Tumor cells escape suicide gene therapy by genetic and epigenetic instability

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Transfer and expression of suicide genes is one cornerstone of cancer gene therapy and is also considered as a proactive tool to enhance the safety of somatic transgenesis. Here we addressed whether retrovirus-mediated suicide gene therapy would result in a predictable antitumor efficiency, given that problems related to gene transfer are solved or that the suicide gene is used in a proactive approach. Using retroviral vectors encoding the thymidine kinase gene of herpes simplex virus, we transduced EL-4 lymphoma cells and induced experimental tumors in congeneic C57Bl/6 mice. Systemic administration of ganciclovir (GCV) resulted in remission of transduced clonal and polyclonal tumors in vivo. However, GCV-resistant relapses occurred and were found to be associated with postinsertional alterations of transgene structure or loss of the entire transgene. Complete loss of a retrovirally marked fusion chromosome was confirmed by spectral karyotyping. Transgene silencing occurred in another clone. We conclude that genetic as well as epigenetic instability related to biologic features of the tumor, the insertion site, and the vector represent relevant limitations of retroviral suicide gene therapy. Considering the mechanisms of escape identified here, the proactive use of suicide genes to prevent complications of insertional mutagenesis may still be efficient. (Blood. 2004;104:3543-3549)

Introduction

Cell death can be induced when proteins that have a toxic or proapoptotic function or that activate precursors of toxic drugs are expressed. Genes encoding such proteins are termed suicide genes.[1-5] Retroviral vectors with their preference for transduction of dividing cells are promising tools for suicide gene transfer into tumor cells.[1,2,6] Several approaches have been proposed to improve the efficiency of gene transfer in vivo and to ensure selective expression of suicide genes in malignant cells.[1-10]

Less well investigated are potential mechanisms of escape from retroviral suicide gene delivery, potentially even occurring after transduction with intact transgenes. Such studies are an important part of the preclinical evaluation of suicide gene therapy, especially since the introduction of suicide genes has been proposed to increase the safety of retroviral vector–mediated gene transfer into postnatal stem cells. In this case, the rationale is that activation of the suicide principle may allow the deletion of a malignant clone that may arise in consequence of protooncogene activation by random vector insertion. Given the demonstration of malignant complications of retroviral vector–mediated gene transfer both in mice and in humans,[11,12] there is an urgent need to evaluate the potential efficiency of such a preventive expression of suicide genes. This "proactive" scenario differs from the "reactive" introduction of suicide genes into pre-existing tumors in 2 major respects. Firstly, the suicide gene would be expected to be present in the same chromosomal position of any cell of the malignant clone, and secondly, the malignant clone would undergo massive clonal expansion before the application of the suicide medication.

Our hypothesis was that alterations in transgene structure or function would promote escape from negative selection. Such alterations do not appear unlikely, since genetic and epigenetic instability is characteristic for malignant tumors[13-16] and independently encountered in experimental transgenesis.[17] To test the type and incidence of such escape mechanisms, we established an experimental tumor, using retrovirally transduced EL-4 lymphoma cells.[18] Our model identified 3 potential mechanisms of escape from negative selection after suicide gene delivery: gene deletion by postinsertional recombination of sequences between the long terminal repeats (LTRs) of the retroviral vector, deletion of the chromosome containing the suicide gene, and transgene silencing. Our observations have significant implications for the design of proactive and reactive scenarios of suicide gene therapy.

Materials and methods

Mice and experimental tumors

C57Bl/6J-Ly5.1 mice obtained from The Jackson Laboratory (Bar Harbor, ME) were maintained pathogen-free at the Heinrich-Pette-Institute (HPI) animal facility and all animal experiments were approved by the regional

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committee. The day before tumor cell inoculation, diameters of the hind legs (5 mm) were determined using a caliper. Animals were then challenged with 1×10^5 congeneic (Ly5.2) EL-4 tumor cells via subcutaneous injection into the right hind flank and analyzed for tumor induction 3 to 4 times weekly. For this purpose, the diameter measurements with a caliper of the cell-injected hind leg (right) were compared with the uninjected one (negative control). At beginning of visible and palpable tumor growth (day 10 after transplantation), mice were treated twice daily with ganciclovir (GCV; Roche, Grenzach-Whylen, Germany; 2×50 mg/kg resuspended in sterile filtrated $1 \times$ phosphate-buffered saline [PBS]) for 7 consecutive days intraperitoneally or intralesionally. After tumor remission, mice were analyzed for up to 6 months for potential tumor relapse.

Plasmids and cells

Vector plasmid pMO3N expressing the neomycin-resistance cDNA (neoR) under control of the LTR of Moloney murine leukemia virus (MLV) has been described.[19] The suicide gene vector plasmid pMO3TIN was constructed by inserting the herpes simplex virus–thymidine kinase (HSV-TK) cDNA as a *Not*I-*Hin*dIII fragment along with the internal ribosomal entry site (IRES) of poliovirus (*Hin*dIII-*Not*I fragment) into the *Not*I site of pMO3N, upstream of neoR. Phoenix-gag-pol packaging cells and human HT-1080 fibrosarcoma cells (American Type Culture Collection [ATCC], Manassas, VA; no. CCL-121) were grown in Dulbecco modified Eagle medium (DMEM), mouse EL-4 lymphoma cells (ATCC; no. TIB-39) in Roswell Park Memorial Institute 1640 (RPMI). All media were supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, and 100 U/mL penicillin/streptomycin.

