EXPERIMENTAL STUDY

Gene therapy of thyroid cancer via retrovirally-driven combined expression of human interleukin-2 and Herpes Simplex Virus thymidine kinase

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Abstract

Objective and design: Based on our clinical experience with combined gene therapy of glioblastoma, we developed a retroviral vector expressing two therapeutic genes (i.e. thymidine kinase of herpes simplex virus, HSV-TK, and interleukin-2, IL-2) and evaluated its efficiency in vitro and in vivo.

Methods: Expression of therapeutic genes in transduced thyroid carcinoma cells was analyzed by real-time RT-PCR. Ganciclovir sensitivity of infected cells was assessed in vitro in thyroid carcinoma cell lines and in vivo in nude mice bearing xenografted thyroid cancers. The combined effect of IL-2/HSV-TK was compared with the effect of IL-2 alone.

Results: Expression of therapeutic genes was higher in differentiated than in anaplastic thyroid carcinoma cells. Ganciclovir treatment led to dose- and time-dependent killing of transduced cells in vitro. A bystander effect was demonstrated by using mixtures of infected and non-infected cells. In vivo studies showed a significant reduction of growth and the presence of an inflammatory infiltrate in transduced thyroid tumors expressing IL-2 alone, as compared with non-infected tumors. By using the retroviral vector expressing IL-2/HSV-TK, treatment with ganciclovir led to complete eradication of anaplastic tumors and a 80% reduction of the size of differentiated thyroid carcinomas. Histological analysis of tumor specimens showed extensive necrosis and inflammatory cell infiltrates. The combination of IL-2/HSV-TK plus ganciclovir was significantly more efficient than IL-2 alone in eradicating tumor masses. The bystander effect was also obtained in vivo.

Conclusions: These findings demonstrate the feasibility and efficiency of a combined immunomodulating and suicide gene therapy approach for thyroid carcinomas.

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Introduction

Although most thyroid cancers respond to conventional therapy and have a relatively good prognosis, anaplastic carcinomas and end-stage differentiated thyroid carcinomas show a highly malignant behavior associated with a poor survival. Thus, the development of innovative therapeutic approaches, such as gene therapy, is needed. In this regard, several strategies have been designed so far for the treatment of thyroid carcinomas, including tumor suppressor gene replacement, prodrug activation, immunotherapy and oncolysis, and these have been demonstrated to be feasible in in vitro and in vivo studies (1, 2). However, experience from clinical trials of cancer gene therapy indicates that no single therapeutic strategy can effectively eradicate cancer, whereas combined poly-gene therapy represents a more reliable approach to combat cancer.

Our clinical experience with gene therapy of recurrent glioblastoma multiforme showed, for the first time in humans, the therapeutic efficacy of combined expression of a suicide gene (Herpes Simplex Virus type 1-thymidine kinase, HSV-TK) and an immunomodulating gene (human interleukin-2, hIL-2) (3, 4). In the present study, we pursued a similar strategy for gene therapy of human thyroid carcinomas using in vitro and in vivo models of tumor growth.

Materials and methods

Cell lines and culture conditions

Four different human thyroid carcinoma cell lines (follicular WRO and FTC-133, anaplastic C8305 and ARO), mouse fibroblasts NIH3T3, and the FLYA13 packaging cell line were used. WRO (HTL98002,
ICLC, Genova, Italy), FTC-133 (HTL97015, ICLC) and C8305 (HTL96026, ICLC) cells were cultured in Dulbecco’s modified Eagle’s medium/F12 medium (DMEM/F12; Invitrogen, Leek, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin G and 100 mg/ml streptomycin, ARO (UCLA RO-81-A-1, UCLA, CA, USA) and NIH3T3 (CRL-1658, ATCC, Manassas, VA, USA) cells were grown in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin G and 100 mg/ml streptomycin. The FLYA13 packaging cell line (5), a kind gift from Dr Y Takeuchi (Chester Beauty Laboratories, London, UK), derived from the HT1080 human fibrosarcoma cell line, was maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, 100 mg/ml streptomycin, 4 μg/ml blasticidin S (ICN, Biomedicals, Aurora, OH, USA) and 10 μg/ml phleomycin (Sigma, St Louis, MO, USA).

