"Suicide" Gene Therapy of Breast Cancer Cells is Only Cytostatic In Vitro But Anti-tumoral In Vivo on Breast MCF7-ras Tumor

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Abstract. Gene therapy with Herpes Simplex Virus thymidine kinase gene (HSV-tk) is effective in various tumor models in vitro and in vivo. We compared the efficacy of the HSV-tk gene therapy in vitro and in vivo in MCF-7 and MCF7-ras cells which form tumor in athymic mice. After viral infection, cells were treated with GCV (Ganciclovir) and live cells were counted. The in vitro treatment significantly inhibited cell growth but did not induce early and late apoptosis, measured, respectively, by annexin or by propidium iodide staining and a significant cell death. The HSV-tk/GCV treatment of MCF7-ras tumor in athymic mice showed a significant inhibition of tumor development until 60 days post-treatment. Some mice showed a complete tumor eradication without tumor regrowth after the end of treatment. In conclusion, we demonstrated that the HSV-tk/GCV system is not very efficient in vitro, but very efficient in vivo in our animal breast cancer model.

Breast carcinoma is one of the most frequent cancers and available treatments (surgery, chemotherapy and/or antiestrogen hormonotherapy with Tamoxifen) are not totally efficient. Chemotherapy and hormonotherapy have side-effects and there is very often a tumor relapse under cancer treatment. Furthermore, hormonotherapy with Tamoxifen is not effective in all breast cancer because some breast cancers are hormone-independent and disease recurrence is commonly observed (1). Gene therapy with Herpes Simplex Virus thymidine kinase gene (HSV-tk), which is effective in various tumor models both in vitro and in vivo (2-4), involves transferring a chemosensitizing "suicide" gene to tumor cells. Tumor cells are exposed to a nontoxic pro-drug such as Ganciclovir (GCV), which is metabolized by HSV-tk into the Ganciclovir monophosphate and, subsequently, converted by host cellular kinases to the di- and triphosphate forms (an analog of the guanine triphosphate). The integration of Ganciclovir triphosphate arrests DNA replication and causes cell death (5). The present study compared the feasibility of a HSV-tk "suicide" gene therapy in vitro and in vivo in the MCF-7 cell model characteristic of hormone-dependent human breast cancer (6). MCF7-ras cells, which overexpress Ras oncogene, are characteristic of hormone-sensitive breast cancer (7, 8) and form tumor in athymic mice without estrogen treatment. The effect on the growth, viability and apoptosis of HSV-tk/GCV-treated cells were compared to the antitumor effect.

Materials and Methods

Cell cultures. The epithelial cell line MCF-7, established originally from a pleural effusion of a breast adenocarcinoma, was obtained from ATCC (Rockville, MD, USA), MCF7-ras, derived from MCF7 by transfection of the H-ras oncogene, was kindly provided by Dr. F. Calvo (Hôpital St. Louis, Paris, France). M11 retrovirus-producing packaging cells (Laboratoire d'Histologie, Bobigny, France) were used to produce virus with a titer from 2×10⁴ to 10⁶ cfu/ml. NIH 3T3 tk- cells did not express endogenous thymidine kinase and were obtained from (UFR Léonard de Vinci, Laboratoire d'Histologie). All cell lines were cultured in Dulbecco's modified Eagle's medium with glutamax I tm (Gibco Invitrogen, Cergy Pontoise, France) supplemented with 10% Fetal Calf Serum (FCS) (Gibco Invitrogen), 50 Ìg/mL streptomycin (Gibco Invitrogen) and 50uI/ml penicillin (Gibco Invitrogen).

Virus and colony-forming assay. M11 cells were seeded in a T 75cm² (Falcon, VWR International, France) in DMEM 10% FCS. When they were at 70-80% of confluence, 5mL medium was replaced by 5mL of fresh medium for 24 hours. Viral supernatant was filtered through 0.45 µm and kept at –80°C. The titer of viral supernatant was determined as follows. NIH 3T3 tk- cells did not express endogenous thymidine kinase and were obtained from (UFR Léonard de Vinci, Laboratoire d'Histologie). All cell lines were cultured in Dulbecco’s modified Eagle's medium with glutamax I tm (Gibco Invitrogen, Cergy Pontoise, France) supplemented with 10% Fetal Calf Serum (FCS) (Gibco Invitrogen), 50 µg/mL streptomycin (Gibco Invitrogen) and 50µl/ml penicillin (Gibco Invitrogen).

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changed the following day. Then 1mL of HAT medium (Gibco Invitrogen) was added and changed every 2 days until colony formation. Colonies were stained with giemsa’s solution for counting.

In vitro sensitivity of GCV. Cells were seeded for 2 days in DMEM supplemented with 10% FCS in 24-well plate (Falcon) at a density of 2.10^4 cells/well. Then cells were infected with 0.4mL/well of viral supernatant plus 8 Î¼g/mL of polybren. After 3 hours, the cells were washed with 1x Phosphate Buffer Saline (PBS) (Gibco Invitrogen) and 1 mL of fresh DMEM 10% FCS was added to each well. Two days later, 1 mL/well of DMEM 10% FCS with GCV (Roche, Neuilly-sur Seine, France) 5 Î¼M was added. After 4 days, the cells were harvested with 0.25% trypsin 0.2% EDTA (Gibco Invitrogen) and counted by trypan blue exclusion assay.