Retrovirus vector production

The day before transfection of Phoenix-gp retroviral packaging cells, 5×10^6 cells were plated in a 9-cm Petri dish. For transfection, the medium was exchanged and 25 μM chloroquine (Sigma, München, Germany) was added. Retroviral vector DNA (3-5 μg) was transfected using the calcium phosphate precipitation method. To generate vesicular stomatitis virus-G (VSV-G)–pseudotyped particles, 2 μg M57 (MLV gag-pol expression plasmid) and 2 to 3 μg M4 (VSV-G expression plasmid) were transfected in addition. Medium was changed after 6 to 8 hours. Supernatants containing viral particles were collected 24 to 84 hours after transfection, filtered through a 0.45-μm filter (Millipore, Schwalbach, Germany), and used to transduce 2×10^5 target cells (EL-4). Transduction was assisted by addition of 4 μg/mL protamine sulfate and centrifugation for 60 minutes at 2000 rpm (200*g*) and 25°C to 32°C. Vector titers were determined by transducing predefined numbers of HT-1080 target cells with serial dilutions of supernatant and analyzing the number of surviving cell clones after geneticin transfer unit (GTU) analysis.

Southern blot

Genomic DNA was isolated from vector-transduced cell clones prior to transplantation into mice and after isolation of tumor cells from relapsed mice, respectively, using the DNazol Genomic DNA Isolation Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. DNA was digested using *Hin*dIII or *Sac*I as restriction enzymes allowing to investigate copy number or integrity of integrated vectors, respectively. Southern blots were performed according to standard protocols. Nylon membranes were hybridized with a radioactively labeled ([alpha ^{32}P] deoxycytosine triphosphate [dCTP]; Hartmann Analytic, Braunschweig, Germany) HSV1-TK full-length probe (*Not*I-*Hin*dIII vector fragment).

PCR

Each polymerase chain reaction (PCR) was performed in a final volume of 50 μL containing 5 μL of $10 \times$ *Taq* buffer (Gibco BRL Life Technologies, Eggenstein, Germany), 5 μL 15 mmol $MgCl_2$ (Gibco BRL Life Technologies), 0.2 μL 10 mM deoxynucleoside triphosphates (dNTPs; Geneo BioProducts GmbH, Hamburg, Germany), 5 μL $10 \times$Yellow Sub (Geneo Bioproducts GmbH), 0.2 μL (5 U/μL) *Taq* polymerase (Gibco BRL Life Technologies), 1 μL 16 μmol for each primer (LTR1, 5′-TGT TTC CAG GGT GCC CCA AGG-3′; LTR2, 5′-CAC TCA GAG GAG ACC CTC CC-3′),[20] and 1 μL (1 μg/μL) genomic DNA as a template (initial denaturation step for 3 minutes at 94°C; 36 cycles of denaturation for 30 seconds at 94°C, annealing for 15 seconds at 52.4°C, and elongation for 15 seconds at 72°C). The resulting LTR-specific PCR product (248 bp) was run on an agarose gel (1.5 g agarose per 100 mL) stained with ethidium bromide. As a control for PCR, additional reactions with murine *Xist* gene–specific primers (X2-R, 5′-GAA GTG AAT TGA AGT TTT GGT CTA G-3′; X2-L, 5′-GGG ACC TAA CTG TTG GCT TTA TCA G-3′; generous gift from Gerald Schumann, HPI) were performed resulting in a 202-bp PCR product (firstly, 2 cycles each of denaturation for 4 minutes at 94°C, annealing for 1 minute at 54.1°C, elongation for 2 minutes at 72°C; secondly, 30 cycles each of denaturation for 1 minute at 94°C, annealing for 1 minute at 54.1°C, elongation for 1 minute at 72°C; and final product extension for 3 minutes at 72°C).

Ligation-mediated PCR (LM-PCR)

LM-PCR was performed essentially according to a published protocol by Schmidt et al.[21] Briefly, genomic DNA isolated from retroviral vector MO3TIN–transduced EL-4 cells was digested with 5 U of restriction enzyme *Rsa*I (New England Biolabs, Frankfurt, Germany) per μg of DNA for 2 hours at 37°C. After standard ethanol precipitation of the heat-inactivated reaction primer, extension with a vector LTR-specific 5′-biotinylated primer (rvLTRI; TIB MOLBIOL, Berlin, Germany) was performed (phycoerythrin [PE], 95°C for 5 minutes, 64°C for 30 minutes, 72°C for 15 minutes). Purified biotinylated PCR products were extracted via streptavidin-labeled magnetic beads (Dynal, Hamburg, Germany). The captured DNA was washed twice with 100 μL of H_2O and resuspended in 7 μL H_2O for overnight ligation at 16°C with 100 pmol of the annealed linker cassette (consisting of oligonucleotides NZ117, 5′-GAC CCG GGA GAT CTG AAT TCA GTG GCA CAG CAG TTA GG-3′; and NZ118, 5′-p CCT AAC TGC TGT GCC ACT GAA TTC AGA TCT CCC G-3′; sequence kindly provided by Nora Zingler, HPI) in the presence of 6 U of T4 DNA ligase (Promega, Mannheim, Germany). A wash step with H_2O preceded first-round PCR using primer combination rvLTRII and OCI (initial denaturation step for 5 minutes at 95°C; 30 cycles of denaturation for 1 minute at 95°C, annealing for 45 seconds at 58°C, and elongation for 1.5 minutes at 72°C; and final product extension for 10 minutes at 72°C). Nested PCR with rvLTRIII and OCII primers was performed under cycling conditions identical to those used for first PCR. Amplified products were separated by preparative agarose gel electrophoresis (2 g agarose per 100 mL), purified using Qiagen Gelelectrophoresis Isolation Kit (Qiagen, Hilden, Germany), and sequenced (SequiServe, Vaterstetten, Germany).

