

## 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 Beatty 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 pMFGIL-2TKSN vector, was the generous gift of Dr R C Mulligan (Dept of Genetics, Harvard Medical School, Boston, MA, USA). The pMFGIL-2TKSN vector was constructed by subcloning into pMFG the 2162 bp IL-2-IRES-TK cassette, which contains the human interleukin-2 (*hIL-2*) gene and the gene encoding for the thymidine kinase 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 neomycine-resistance gene under the control of SV40 early promoter (*SV40neo*), obtained from pLXSN (7) by NheI and XhoI digestion, was blunt-ended and ligated into the BamHI site of the vector. A retroviral vector containing the *hIL-2* gene alone (pMFGIL-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 with virus-containing supernatants from single-cell-derived clones of FLYA13 producer cells as described previously (8), ranged from  $2 \times 10^5$  to  $3.5 \times$

$10^6$  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 guanidium 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 pMFGIL-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  $5 \times 10^3$  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-diphenyltetrazolium 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  $8 \times 10^6$  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

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  $\mu$ m) 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.

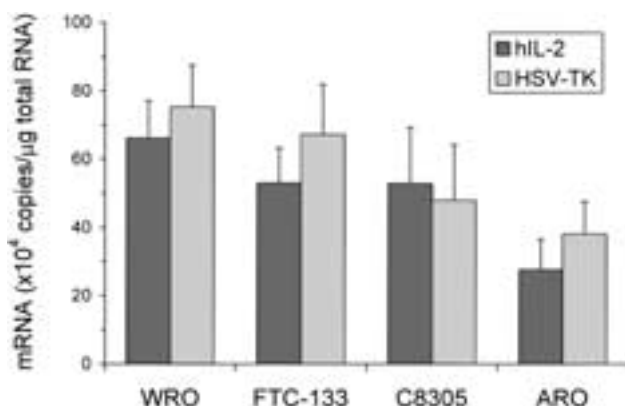
### Statistical analysis

Results are given as means  $\pm$  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).



**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  $\beta$ -actin, as quantitated by real-time RT-PCR analysis. Data are representative of at least three separate experiments performed in triplicate.

### Ganciclovir sensitivity of infected cells

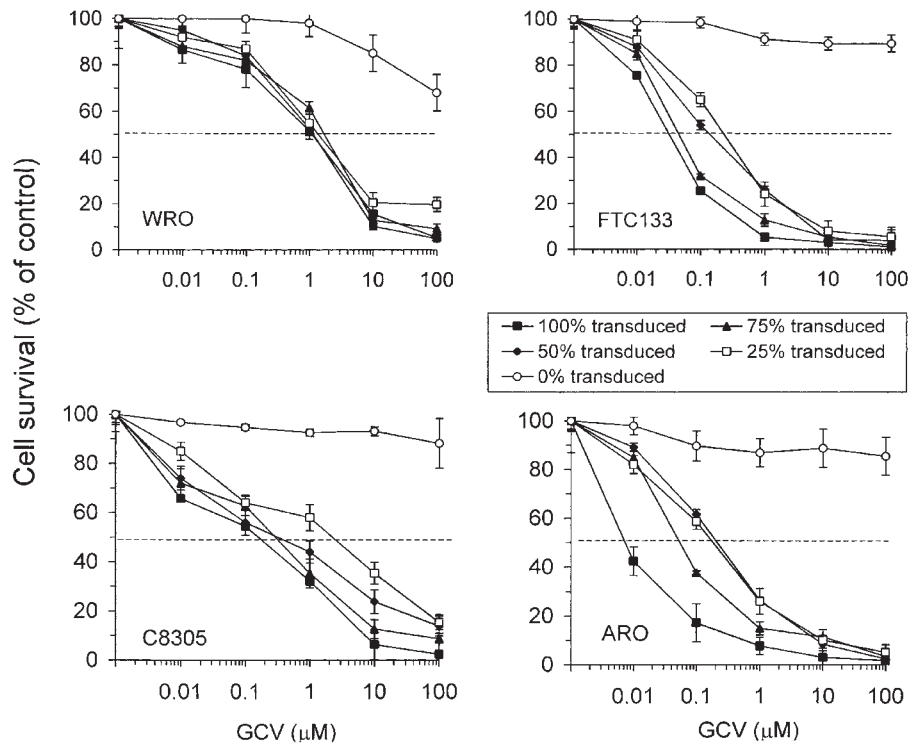
Morphological characteristics and proliferation rates of transduced cells remained the same as those of wild-type cells. Sensitivity to GCV of parental and infected thyroid cells was assessed by determining the  $IC_{50}$  by the MTT assay (Fig. 2). Infection with the MFGIL-2TKSN vector conferred sensitivity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

