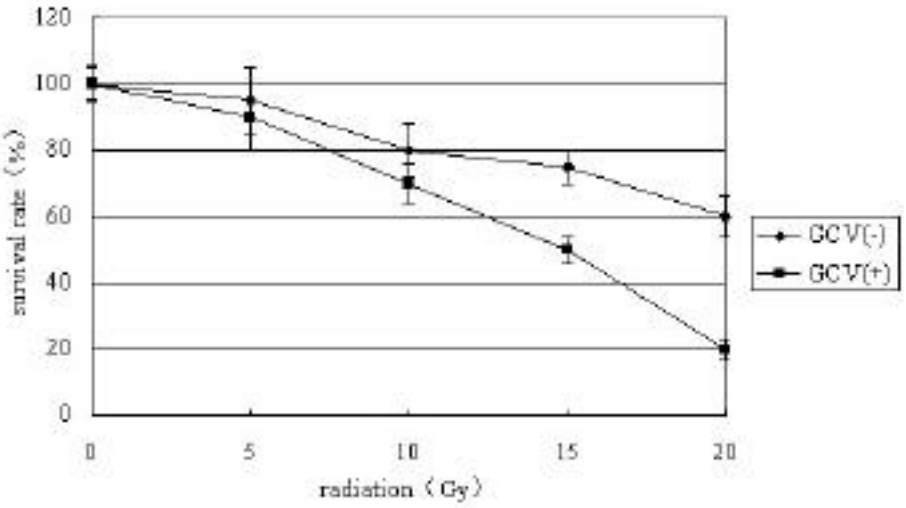

Figure 5. Survival cell fraction-effect curve. GCV 0.1μg/ml was added into the GCV(+) groups for 48 hours. Two weeks later, the survival-effect curve was drawn according to the survival cell clone and the dosage of radiation. SER (sensitizer enhancement ratio) is 3.48 calculating from the computer. It demonstrated that pIRES-tk-fl transduced MCF-7 could increased the radiosensitization of tumor cells. Results are the mean±SD of triplicate samples.

F. DC in contact with apoptotic cells could efficiently stimulate allogeneic T lymphocytes proliferation

As described previously, apoptosis could be induced in MCF-7/FL-IRES-TK cells by GCV treatment or assisting with ^{60}CO(20Gy) radiation. After DC came into contact with apoptotic tumor cells in different groups, we determined the capacity of DC to stimulate allogeneic T lymphocyte. As shown in **Figure 7**, DC contacting with more apoptotic tumor cells were potent stimulators of allogeneic T lymphocyte, mean while DC meeting fewer apoptotic tumor cells had poor immuno-stimulatory activity ($P<0.01$). Thus, apoptosis significantly increased the stimulatory capacity of DC in T lymphocytes proliferation, indicating it is an effective method for enhancing DC function.

IV. Discussion

Dendritic cells (DC) are considered the most effective antigen-presenting cells (APC) for primary immune responses (Steinman and Anchereau, 1998). Since presentation of antigen to the immune system by appropriate professional APC is critical to elicit a strong immune reaction and DCs seem to be quantitatively and functionally defective in the tumor host, DCs hold great promise to improve cancer vaccines (Girolomoni and Castagnoli, 1997). In order to stimulate an anti-tumor immune response, the use of transferring gene to tumor