Chromosome preparation and spectral karyotyping (SKY analysis)

For chromosome preparation, cultured EL-4 cells were treated with colcemid for 1 hour in a concentration of 0.035 μg/mL, incubated in 0.075 M KCl for 20 minutes at 37°C, and fixed in freshly prepared methanol–acetic acid (3:1) at room temperature. Cell suspension was dropped onto glass slides in a climate chamber (Polymehr, Kassel, Germany) at 22°C and 48% humidity. Metaphase chromosomes were denatured in 70% formamide and $2 \times$ standard saline citrate (SSC) for 1.5 minutes and dehydrated. The mouse SKY probe mixture (Applied Spectral Imaging, Migdal Ha'Emek, Israel) was denatured at 80°C for 7 minutes and preannealed at 37°C for 1 hour. Hybridization took place for 48 to 72 hours at 37°C in a humidified chamber. Signal detection procedure was carried out according to the manufacturer's instructions (Applied Spectral Imaging). Chromosomes were counterstained with DAPI (4,6 diamidino-2-phenylindole) and mounted with antifade solution. For image acquisition, the SpectraCube system (Applied Spectral Imaging) coupled to an epifluorescence microscope (Carl Zeiss Jena GmbH, Jena, Germany) was used. Chromosome analysis was performed using the SKYView software package (Applied Spectral Imaging).

Statistical analyses

On the basis of the measured mean diameter of the injected hind legs, we calculated the mean number of EL-4 cells present in the tumors at the beginning of GCV treatment (day 10 after transplantation). With 1×10^5 originally injected cells and an in vivo doubling time of 38.4 hours (see "Results", Figure 1B), this corresponded to approximately 6 doublings. On the basis of the actual sizes of tumors, the numbers of EL-4 cells in each tumor at the beginning of GCV treatment were determined. This was possible by calculating the doublings needed to reach a particular tumor size (eg, ~8 doublings in a tumor of 8.5-mm diameter) and apply this value (n) to the formula $f_{(x)} = y \times 2^n$, where y is the number of originally transplanted EL-4 cells (1×10^5). Taking into account the time period needed from beginning of GCV treatment to relapse, we were thus able to compute the numbers of GCV-resistant cells existent at the start of treatment. To obtain the mutation frequencies for a particular resistance mechanism in a given clone, the number of GCV-resistant cells existent at the start of treatment was divided by the total number of tumor cells present at this time point. Statistical analysis was performed with SAS software (release 8.02; SAS Institute, Cary, NC).

Results

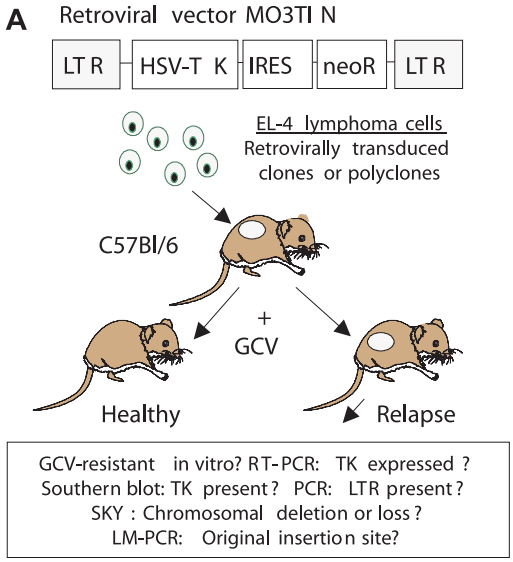
Retroviral transduction, selection, and inoculation of EL-4 cells

The experimental setup is shown in Figure 1A. The retroviral vector MO3TIN, expressing the genes for HSV-TK and neoR linked by an IRES of poliovirus, was produced as cell-free supernatant of safety-modified packaging cells and titrated on human HT-1080 cells (determining GTU). For tumor induction experiments, we chose EL-4 lymphoma cells that revealed no evidence of replicating retrovirus (data not shown) and induced a malignant tumor when injected into congeneic immunocompetent C57Bl/6 mice. EL-4 cells were transduced in vitro with a multiplicity of infection less than 1, resulting in a single transgene copy in the majority of transduced cells. This mimics potential limitations encountered in retroviral transgene delivery into tumor cells in vivo and also represents the desired outcome of retroviral gene transfer in the genetic modification of somatic stem cells.[22] MO3TIN-transduced cells were selected in vitro using G418 for 14 days, using concentrations of 1.2 mg/mL. This ensured complete elimination of untransduced cells, as previous dose-finding experiments revealed that concentrations of 0.6 to 0.8 mg/mL were sufficient to kill untransduced EL-4 cells.[23]

Subcutaneous transplantation of uncloned, neoR-resistant EL-4 cells reproducibly resulted in rapid tumor formation in C57Bl/6 mice. The in vivo doubling time was in the range of 40 hours (Figure 1B).[24] Thus, transgene expression did not give rise to immunologic rejection of EL-4 cells.[18] Subsequent experiments addressed whether local or systemic treatment with GCV could reverse the EL-4 tumors. In the case of relapse, detailed analyses were performed to identify the underlying mechanisms (Figure 1A).

GCV ("suicide") treatment and relapse of polyclonal tumors

At the beginning of visible tumor growth (day 10, equivalent to ~6 population doublings), mice were injected twice daily for 7 days with GCV (50 mg/kg body weight), given either intraperitoneally or intralesionally. Both regimens resulted in remission of established lesions. However, prolonged observation (for 4 weeks) of mice that received transplants of uncloned putative MO3TIN-transduced EL-4 cells showed local relapse with an incidence of 100%. When material from preliminary tests was analyzed by PCR and Southern blot, the transgene could not be detected in relapsed tumors (data not shown).[23] As survival of untransduced EL-4 cells during G418 selection in vitro was unlikely due to the stringency of G418 selection, this indicated genetic instability occurring during in vivo passage, resulting in loss (partial) of the transgenic allele.