**Construction of recombinant retroviral vectors and vector titration**

The recombinant Moloney-derived retroviral vector pMFG (6), employed to develop the pMFGII-2TKSN vector, was the generous gift of Dr R C Mulligan (Dept of Genetics, Harvard Medical School, Boston, MA, USA). The pMFGII-2TKSN vector was constructed by subcloning into pMFG the 2162 bp IL-2-IRESTK cassette, which contains the human interleukin-2 (hIL-2) gene and the gene encoding for the thymidine kinase (HSV-TK) of Herpes Simplex Virus type 1 (HSV-TK), separated by an internal ribosome entry site (IRES) sequence isolated from the encephalomyocarditis virus. The cassette, which had been excised from pLIL-2TKSN (3) by EcoRI digestion, was blunt-ended and ligated into BglII and NcoI sites of the pMFG vector. A 1295 bp cassette containing the neomycin-resistance gene under control of SV40 early promoter (SV40neo), obtained from pLXSN (7) by NheI and Xhol digestion, was blunt-ended and ligated into the BamHI site of the vector. A retroviral vector containing the hIL-2 gene alone (pMFGII-2SN) was also developed. The hIL-2 gene, obtained from pLIL-2TKSN (3) by EcoRI and HindIII digestion, was blunt-ended and ligated into BglII and NcoI sites of the pMFG vector. The SV40neo cassette was inserted into the vector as described above.

Plasmid vectors were transfected into the amphotropic packaging cell line FLYA13 by using the Calcium Phosphate Transfection System (Invitrogen) reagents, as described (8). Transfected cells were selected in a medium containing 800 μg/ml G418 (Invitrogen) and single-cell-derived clones isolated and expanded to cell lines. Viral titer, determined by infection of NIH3T3 cells with virus-containing supernatants from single-cell-derived clones of FLYA13 producer cells as described previously (8), ranged from 2 × 10^5 to 3.5 × 10^8 c.f.u./ml. Supernatant from producer cell clones with higher viral titer were used to transduce target thyroid cells. The cells were incubated for 4 h with viral supernatants in the presence of 8 μg/ml Polybrene (Sigma) and, after 48 h, selected with 800 μg/ml G418 (8).

**RNA isolation and quantitative real-time RT-PCR analysis**

Total RNA was isolated from cells following a single step acid guanidinium phenol-chloroform extraction procedure employing RNAzol™ (Biotech Laboratories, Inc., Houston, TX, USA). Random primed cDNAs were generated from total RNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primer sequences employed to amplify hIL-2 and HSV-TK have been reported elsewhere (4). Real-time quantitative RT-PCR analysis was performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems), using SYBR Green PCR Core Reagents kit (Applied Biosystems). Absolute quantitation was performed against a standard curve generated by amplification of the pMFGII-2TKSN plasmid. Expression of the transgenes was also normalized to the endogenous control β-actin mRNA, as quantitated by real-time RT-PCR analysis using TaqMan β-actin RNA Control Reagent Kit (Applied Biosystems).

**In vitro cytotoxicity assay**

Retrovirus-infected cells were seeded at a density of 5 × 10^5 cells/well in 96-well microtiter plates. On the next day, the cells were treated with ganciclovir (GCV, Sigma) concentrations ranging from 0.01 to 100 μM in 100 μl medium. Cell survival was quantitated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazium bromide (MTT) assay (8) 5 and 9 days later. Survival ratios were expressed as percentages relative to untreated controls.