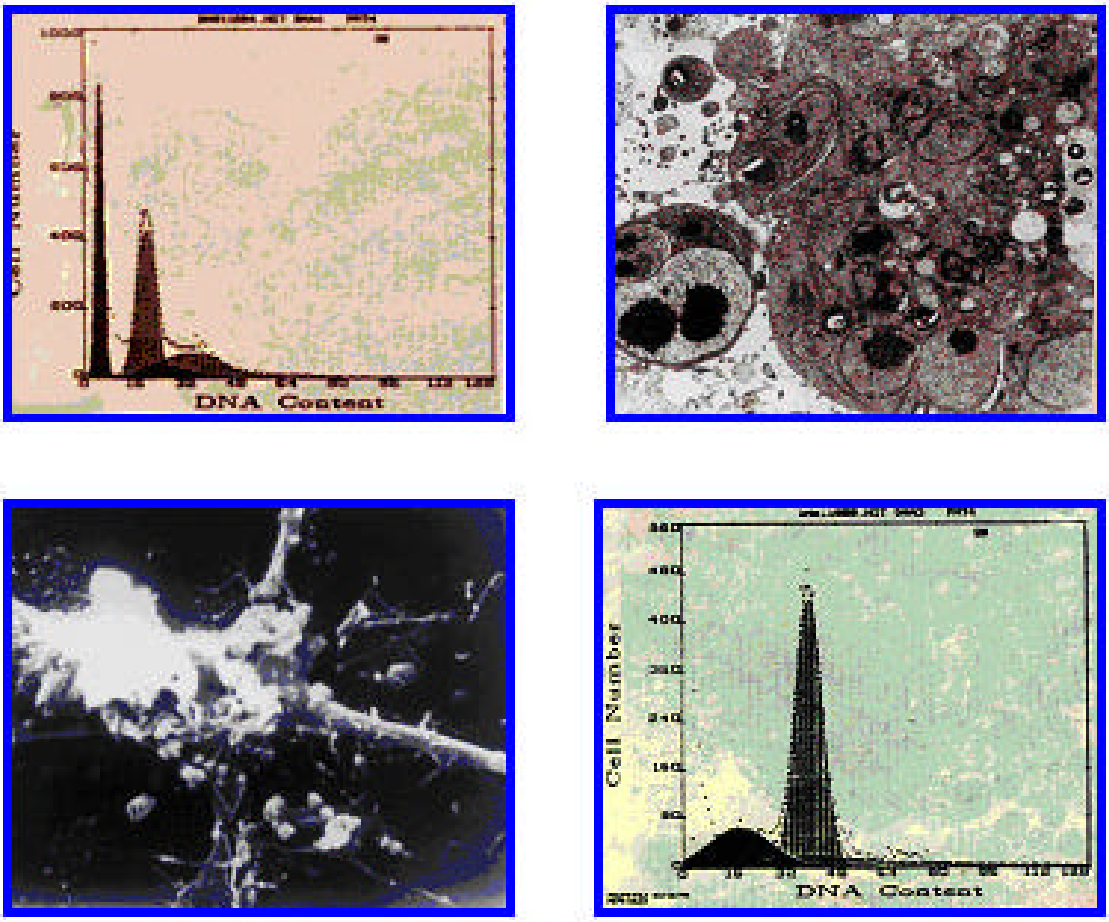

Figure 6. Apoptotic cell death induced by GCV in MCF-7/FL-IRES-TK cells. The MCF-7/FL-IRES-TK cells were plated at a density of $3\mu10^4$ cells/well in six-well culture dishes. Four groups were designed (1)GCV(-), (2)GCV(+) means presenting of the IC_{50} of GCV, (3)GCV(+) group assisting with $^{60}C_O$ radiation(20Gy) and (4)only with $^{60}C_O$ radiation (20Gy). Four days later, apoptosis phenomena in the cells was detected .DNA content was assessed by FACScan. Numbers of cells with sub-G_1 DNA content were determined with a program.

A: GCV(+) group, the percentage of apoptosis cells in MCF-7/FL-IRES-TK is 19.3±1.0% ($P<0.01$).

B:GCV(+) group assisting with $^{60}C_O$ radiation(20Gy) , radiation further increased the rate of apoptosis in GCV treatment (31.3±1.2%; $P<0.01$).

Morphological changes of the cells were observed under the electron- microscope.1:Apoptosis phenomena in GCV treating group under transmission eletron microscope (10000μ). 2: Apoptotic bodies under TEM (8000μ).