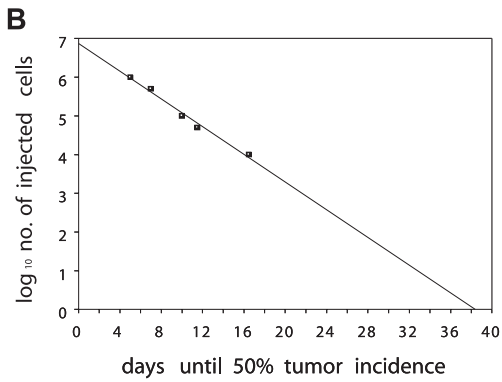


Figure 1. Experimental outline. (A) Schematic representation of the retroviral suicide gene vector MO3TIN and the EL-4 tumor model used in this study. The experiments performed to address the potential mechanisms of escape from ganciclovir (GCV) suicide treatment are summarized in the box. (B) Determination of the in vivo doubling time of tumorigenic EL-4 cells in C57Bl/6 mice. A regression line was extrapolated after plotting on the y-axis the values of the logarithms$_{10}$ for administered EL-4 cells with numbers of 1×10^4 (n = 7); 5×10^4 (n = 5); 1×10^5 (n = 12); 5×10^5 (n = 5); and 1×10^6(n = 12) versus days until 50% tumor incidence (according to the method of Skipper).[24]

Relapse after GCV ("suicide") treatment even occurs in clonal tumors

To fully exclude that relapse occurred from residual cells surviving G418 selection and to analyze potential clonal variability in escape mechanisms, 6 of 9 independent G418-resistant MO3TIN-transduced EL-4 clones with single vector insertions (confirmed by Southern blot; data not shown)[23] were used in further investigations. To verify intact TK function prior to tumor inoculation, cells from each of the 6 clones were treated with GCV. More than 95% cell death was observed in all cultures. Thus, the TK gene was intact and functionally expressed in the vast majority of independent clones. This indicated that retroviral transduction of truncated versions of TK was not a frequent event in our experimental setting, in line with recent findings.[10] However, despite high rates of initial cell killing, GCV-resistant subclones from 3 of the

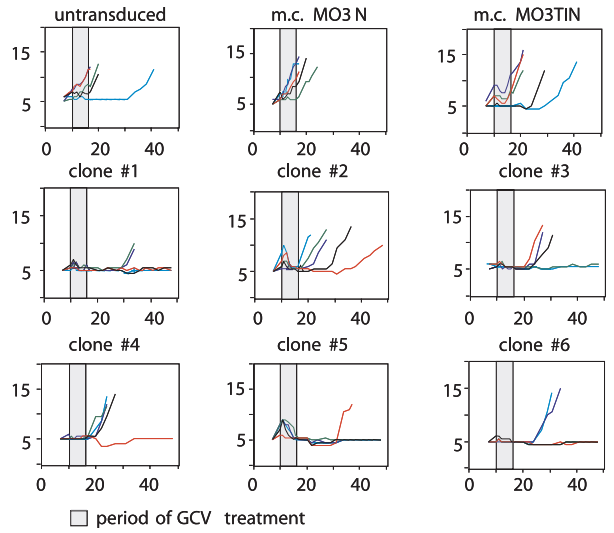


Figure 2. Tumor relapses after suicide gene therapy in animals that received transplants of either uncloned or cloned MO3TIN cells occur with variable kinetics. The tumor size (y-axis, diameter of the hind leg in mm) was determined in mice (n = 5 per group) at regular intervals (days after inoculation, x-axis). m.c. indicates mass culture; all clones are transduced with a single copy of the MO3TIN vector.

analyzed clones could be recovered, indicating a low but detectable mutation frequency to GCV resistance in vitro. Control G418-resistant EL-4 cells transduced with a similar retroviral vector lacking TK (MO3N) remained resistant to GCV toxicity in culture, as wild-type EL-4 cells.

Six clones containing an intact MO3TIN transgene as well as 2 uncloned G418-selected mass cultures (transduced with either MO3TIN or MO3N) were inoculated into 5 mice per group. Rapid tumor outgrowth was observed in animals that received transplants of MO3N-transduced cells with or without GCV administration. Again, initial tumor remission was observed after administration of GCV in all animals that received transplants of MO3TIN-transduced cells. However, relapses occurred in all animal groups that received transplants of either cloned or uncloned MO3TIN cells. Three of the 5 recipients of cells derived from the MO3TIN mass culture showed onset of relapse already at the end of the GCV treatment period, indicative of a relatively high frequency of escape mutants. Between the different MO3TIN clones, the incidence and kinetics of relapse showed a strong variability (Figure 2; Table 1).

Clone no. 2 showed the greatest incidence of relapse (5/5) and the greatest extent of subclonal variability (Figure 2). For clone nos. 1, 3, 4, and 5, the experiment was repeated. Among these 4 clones, no. 5 showed the lowest incidence of relapse (1/10 animals) and no. 4 the highest (9/10 animals; Table 1). The average incidence of tumor relapses from clonal tumors was 48.89% (95% confidence limit, 0.3370-0.6423). By using Fisher exact test we found a highly significant association between the incidence of tumor relapses and the respective clone ($P = .0001$), suggesting an important role of the vector insertion site.

Genetic analysis of escape mutants

To analyze the mechanisms of escape from GCV selection, we isolated relapsed tumor cells. In contrast to their clonal progenitors, all relapsed cells were resistant to GCV in vitro, ruling out that the drug regimens were inefficient in dose, penetration, or timing. Based on Southern blot analysis (data not shown),[23] relapsed tumor cells originating from MO3TIN-transduced mass cultures showed a monoclonal or oligoclonal pattern with respect to the vector insertion site. In relapsed tumors originating from defined clones, neither neoR nor TK transcripts were detected by reverse transcriptase–PCR (RT-PCR; data not shown). For a more detailed analysis, we used a PCR to detect residual LTR sequences of the vector[20] and checked all relapsed clones using this PCR in addition to the Southern blot that only detected the presence of TK. Southern blot (data not shown)[23] and PCR data (Figure 3) revealed that the GCV-resistant subclones of clone no. 4 (high incidence of relapse with relatively early manifestation) still showed the presence of an intact transgene. In contrast, in all other relapsed tumors we observed either a partial loss of transgene (ie, Southern blot TK negative, PCR LTR positive, as in the single GCV-resistant subclone of no. 5 and the GCV-resistant subclones nos. 2-1, 2-5, and 3-4) or a complete loss of transgene (ie, both LTR-PCR and TK-Southern blot negative as in all other GCV-resistant subclones of nos. 1, 2, 3, and 6).