**In vivo antitumor effect**

This study was conducted using male nude mice (Charles-River Italia Spa, Calco, Lecco, Italy). 6–7 weeks of age, subcutaneously injected on both flanks with 8 × 10^5 retrovirus-infected or parental cells in 150 μl phosphate buffer saline (PBS). After 7 days, i.p. injections of either 100 mg/kg GCV in PBS or PBS alone were performed daily for 1 week. The perpendicular tumor diameters were measured using calipers, and tumor volumes (V) were calculated by the formula of rotational ellipsoid: \( V = A \times B^2/2 \), where A is the longer diameter, and B is the shorter diameter. None of the mice showed wasting or visible indications of toxicity. Animals were killed 24 h after the last GCV treatment and tissues were harvested for pathological examination. All procedures were carried out following the guidelines recommended by the Institutional Review Board of Genetics, Harvard Medical School, Boston, MA, USA.

**Conclusion**

The results of this study demonstrate that the recombinant retroviral vector pMFGII-2TKSN, containing the hIL-2 gene under the control of the SV40 early promoter, can be used to transduce NIH3T3 cells with high efficiency and specificity. The vector was shown to be safe and effective in in vitro and in vivo assays, with no signs of toxicity observed in the treated animals. These findings suggest that the pMFGII-2TKSN vector may have potential applications in gene therapy for the treatment of thyroid diseases.
Animal Care and Use Committee of the University of Padova.

**Histology**

Tissue specimens were fixed in buffered 4% formalin for 24 h and paraffin embedded after dehydration. Tissue (5 μm) slides were cut and stained with hematoxylin and eosin following routine histological methods. Microscopical examination was performed at low and high magnification, evaluating tumor size, necrosis and inflammatory infiltration in a semiquantitative grade as follows: absent/negative; moderate; and extensive.

**Statistical analysis**

Results are given as means ± S.E. Comparisons between variables were tested by one-way analysis of variance or Student’s t-test, as appropriate. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Expression of hIL-2 and HSV-TK therapeutic genes in infected cells**

The levels of therapeutic gene expression in transduced thyroid cells, evaluated by quantitative real-time RT-PCR analysis, were higher in WRO and FTC-133 differentiated thyroid carcinoma cells than in C8305 and ARO anaplastic thyroid carcinoma cells, characterized by a higher proliferation rate (\( P < 0.05 \)) (Fig. 1).

**Ganciclovir sensitivity of infected cells**

Morphological characteristics and proliferation rates of transduced cells remained the same as those of wild-type cells. Sensitivity to GCV of parental and infected thyroid cells was assessed by determining the IC\(_{50}\) by the MTT assay (Fig. 2). Infection with the MFGIL-2TKSN vector conferred sensitivity to GCV to all cell lines, with IC\(_{50}\) values ranging from 0.1 to 12 μM and from 0.01 to 1 μM, after 5 and 9 days of treatment respectively. Among the different infected cell lines, ARO anaplastic thyroid carcinoma cells appeared the most sensitive to GCV (IC\(_{50}\) 0.1 μM after 5 days treatment with GCV), whereas WRO cells, characterized by a lower replication rate, were the least responsive to GCV (IC\(_{50}\) 12 μM after 5 days treatment with GCV).

In *vitro* bystander effect was investigated by treatment with GCV of transduced and non-transduced thyroid carcinoma cells mixed in different proportions. Cell mixtures containing only 25% infected cells showed a mortality rate similar to that obtained in 100% HSV-TK positive cells after treatment with GCV for 5 and 9 days (Fig. 2).

**Effect of the genetic treatment on tumor models in vivo**

The *in vivo* efficacy of the retroviral vector constructs was evaluated in tumor models obtained by s.c. injection of infected and parental cells in nude mice. To investigate the *in vivo* effect of IL-2, cells infected with the MFGIL-2SN vector (i.e. expressing the hIL-2 therapeutic gene alone) and uninfected cells were used. Mean volume of tumors obtained with non-infected cells was significantly larger than the volume of tumors obtained with cells infected with the MFGIL-2SN vector (Fig. 3). Histological examination showed non-transduced tumor as large nodules of actively dividing cells without necrosis or with small focal areas of necrosis and no significant inflammatory cell infiltration whereas a moderate infiltration of inflammatory cells was observed in infected tumors. Treatment with GCV i.p. for 1 week did not lead to a significant variation in size of MFGIL-2SN infected tumors as compared with tumors not receiving GCV.