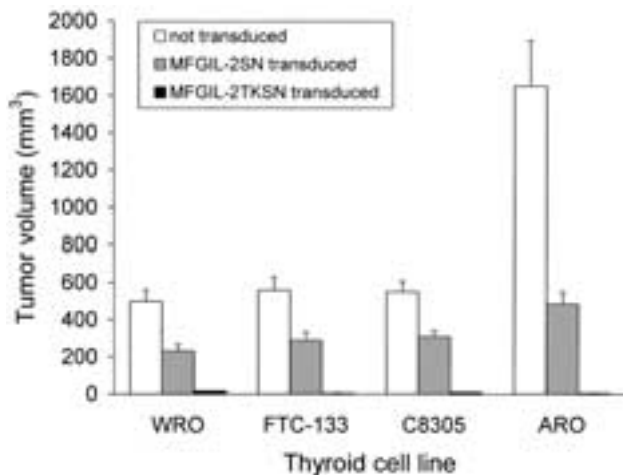
### Effect of the genetic treatment on tumor models in vivo

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 2** *In vitro* cytotoxicity and bystander effect of GCV in parental cells and cells transduced with the MFGIL-2TKSN vector. Transduced and non-transduced cells mixed in different proportions were incubated with various concentrations of GCV for 9 days, followed by cell survival quantitation by MTT assay. IC<sub>50</sub> was calculated as the concentration of drug which inhibits cell growth by 50%. Data are representative of at least three separate experiments performed in sextuplicate; each point represents the mean ± s.e. and is expressed as a percentage relative to drug-free cells.