Three mechanisms of escape: chromosomal deletion, postinsertional recombination, and gene silencing

To address how the complete loss of a retrovirally transduced transgene could be explained, we analyzed the karyotypes of clone no. 1 and a relapsed subclone thereof (no. 1-3) recovered after in vivo GCV treatment. Highly sensitive SKY analysis of subclone

Table 1. Differences in the incidence of tumor relapses following GCV treatment in vivo

Transplanted cells	No. of mice with complete remission, +GCV	No. of mice with tumor relapse after GCV treatment	Mean survival time after tumor relapse, d	Escape mechanism of independent tumors
EL-4, mock	1/5	5/5	23.0 ± 7.2	NA
Mass culture, MO3N	0/5	5/5	19.0 ± 2.4	NA
Mass culture, MO3TIN	2/5	5/5	26.6 ± 6.7	ND
Clone no. 1, MO3TIN; Exp 1	5/5	2/5	35.5 ± 1.5	2 CD/CL
Clone no. 1, MO3TIN; Exp 2	5/5	0/5	NA	NA
Clone no. 2, MO3TIN	4/5	5/5	31.8 ± 8.2	3 CD/CL, 2 PR
Clone no. 3, MO3TIN; Exp 1	5/5	3/5	28.3 ± 1.8	2 CD/CL, 1 PR
Clone no. 3, MO3TIN; Exp 2	5/5	3/5	34.3 ± 6.4	ND
Clone no. 4, MO3TIN; Exp 1	5/5	4/5	24.8 ± 1.1	4 GS
Clone no. 4, MO3TIN; Exp 2	5/5	5/5	27.2 ± 3.4	5 GS
Clone no. 5, MO3TIN; Exp 1	5/5	1/5	37.0	1 PR
Clone no. 5, MO3TIN; Exp 2	5/5	0/5	NA	NA
Clone no. 6, MO3TIN	5/5	2/5	32.5 ± 1.5	2 CD/CL

± indicates SEM; NA, not applicable; ND, not determined; CD/CL, chromosomal deletion and/or chromosomal loss; PR, postinsertional recombination; and GS, gene silencing.

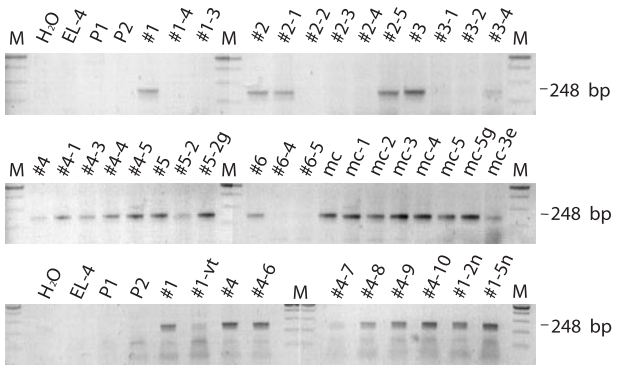


Figure 3. PCR analysis of GCV-sensitive and GCV-resistant cells. The PCR reaction detects LTR sequences of the retroviral vector.[20] Tumors with a negative Southern blot (TK probe; data not shown)[23] and a positive PCR reaction were numbers 2-1, 2-5, 3-4, and 5-2. Clones with ambiguous results were repeatedly analyzed. Starting material for PCR was gDNA; M, 100-bp ladder DNA marker; EL-4, untransduced EL-4 cell gDNA; P1 and P2, primers LTR1 and LTR2 tested on number 1 gDNA; g, a split from isolated tumor cells had been cultivated further and was selected additionally with G418 before gDNA extraction; mc-1 to mc-5, tumor cells isolated from relapsed mice that originally received transplants of vector-transduced mass culture (mc) cells; e, tumor cells had been isolated from mice that were treated with GCV beginning one day after tumor cell inoculation; vt, EL-4 cells isolated after GCV selection in vitro; and n, tumor cells isolated from mice that had not been treated with GCV. DNA from clones with a negative result in the LTR-PCR was successfully amplified using primers annealing to the endogenous *Xist* gene (see "PCR" in "Materials and methods").

no. 1-3 showed the loss of regions A1 to B2/3 from chromosome 6 of one defined derivative fusion chromosome [Der(19)T(6B;19D)] (Figure 4; Table 2). The same chromosomal loss was observed (SKY data not shown) in a GCV-resistant subclone recovered after treating clone no. 1 with GCV in vitro (see "Relapse after GCV ("suicide") treatment even occurs in clonal tumors").

For each of the 6 clones, the insertion site of the retroviral vector (Table 3) was identified by LM-PCR, sequence analysis, and databank search.[21] None of the loci coincided with potential or established oncogenes characterized by studies with murine replication-competent viruses.[26] Therefore, it is unlikely that insertional activation of oncogenes or disruption of tumor suppressor genes were the cause of the differential resistance patterns of the clones. In clone no. 1, sequence data were consistent with integration in the above-mentioned fusion chromosome (region B3). Therefore, we conclude that loss of the retrovirally delivered transgene cassette by chromosomal deletion and/or chromosomal loss was the underlying mechanism of escape from suicide gene therapy in the tumor subclones of clone no. 1 (eg, no. 1-3) and possibly other relapsed tumors of clone nos. 2, 3, and 6, where no vector sequences could be detected by Southern blot and PCR analysis.