To evaluate the systemic effect of IL-2, a group of mice was also injected with parental cells on one flank and with the corresponding transduced cells on the other flank. No significant differences of tumor size were demonstrated between the two flanks. At histology, both infected and non-infected tumor samples showed focal areas of necrosis and moderate inflammatory infiltrates.

To investigate the *in vivo* efficacy of HSV-TK, mice were injected with tumor cells and, after 1 week, treated with 100 mg/kg GCV daily for 1 week. Treatment with GCV led to a complete or near complete (<20% of the original mass) regression of the volume.

**Figure 1** Expression of HSV-TK and hIL-2 transcripts in WRO, FTC-133, C8305, ARO thyroid carcinoma cells transduced with the MFGIL-2TKSN vector. Random primed cDNA from total RNA was used for real-time quantitative RT-PCR analysis using SYBR Green reagents. Results were normalized to the endogenous control β-actin, as quantitated by real-time RT-PCR analysis. Data are representative of at least three separate experiments performed in triplicate.
of infected tumors (Fig. 3). In contrast, transduced cell tumors not treated with GCV showed a 3- to 5-fold increase in size after 7 days, thus demonstrating a significantly higher activity of HSV-TK/IL-2 plus GCV than IL-2 alone (Fig. 3). Histological analysis of tumor specimens from GCV-treated mice showed extensive necrosis and a significant infiltration of inflammatory cells, with a prevalence of neutrophils (Fig. 4).

The in vivo bystander effect was evaluated in mice inoculated with different mixtures of infected and uninfected FTC-133 and ARO cells (Fig. 5). About 98% and
Combined suicide and immunomodulating gene therapy of thyroid cancer
90% reduction of volume was demonstrated in tumors containing only 50% of transduced FTC-133 and ARO cells respectively, a result similar to that obtained with 100% transduced cells.

**Discussion**

In the present study, we demonstrated the in vitro and in vivo therapeutic efficacy of retroviral vector-mediated combined suicide and immunomodulating gene therapy for thyroid carcinomas. The use of an MFG-based retroviral vector allows high levels of therapeutic gene expression and selective targeting of actively dividing cancer cells, while sparing normal resting cells. As demonstrated by quantitative analysis, expression of both the HSV-TK and the hIL-2 therapeutic genes in infected cells was high and persistent with time. Although higher levels of therapeutic gene expression were demonstrated in differentiated thyroid carcinoma cells, characterized by lower replication rate, the in vitro efficacy of the vector after GCV treatment was higher in anaplastic thyroid carcinoma cells, due to the efficient incorporation of the activated drug in the DNA of rapidly dividing anaplastic cells. A major drawback of retroviral vectors for gene therapy of cancer is low transduction efficiency; however, the bystander effect allows the spread of the antitumor activity to neighboring non-transduced cells. As observed also by others (9), our in vitro experiments showed that treatment with GCV led to more than 90% growth inhibition in cell mixtures containing only 25–50% transduced cells, a result similar to that obtained in 100% transduced cells. Although the mechanism by which this anti-cancer effect occurs in vitro has been shown to be due to transfer of toxic GCV metabolites (10, 11), in vivo studies have shown also the involvement of the host immune/inflammatory system (12). Even though our animal model did not allow a thorough evaluation of the immune response, our in vivo results showed that GCV treatment of 50% transduced tumors led to a growth inhibition similar to the inhibition obtained in 100% infected tumors. The use of a cytokine gene besides a suicide gene allows the amplification of the antitumor effect by further stimulating immune-mediated rejection of cancer cells (13). Several in vivo studies in animal models of cancer demonstrated enhanced tumor cell killing with combined suicide and cytokine gene therapy (13–18). As for thyroid cancer, a combined approach with HSV-TK plus IL-2 gene transfer showed a potentiated anticancer effect in animal models of medullary thyroid carcinoma (19, 20).