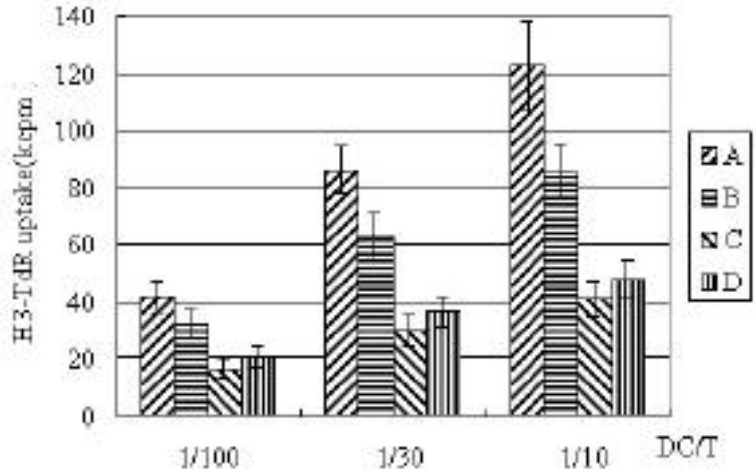

Figure 7. The stimulatory capacity of DCs contacting with apoptotic bodies in proliferation of T lymphocytes. DCs firstly contacted with different treating tumor groups, then DCs were separated to stimulate T lymphocytes. Responder cells (T lymphocytes) were $1\mu10^5$/well, stimulator cells (DCs)were in different numbers $1\mu10^3$, $3\mu10^3$, $1\mu10^4$/well (DC:T 1:100,1:30,1:10).[3][H]TdR was added into medium 16h before ending the experiment. A,B,C,D represented DCs contacting different treating tumor groups. One-way ANOVA analysis was adopted in this experiment. All results represent the means of triplicates, with standard deviations shown by error bars.

cells emerged. To date, this has been successfully achieved in animal models using a variety of strategies, including transfer of MHC, co-stimulatory, cytokine, chemokine or other immune stimulatory genes (Tuting et al, 1997). One of the most effective ways to stimulate anti-tumor immunity has been to express genes that promote the cross priming of host professional antigen-presenting cells (APC) by enhancing either direct release of tumor antigens from tumor cells in vivo or APC-recruitment and activation. HSVtk/GCV system-mediated tumor killing can occur via apoptotic mechanism, and apoptotic tumor cells can provided tumor antigens to APC, so combining the two ways would be a novel approach for tumor therapy. Cytokine gene therapy was in common use to generation of anti-tumor immunity, and FL had many advantages in DC-based therapy, so it was a good candidate (Shurin et al, 1997). In this study, the effects of multi-component vaccine by using DC combined with suicide gene tk and cytokine gene fl was investigated.

It is important to expansion sufficiently large numbers of functional DC for our study. Many investigators have testified that DC can be generated in vitro from hematopoietic progenitors in peripheral blood, cord blood or bone marrow by different cytokine combinations (Caux et al, 1996). In this paper we investigated cord blood $CD34^+$ cell and depleting T and B lymphocyte PBMC as potentially more available sources of DC precursors for research use in cancer immnuotherapy. Our experiment indicated that: FL acts synergistically in vitro with GM-CSF, TNF-α and SCF in DC generation from $CD34^+$ cells of cord blood. The result showed that GM-CSF, TNF-α, SCF and FL could induce 27.18%±1.56% $CD1a^+$ cells, much higher than that in other groups($P<0.01$). It demonstrated the expansion efficiency of DC depended on the combinations of cytokines, and different sources required different cytokines, which maybe related to the DC origin and differentiation (Gliboa et al, 1998). The dosage of IL-4 play key role in DC expansion. DCs were generated from adult peripheral blood and required IL-4 in addition to GM-CSF to maintain the CD1a expressing and antigen presentation competent state. When IL-4 was under 400U/ml, the percentage of $CD1a^+$ cells decreased to 3.30%±0.16% from 21.8%±0.32% of GM-CSF+IL-4 (1000U/ml) group ($P<0.01$). It is not clear what role IL-4 may play, but many data suggested that IL-4 is the desired exogenous cytokine that is to be applied in combination with GM-CSF. We suspect that IL-4 acts by suppressing the monocyte differentiation potential of the DC progenitor (Chapuls et al, 1997). Functionally, from our results, $CD34^+$ cells-derived DC and PBMC-derived DC were similar in stimulating T lymphocytes. The stimulating capacity was then increased five- to six-fold relative to their control groups. Together, these data indicate that both of the cord blood and peripheral blood were also the useful sources for the generation of sufficiently large numbers of typical DC, and could be adopted for the application according to different purposes.