As mentioned in "Genetic analysis of escape mutants," the PCR analysis detected LTR sequences in other subclones that showed no TK transgene in the Southern blot (eg, nos. 2-1 and 5-2). Such a result was consistent with postinsertional recombination as the mechanism of escape, possibly involving the LTR sequences.[27,28] Interestingly, the strongest interanimal variability with respect to the latency of relapse was observed in clones showing 2 different mechanisms of escape in their subclones, either chromosomal deletion and/or chromosomal loss, or postinsertional recombination (Table 1; Figure 2). Statistical analyses (Fisher exact test) revealed a highly significant association between the investigated resistance mechanism against GCV and the various clones ($P = .000\,006$).

The earliest relapse, indicating a relatively high mutation frequency, was observed with clone no. 4, in which silencing of expression of the intact transgene occurred in all subclones analyzed (Table 1). The known tumor doubling time, the size of the tumor at the beginning of GCV exposure, and the latency of relapse allowed us to determine the mutation frequency to GCV resistance per cell (Table 3). As it remained unknown which fraction of the GCV-resistant cells might have been eliminated by bystander effects or a potential immune response, the actual mutation frequencies may even have been higher. Thus, the mutation rate of the clone escaping by gene silencing was at least 9×10^{-2}. Possibly depending on the insertion site, postinsertional recombination occurred with frequencies ranging from 3×10^{-2} to 2×10^{-5} and chromosomal deletion and/or chromosomal loss with frequencies ranging from 2×10^{-3} to 8×10^{-5}. If similar mutation rates would occur in the scenario of "proactive" suicide gene delivery prior to clonal expansion in vivo, any of these mechanisms would produce a relatively high number of potential suicide-resistant cells.

Discussion

The present study reveals that both genetic and epigenetic instability limit the efficiency of retroviral suicide gene therapy of malignant tumors. Besides epigenetic gene silencing, we identified 2 genetic mechanisms of escape: postinsertional recombination, an event that can be triggered by the sequence repetitions in the LTRs,[27,28] and deletion of neighboring chromosomal sequences along with the retroviral insertion site. Escape from suicide gene therapy was observed in all uncloned and cloned tumor populations analyzed. The type and incidence of escape mutants were probably related to the insertion site of the vector, as independent clones differed with respect to the mechanisms of relapse ($P = .000\,006$). Importantly, as killing of mutant bystander cells[1,4] and systemic immunity[6,29,30] are typically extending the efficiency of the GCV-mediated suicide to neighboring cells not actively expressing TK, the incidence of escape variants may have been even higher than detected in our study (range, 3×10^{-2} to 8×10^{-5} depending on clone and mechanism).

Besides the dependence on the transgene insertion site, the incidence and type of escape variants is probably influenced by additional factors, such as the type of genetic alterations underlying tumor development, the clonal expansion of tumor cells following gene transfer prior to activation of the suicide mechanism, and the type of vector system used. Additional possibilities for escape

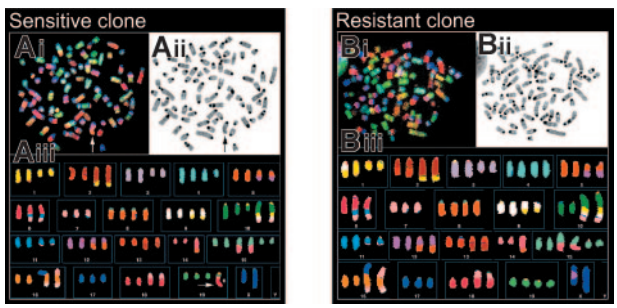


Figure 4. Spectral karyotyping shows loss of transduced chromosome in relapsed tumor. Spectral karyotyping (SKY analysis) of metaphases from a GCV-sensitive and a GCV-resistant clone. (Ai, Bi) Original RGB (red-green-blue) image after acquisition; (Aii, Bii) inverted DAPI image showing chromosome banding; and (Aiii, Biii) SKY karyotype after chromosome classification. (Aiii) The sensitive cell showed 3 normal chromosomes 19 and a Der(19)T(6B;19D) (arrow); (Biii) the resistant cell revealed 4 normal chromosomes 19 and has thus lost the marker Der(19)T(6B;19D). The complete karyotype is provided in Table 2.

Table 2. Karyotypes of GCV-sensitive clone no. 1 and GCV-resistant subclone no. 1-3

Karyotype of clone no. 1	Karyotype of GCV-resistant subclone no. 1-3
74-77, X,	73-77, X,
Dup(XF4-5D),	Dup(XF4-5D),
Del(1F)×2,	Del(1F)×2,
T(2E;13A)×2,	T(2E;13A)×2,
Der(2)T(1F;2H)×2,	Der(2)T(1F;2H)×2,
Del(3F)×2,	Del(3F)×2,
Del(4D),	Del(4D),
T(5D;12C)×2,	T(5D;12C)×2,
−6,	−6,
Der(6)T(6D;11B2)T(11D;7F)×2,	Der(6)T(6D;11B2)T(11D;7F)×2,
Del(7E),	Del(7E),
Der(7)T(7F;11D)×2,	Der(7)T(7F;11D)×2,
Der(9)T(1F;9E)×2,	Der(9)T(1F;9E)×2,
+10,	+10,
?T(10D;10B),	?T(10D;10B),
Der(10)T(10D;1D)T(1F;9D)T(9F;7F)×2,	Der(10)T(10D;1D)T(1F;9D)T(9F;7F)×2,
Der(11)T(6D;11B2)×2,	Der(11)T(6D;11B2)×2,
Del(13A2A5)×2,	Del(13A2A5)×2,
−14,	−14,
Del(14B)×2,	Del(14B)×2,
Der(14)T(8E;14E),	Der(14)T(8E;14E),
+Del(15B)×2,	+Del(15B)×2,
Der(15)T(14B;15E)×2,	Der(15)T(14B;15E)×2,
RT(16;17)T(16C1;7F?2)T(7F?4;9F),	RT(16;17)T(16C1;7F?2)T(7F?4;9F),
Dic(7;16)(7B;16cen)T(16C1;7F?2)T(7F?4;9F),	Dic(7;16)(7B;16cen)T(16C1;7F?2)T(7F?4;9F),
Dic(7B;18cen)×2,	Dic(7B;18cen)×2
Der(19)T(6B;19D)	Not detected