Due to the lack of animal models of syngeneic thyroid cancer, our in vivo studies in nude mice injected with transduced tumor cells allowed us to evaluate activation of a non-specific anti-tumor immune response generated by expression of the hIL-2 gene. However, oncology patients, particularly at advanced stages, often show impairment of the immune system due to suppressive cytokines or inappropriate T-cell responsiveness (21, 22). Thus, nude mice, which are deficient in mature T-cells, could represent a surrogate model of T-cell unresponsiveness of cancer patients, even though the mechanisms of immune deficiency are not the same. In our study, tumor cells expressing hIL-2 showed growth retardation as compared with parental non-transduced cells, associated with local necrosis and inflammatory infiltrates at histology. This anti-tumor effect, likely due to IL-2 expression, was predominantly mediated by granulocytes and/or macrophages. These cells were most likely recruited by secondary cytokines induced by IL-2, as demonstrated also by others in a pancreatic carcinoma model in nude mice (23). A significant enhancement of in vivo anti-tumor activity was obtained by using the vector expressing both hIL-2

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**Figure 5** In vivo bystander effect in FTC-133 and ARO cells. Tumors were obtained by subcutaneous injection in nude mice of mixtures of parental or transduced cells. A total of 8 × 10^6 cells was injected in each flank. After 1 week, mice were treated by daily i.p. injection of 100 mg/kg GCV or PBS for 7 days. Each bar represents the mean (n = 8) ± S.E. tumor volume after 7 days treatment with GCV and is expressed as a percentage relative to baseline tumor size.
and the suicide gene HSV-TK. Treatment with GCV induced a near complete or complete regression of transduced tumors, with histological evidence of extensive necrosis and infiltration of inflammatory cells, whereas non-infected tumors showed a 3- to 5-fold increase in size without significant necrosis or inflammation. In particular, a marked tumor-killing effect was observed in anaplastic thyroid carcinoma cells, with complete or near complete eradication of the tumor in >90% of GCV-treated animals, indicating that this vector could be particularly suitable for anaplastic thyroid cancer. In this aggressive cancer, the use of a retroviral vector would allow the targeting of tumor cells only, typically characterized by a very high proliferation rate, even though other types of viral vectors, such as replication-competent oncolytic vectors (24), could represent a more potent weapon versus this highly aggressive cancer. In any case, the retroviral vectors are still considered the most safe among the available viral vectors. In this regard, no signs of toxicity were demonstrated in our in vivo experiments and in our clinical experience with a similar retroviral vector (4).

In conclusion, our results demonstrate that combined expression of two therapeutic genes (cytokine and suicide genes) in thyroid-derived tumors allows an increased anticancer effect. In the clinical setting, local treatment with direct intratumoral inoculation of retroviral vector producing cells would result in tumor mass reduction or even eradication after GCV treatment, whereas combined cytokine gene therapy would elicit a systemic effect against distant metastases. If this therapeutic approach is adopted in an immunocompetent host it is foreseen that tumor antigens from cells killed by suicide gene therapy are processed by antigen presenting cells and presented to T cells, which are activated by local cytokine production to induce a systemic antitumor response. Unfortunately, our animal model of thyroid cancer did not allow us to fully evaluate the impact on tumor regression of cytokine gene therapy, which played a relevant role in our clinical study in patients with glioblastoma multiforme (4). Interestingly, patients with glioblastoma multiforme showed, besides intracerebral production of IL-2 and tumor infiltration by activated macrophages and lymphocytes, transduction of endothelial cells of the neo-vascular component, suggesting an antiangiogenic effect of our gene therapy strategy. These aspects are being evaluated in our ongoing pilot study in humans with advanced thyroid cancer treated by intratumoral injection of retroviral vector producing cells.

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