Suicide gene system is such an approach which involves the transfer of a suicide gene into tumor cells resulting in sensitization to the nontoxic prodrug. Herpes simplex virus thymidine kinase (HSV-tk), widely used in experimental gene therapy studies and also human clinical trials (Kato, 1994), converts the antiviral prodrug ganciclovir (GCV) to the toxic phosphorylated form. In this study, tk gene expressing MCF-7 cell line resulted in the sensitivity of MCF-7/FL-IRES-TK to GCV, and IC_{50} (0.5 μg/ml) was much lower than that in non-transduced parental cells. This demonstrated that HSVtk/GCV system would be a useful way for breast carcinoma treatment. In the further experiment, we found that apoptotic cell death induced by GCV could be detected by FACS or electron-microscope. The percentage of apoptotic cells reached to 19.3%±1.0%. Apoptosis has been suggested as a mechanism by which bystander cell death is mediated. There was an important and interesting effect in suicide gene system, treatment of cells expressing the suicide gene with appropriate prodrug can lead to the death of neighboring cells that do not express the gene. This has been termed the bystander effect. However, there is uncertainty about bystander effect mechanisms involved. Investigations using cultured cells have led to the suggestion that toxic metabolites are transferred via a gap junctional pathway (Kaneko and Tsukamoto, 1995; Mesnil et al, 1996), but it has also been suggested that phagocytosis of apoptotic vesicles, derived from the tk^+ cells, leads to death of unmodified cells. Our data are not sufficient for explaining that the apoptosis is the result or the cause of the bystander effect, why and how the apoptosis existed in this system, but we just know the apoptotic phenomena is greatly advantageous for the DC function. As studies previously reported, DC could phagocytize apoptotic bodies and present antigens from them to T lymphocyte. From our result, after DC came into contact with apoptotic cells, they did further stimulate the proliferation of T lymphocytes comparing to the DC contacting with fewer apoptotic cells or no apoptotic cells ($P<0.01$). Together, it is possible that combining DC-based immunotherapy with other antineoplastic therapies that induced apoptosis, such as HSVtk/GCV suicide gene therapy and irradiation, may lead to synergistic anti-tumor activity, because all appear to activate a common pathway.

It is well documented that DC play a pivotal role in the induction of anti-tumor immunity. But one of the limiting factors in using these professional APC for human DC-based immunotherapy is the relative low yield of functionally active DC *in vitro* either in the lymphoid tissues or in blood (Steinman, 1996; Marina et al, 1998; Steinman and Banchereau, 1998). *In vitro* generation of large numbers of autologous DC from either monocyte or $CD34^+$progenitors is time consuming and requires special equipment. Recently, many groups researched for the optimal culture conditions and achieved some common opinion. GM-CSF has been shown to enhance the yield of DC generated *in vitro* from peripheral blood. Other cytokines such as SCF, TNF-α, and IL-4 cooperate with GM-CSF in *in vitro* generation of DC. Interestingly, FL has been used as a component of multi-cytokine supported culture (IL-1, IL-7, IL-3, IL-6, SCF, TNF-α, GM-CSF and FL) medium for short-term DC cultures (Maraskovaky et al, 1996; Celluzzi and Falo, 1998). Our results showed

that, FL or (FL+SCF) significantly enhanced (GM-CSF+TNF-α) dependent generation of DC from cord blood $CD34^+$ cells. The percentage of $CD1a^+$ cells increased to 18.0%±0.11% (FL+GM-CSF+TNF-α) or 27.18%±1.56% (FL+SCF+GM-CSF+TNF-α) from 4.12%±0.6% (GM-CSF+TNF-α) ($P<0.01$). As described in previous results, MCF-7/FL-IRES-TK tumor cells-derived FL had bio-activity in proliferation of $CD34^+$ cells and generation of DCs. The percentage of $CD1a^+$ cells decreased to 8.17%±0.32% when supernatant FL replacing standard FL, but it was higher than that in control group. This is very important for the generation of DCs *in vivo*. It demonstrated that tumor-derived cytokine might play some roles in DC generation and function. In summary, combining fl and tk genes to transduce tumor cells was not only a method for providing apoptotic bodies to DCs, but also a good way to the generation of DCs, which was significant for the DC-based immunotherapy. Taken together, our findings suggested that multi-component vaccine might improving the T cell proliferation by enhancing DC functions and thus play an important role in tumor immnunotherapy.

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