The deletion of one of the chromosomes 4 described in the karyotype for clone no. 1 is unfortunately not as clearly recognizable in the resistant clone as in the sensitive clone shown in Figure 4, although it was detected in both clones. The additional chromosome 10 of clone no. 1 described is only absent in the metaphase shown in Figure 4. We analyzed 10 metaphases of each clone.

mechanisms (not investigated by us) exist in transgene recombinations occurring prior to incorporation into vector particles[10] or at the level of gene transfer, in the response of tumor cells to the suicide mechanism, and in insufficient drug delivery.

It is likely that higher transgene copy numbers per single cells, or the combined transfer of different suicide transgenes,[6] will decrease the risk for genetic or epigenetic escape from negative selection. However, increased transgene copy numbers of integrating vectors may be difficult to achieve or result in a higher risk for healthy tissues, given the current limitations in tumor cell targeting in vivo.[22] More promising options to reduce the incidence of escape variants in reactive scenarios of suicide gene therapy are an early application of gene transfer in younger stages of tumor development and activation of the suicide mechanism shortly after (retroviral or episomal) gene transfer, before variants with genetic or epigenetic instability develop.

When considering the proactive use of suicide genes to delete malignant clones with insertional up-regulation of protooncogenes,[11,12,22] the escape mechanisms of gene silencing or chromosomal deletion/chromosomal loss could also involve the activated protooncogene. If this represented a necessary signal alteration for tumor cell survival, such clones would be spontaneously extinguished shortly after their occurrence. However, postinsertional recombination may preserve the activated protooncogene while deleting the suicide gene, given that

Table 3. Clonal variation of tumor cell escape due to genetic and epigenetic instability

Clone no.	Postinsertional recombination	Chromosomal deletion and/or chromosomal loss	Gene silencing	Vector integration site	Next flanking genes (start of transcription)
1	ND ($< 2.3 \times 10^{-8}$)	8.2×10^{-5}	ND ($< 2.3 \times 10^{-8}$)	Chromosome 6 (B3; start 49122055 bp, end 49122282 bp)	AI854703 (49074600 bp); NM_174960 (49122891 bp)
2	2.9×10^{-2}	6.4×10^{-3}	ND ($< 2.0 \times 10^{-8}$)	Chromosome 3 (H2; start 141965299 bp, end 141965563 bp)	Dapp1 (141855603 bp); NM_177860 (141972963 bp)
3	3.2×10^{-3}	8.3×10^{-3}	ND($< 4.7 \times 10^{-8}$)	Chromosome 13 (D2.1; start 107923565 bp, end 107924277 bp)	9630011N22Rik (107807963 bp); Pde4d (108438579 bp)
4	ND ($< 2.9 \times 10^{-8}$)	ND ($< 2.9 \times 10^{-8}$)	8.9×10^{-2}	Chromosome 11 (E2; start 120811840 bp, end 120811863 bp)	4430402O11Rik (120790163 bp); 1110054H05Rik (120849830 bp)
5	1.8×10^{-5}	ND ($< 8.1 \times 10^{-9}$)	ND ($< 8.1 \times 10^{-9}$)	Chromosome 1 (E3; start 129935602 bp, end 129935674 bp)	D130011D22Rik (129221654 bp); Mgat5 (130003677 bp)
6	ND ($< 6.3 \times 10^{-8}$)	2.4×10^{-3}	ND ($< 6.3 \times 10^{-8}$)	Chromosome 6 (B2; start 47840185 bp, end 47840326 bp)	Cull (47797245 bp); Ezh2 (47842412 bp)

Frequency values are the ratio of the calculated number of initial GCV-resistant cells (based on the size and latency of the relapsed tumor) to the total number of tumor cells at the onset of GCV therapy ("Materials and methods"). The localisations of the vector insertion sites were mapped with reference to the ENSEMBL database[25] using the BlastSearch function. Next flanking genes with respect to the insertion site contain those which are ENSEMBL-predicted and known. ND indicates not detectable. In the "Vector integration site" column, the information in parentheses describes the chromosomal region and the matching sequences.

those cis-active elements of the LTR, which were involved in the activation of a protooncogene, remain intact. Postinsertional recombination may also maintain the potential disruption of a tumor suppressor gene. If, however, tumor progression became independent of the initial insertional lesion, all 3 escape mechanisms may foster the survival of mutants.

The experimental system established here may be modified to address the relevance of these different obstacles and develop possible solutions. In this context, it will also be interesting to test the impact of the vector backbone on the incidence of escape from suicide gene therapy. Lentiviral vectors as opposed to the MLV-based vectors studied here not only show a stronger trend to insert into transcribed regions of cellular genes[31] but also are typically constructed with self-inactivating LTRs.[32] Both features may reduce the incidence of suicide escape mechanisms. Specifically, insertion into active genes may lower the rate of gene silencing, and the shorter regions of sequence identity found in self-inactivating LTRs may prevent postinsertional recombinations within the integrated transgene.

Our ongoing experiments in the mouse model have revealed the risk of leukemia induction by combinatorial protooncogene activations from independent retroviral insertions in single hematopoietic cells.[33] For the proactive use of suicide genes in experimental conditions where cells will need to be transduced with more than one transgene copy to overcome a therapeutic threshold, our data suggest a positive conclusion. In such a case, the incidence of escape would be expected to be too low to allow the survival of mutants with 2 or more spontaneous recombination or silencing events.