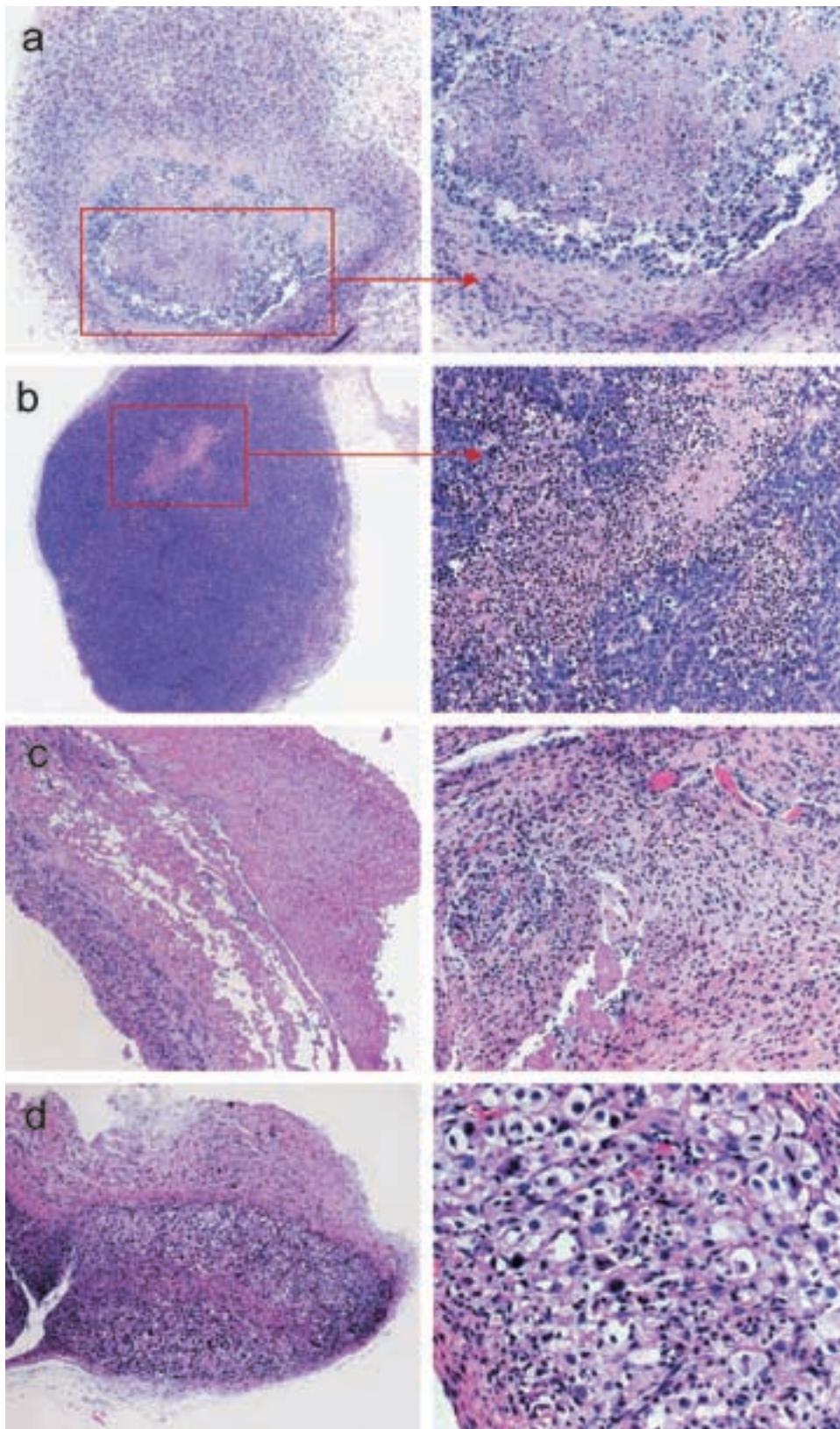


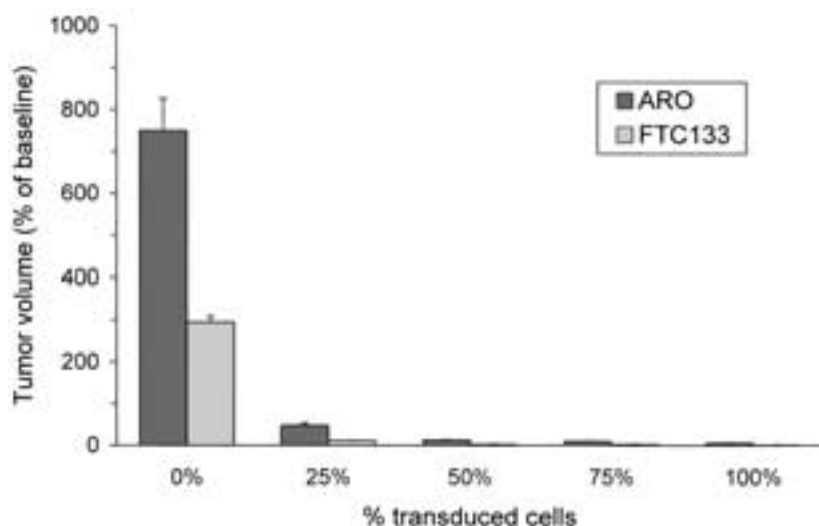
**Figure 3** *In vivo* efficacy of MFGIL-2SN and MFGIL-2TKSN vectors in tumors obtained by subcutaneous injection of parental or transduced cells in nude mice. A total of  $8 \times 10^6$  cells was injected in each flank. After 1 week, mice were treated by daily i.p. injection of 100 mg/kg GCV or PBS for 7 days. Each bar represents the mean ( $n = 6-8$ ) ± s.e. tumor volume after 7 days treatment with GCV.

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

**Figure 4** Histopathological analyses of thyroid tumor specimens obtained by s.c. inoculation of cells in nude mice. Formalin-fixed and paraffin-embedded sections were stained with hematoxylin and eosin. Low (left panels) and high (right panels) magnifications of representative sections in MFGIL-2TKSN-infected (a) and non-infected (b) ARO cells after GCV treatment, showing a large area of tumor necrosis with remarkable infiltration of inflammatory cells in MFGIL-2TKSN-treated mice and a small and focal spontaneous necrosis without inflammatory cell infiltration in the control mice. Low (left panels) and high (right panels) magnification of representative sections in infected FTC-133 tumors after GCV (c) or PBS (d) treatment, showing, respectively, extensive and moderate necrosis and inflammatory infiltrates.





**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  $8 \times 10^6$  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 ( $n = 8$ )  $\pm$  s.e. tumor volume after 7 days treatment with GCV and is expressed as a percentage relative to baseline tumor size.

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 focal 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*

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 against 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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