Annexin V-FITC labelling. Cells were seeded in 6-well plate (Falcon) in DMEM 10% FCS at a density of 1.10^5 cells/well. At the indicated time after infection and treatment with GCV, the cells were harvested, counted (plasma membrane integrity was estimated by trypan blue exclusion assay) and apoptosis was detected and quantified by staining with annexin V-FITC (Clontech, CA, USA). Soon after the initiation of apoptosis, most cell types translocated phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface and PS could easily be detected by staining with the FITC conjugate of annexin V. The cells were incubated in the dark for 15 minutes at room temperature with annexin V-FITC and/or propidium iodide as described in the kit user manual. Five thousand cells were analyzed by flow cytometry (FACScan®, Becton Dickinson, France S.A.).

Animal studies. For animal injections, culture cells in the proliferative stage were changed with fresh medium 24 hours before harvesting, then trypsinized, centrifuged at low speed, washed once with 1x PBS buffer, and then resuspended in DMEM without serum at a density of 10.10^6 cells/ml. For subcutaneous injection, 2.10^6 MCF7-ras cells in 200 Î¼l were injected into the flanks of athymic mice (Harlan, France). When the tumors had reached a volume of about 200 mm^3, 5.10^6 retrovirus-producing cells M11 (Laboratoire d’Histologie) in 200 Î¼l were injected into...
the tumor bed. Before GCV injection, the animals were randomly separated into 2 groups after tumor formation and M11 injection into the tumor bed. Mice in group 1 (n=6) received 1x PBS administration (in the volume corresponding to GCV administration) and mice in group 2 (n=6) received GCV treatment. Five days later, the animals started to be treated with GCV, which was administered twice a day for 14 days at a dose of 150 mg/Kg/day (15). Tumor volumes were regularly measured twice a week with an external caliper. Tumor volumes were calculated as follows: $4/3 \times \pi \times R_1^2 \times R_2$, where $R_1$ and $R_2$ are two diameters of the tumor and $R_1 < R_2$.

**Results**

In vitro treatment of HSV-tk-infected MCF7 and MCF7-ras cells with GCV-induced cell growth arrest but not a significant cell death (cytostatic effect). Before investigating the HSV-tk "suicide" gene effect with GCV prodrug, we tested the effect of GCV alone on MCF7 and MCF7-ras proliferations. Figure 1 shows that, with 10 μM of GCV, the cell growths of MCF7 and MCF7-ras lines slowed down in a dose-dependent manner. We obtained 65% and 70% of cell growth inhibition for MCF7 and MCF7-ras, respectively, with 50 μM of GCV. Since we observed that a high concentration of GCV (50 μM) increased the cell death rate measured by trypan blue exclusion test (data not shown), we used a concentration of 5 μM of GCV in order to avoid a masking effect of the in vitro "suicide" gene therapy.

When the two cell lines were exposed to virus supernatant obtained as described in the Materials and Methods section, we observed that the HSV-tk infection was not responsible for a significant decrease of growth and viability as compared to the control (Figure 2, Table I). In order to evaluate the effect of our HSV-tk virus infection protocol on early and late apoptosis, as well as the death rate, we performed an annexin V-FITC and propidium iodide labelling of infected and non-infected cells. The calculated percentages of cells in early (annexin V-FITC-positive cells) or late (propidium iodide-positive cells) apoptosis phase indicated that the HSV-tk virus did not induce apoptosis for the two cell lines, 1, 2.5, 24 and 48 hours after the beginning of the infection (data not shown). Thus viral infection by HSV-tk does not affect the cell growth and viability of MCF7 and MCF7-ras cell lines.

HSV-tk infected-cells in contact with GCV (5 μM) for 4 days showed an important growth inhibition (Figure 3). MCF7 and MCF7-ras growth were inhibited by 53%±1 and 35%±1, respectively. When GCV was added, the death rate normally induced by GCV was not very important for the MCF7 cell line (Table I). The viability of MCF7-ras cells was the same as the MCF7-ras control (Table I). We confirmed these results for the MCF7 and MCF7-ras cell lines by annexin V-FITC labelling in vitro (Figure 3). This experiment indicated, for the MCF7 cell line, that the "suicide" gene therapy was able to induce significant, but not very important, apoptosis. One hour after GCV treatment, we did not find significant apoptosis (annexin V and propidium iodide staining) induced by HSV-tk/GCV therapy for both MCF7 and MCF7-ras cells. After 24 hours of incubation with GCV, a significant increase of the infected MCF7 cell apoptosis-GCV mediated of 8%±1 as compared to the apoptotic MCF7 control cells was detected.
After 48 hours of treatment, almost the same difference (7%±1) between apoptotic MCF7 control cells and HSV-tk/GCV MCF7 cells was found.