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References

1. Moolten FL. Drug sensitivity ("suicide") genes for selective cancer chemotherapy. Cancer Gene Ther. 1994;1:279-287.
2. Mullen CA. Metabolic suicide genes in gene therapy. Pharmacol Ther. 1994;63:199-207.
3. Spencer DM. Developments in suicide genes for preclinical and clinical applications. Curr Opin Mol Ther. 2000;2:433-440.
4. van Dillen IJ, Mulder NH, Vaalburg W, de Vries E F, Hospers GA. Influence of the bystander effect on HSV-tk/GCV gene therapy: a review. Curr Gene Ther. 2002;2:307-322.
5. Fillat C, Carrio M, Cascante A, Sangro B. Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. Curr Gene Ther. 2003;3:13-26.
6. Uckert W, Kammertons T, Haack K, et al. Double suicide gene (cytosine deaminase and herpes simplex virus thymidine kinase) but not single gene transfer allows reliable elimination of tumor cells in vivo. Hum Gene Ther. 1998;9:855-865.
7. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. Hum Gene Ther. 2001;12:861-870.
8. Ogris M, Wagner E. Targeting tumors with non-viral gene delivery systems. Drug Discov Today. 2002;7:479-485.
9. Schatzlein AG. Non-viral vectors in cancer gene therapy: principles and progress. Anticancer Drugs. 2001;12:275-304.
10. Ebeling SB, Eric Borst HP, Simonetti ER, et al. Development and application of quantitative real time PCR and RT-PCR assays that discriminate between the full-length and truncated herpes simplex virus thymidine kinase gene. J Virol Methods. 2003;109:177-186.
11. Li Z, Düllmann J, Schiedlmeier B, et al. Murine leukemia induced by retroviral gene marking. Science. 2002;296:497.
12. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science. 2003;302:415-419.
13. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer. 2001;1:157-162.
14. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature. 1998;396:643-649.
15. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene. 2002;21:5400-5413.
16. Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol. 2002;196:1-7.
17. Zhou ZH, Akgün E, Jasin M. Repeat expansion by homologous recombination in the mouse germ line at palindromic sequences. Proc Natl Acad Sci USA. 2001;98:8326-8333.
18. Stripecke R, Carmen Villacres M, Skelton D, Satake N, Halene S, Kohn D. Immune response to green fluorescent protein: implications for gene therapy. Gene Ther. 1999;6:1305-1312.
19. Hildinger M, Eckert HG, Schilz AJ, John J, Ostertag W, Baum C. FMEV vectors: both retroviral long terminal repeat and leader are important for high expression in transduced hematopoietic cells. Gene Ther. 1998;5:1575-1579.
20. Morgan RA, Cornetta K, Anderson WF. Applications of the polymerase chain reaction in retroviral-mediated gene transfer and the analysis of gene-marked human TIL cells. Hum Gene Ther. 1990;1:135-149.
21. Schmidt M, Hoffmann G, Wissler M, et al. Detection and direct genomic sequencing of multiple rare unknown flanking DNA in highly complex samples. Hum Gene Ther. 2001;12:743-749.
22. Baum C, Düllmann J, Li Z, et al. Side effects of retroviral gene transfer into hematopoietic stem cells. Blood. 2003;101:2099-2114.
23. Frank O. Abschaltungsmechanismen retroviraler Gensequenzen: Konstruktion verbesserter Vektoren für die somatische Gentherapie beim Menschen: Thesis 2002 at University of Hamburg. http://www.sub.uni-hamburg.de/opus/volltexte/2002/788/pdf/dissertation.pdf. Accessed January 2, 2003.
24. Golumbek PT, Hamzeh FM, Jaffee EM, Levitsky H, Lietman PS, Pardoll DM. Herpes simplex-1 virus thymidine kinase gene is unable to completely eliminate live, nonimmunogenic tumor cell vaccines. J Immunother. 1992;12:224-230.
25. The Wellcome Trust Sanger Institute. ENSEMBL database. http://www.ensembl.org/Mus_musculus/. Accessed April 15, 2004.
26. Akagi K, Suzuki T, Stephens RM, Jenkins NA, Copeland NG. RTCGD: retroviral tagged cancer gene database. Nucleic Acids Res. 2004;32:D523-D527.
27. Copeland NG, Hutchison KW, Jenkins NA. Excision of the DBA ecotropic provirus in dilute coat-color revertants of mice occurs by homologous recombination involving the viral LTRs. Cell. 1983;33:379-387.
28. Stoye JP, Fenner S, Greenoak GE, Moran C, Coffin JM. Role of endogenous retroviruses as mutagens: the hairless mutation of mice. Cell. 1988;54:383-391.
29. Zhang L, Wikenheiser KA, Whitsett JA. Limitations of retrovirus-mediated HSV-tk gene transfer to pulmonary adenocarcinoma cells in vitro and in vivo. Hum Gene Ther. 1997;8:563-574.
30. Vile RG, Nelson JA, Castleden S, Chong H, Hart IR. Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. Cancer Res. 1994;54:6228-6234.
31. Wu X, Li Y, Crise B, Burgess SM. Transcription start regions in the human genome are favored targets for MLV integration. Science. 2003;300:1749-1751.
32. Ailles LE, Naldini L. HIV-1-derived lentiviral vectors. Curr Top Microbiol Immunol. 2002;261:31-52.
33. Modlich U, Meyer J, Rudolph C, et al. Reproducible insertional leukemogenesis with evidence for chromosomal instability following dose-escalated retroviral vector transfer into murine hematopoietic cells [abstract]. Blood. 2003;102:200a. Abstract 699.