Results for the MCF7-ras cell line, indicated and confirmed that the "suicide" gene therapy was not able to induce cell death. GCV treatment could not induce significant apoptosis between control MCF7-ras cells and HSV-tk/GCV MCF7-ras cells after 1, 24 or 48 hours of GCV treatment (Figure 3). In conclusion, it appears that the MCF7 cell line, but not MCF7-ras, was slightly more sensitive in vitro to HSV-tk/GCV "suicide" gene therapy.

**HSV-tk/GCV therapy induced a strong MCF7-ras tumor regression in nude mice.** Furthermore, we wanted to know if MCF7-ras tumor-induced cells could be destroyed by the HSV-tk/GCV "suicide" gene therapy in vivo in spite of lack of sensitivity in vitro. So we used immune-depressed mice or athymic mice for the development of the MCF7-ras tumor. As shown in Figure 4, from day 0 to day 6, the MCF7-ras tumor volumes of treated and untreated groups were not significantly different. However, these differences were significantly enlarged from day 6. In the GCV -treated group, tumors started to regress from day 6 and their growth was completely inhibited by day 14, until day 63 (data not shown).

Even after the end of GCV treatment at day 19, we did not observe a relapse in GCV-treated mice and two mice showed a complete tumor regression. In contrast, in the control group, all tumors were exponentially growing. On day 63, all control mice bearing huge tumors were sacrificed. Thus, HSV-tk/GCV treatment of MCF7-ras tumors blocked tumor development and irreversibly induced a tumor regression.

**Discussion**

The HSV-tk/GCV "suicide" gene therapy has been widely shown to be very efficient for the in vitro and in vivo treatment of various cells lines and transplanted tumors (2-4). In this study, we assessed the feasibility of a HSV-tk-mediated gene therapy for the treatment of a hormone-sensitive breast adenocarcinoma (6-8). In vitro MCF7 and MCF7-ras proliferations were compared for HSV-tk/GCV "suicide" gene therapy. We also compared the effects of HSV-th/GCV therapy on the in vitro proliferation of MCF7-ras to the MCF7-ras xenografted growth in nude mice.

We showed that direct injection into the MCF7-ras tumor bed with retroviral vector-producing cells followed by GCV injections was efficient to cure MCF7-ras xenografted tumors. This efficacy was so important that in 2 mice of the...
GCV-treated group, tumors were completely eradicated without tumor relapse. However, in the other 4 GCV-treated mice, tumor growth was completely inhibited even after the end of GCV injections (day 19). Tumor regression was not due to the retroviral vector-producing cells because no tumor size variation was observed as compared to the control. This tumor regression under HSV-tk/GCV treatment could be due to the "bystander" effect, first demonstrated by Moolten et al. (9), which consists of the transfer from cell to cell, infected or not, of phosphorylated GCV by gap junction (10) and/or apoptotic core (11). In our study, due to the fact of complete tumor regression even after GCV treatment, HSV-tk/GCV therapy might have a "memory" effect to prevent tumor regrowth.

In vitro results showed that the MCF7-ras cell line was not sensitive to HSVtk/GCV therapy as far as toxicity and early apoptosis induced by phosphorylated GCV. The MCF7 cell line was not very sensitive to this effect induced by GCV. However, for these two cell lines, we found that HSVtk/GCV "suicide" gene therapy could induce an important antiproliferative cytostatic effect (12, 13). Treatment of MCF7 cells was more efficient than MCF7-ras cell treatment: 53% of growth inhibition with a weak toxicity and weak early and significant late apoptosis for MCF7 cells, and 36% of growth inhibition, no toxicity and early and late apoptosis for MCF7-ras cells. These differences can be explained by the fact that the MCF7 and MCF7-ras cell lines were not representative of the same cancer grade progression: grade II for MCF7 which is not tumorigenic in nude mice and grade III for MCF7-ras. Overexpression of the ras oncogene could also confer to MCF7-ras its resistance to in vitro "suicide" gene therapy. Perhaps the slow down in growth without cell death could be explained by too short an exposure time to GCV and/or to an insufficient GCV dose. However, we know that this "suicide" gene therapy induced a fast cell death and that it is not necessary to use a high dose of GCV to obtain cell destruction (5).

Taking all these results together, we can conclude that the immune system was absolutely necessary for the total destruction of MCF7-ras neoplastic cells (14, 15). In vitro cell growth was slowed down without cell death or apoptosis induced by HSV-tk/GCV, but in vivo we observed a total destruction of these cells. Thus, we can postulate that the "bystander" cell killing effect is mostly immune-mediated (16, 17).

In contrast to our in vitro results, a clear conclusion can be drawn that HSV-tk gene therapy was efficient in treating experimental breast cancer in athymic mice. These results would suggest that the immune system might play an important role in tumor regression in vivo, explaining, in part, the discrepancy between the in vitro and in vivo results (18).

In conclusion, in this breast cancer model the immune system plays an important role for total tumor cell destruction. These results suggested that the distant "bystander" effect is immune-mediated, but its precise mechanism remains to be elucidated.

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References


