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Gene Therapy for Sarcoma

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Key Words
Sarcoma · Myeloprotective gene transfer · Multidrug resistance 1 gene · Retroviral vector · Suicide gene transfer · Thymidine kinase gene · Adenoassociated virus-2 vector

Abstract
Soft tissue sarcomas are mesenchymal tumors which respond poorly to systemic therapy. Recent studies suggest a higher response rate with an increased doxorubicin dosage. However, this was parallel with a profound hematotoxicity in 75% of patients. Transfer of the human *multidrug resistance* 1 (*MDR*1) gene to normal hematopoietic stem cells and transplantation may significantly reduce the hematotoxicity of anthracyclin-based chemotherapy. To test this concept of supportive gene therapy in advance of a clinical study, we transduced mobilized peripheral blood progenitor cells (PBPC) with the retroviral vector SF91m3 containing the human *MDR*1 gene, transplanted these cells to immune-deficient mice, allowed 6 weeks for engraftment to occur and treated the animals with *MDR*1-based chemotherapy. In the *MDR*1-transduced group the human leukocytes were significantly protected from the toxicity of chemotherapy ($p < 0.05$). While the gene transfer rate was in the range of 10% and thus comparable to recent clinical trials, the gene expression was 59% of transduced cells and thus significantly higher than previously reported for less-advanced vectors. On the other hand, ifosfamide, a drug which has been used successfully for stem cell mobilization, is active in soft tissue sarcoma. Due to these favorable characteristics sarcoma is an attractive target to test the efficacy of *MDR*1 gene therapy in a clinical setting.

Abbreviations used in this paper

AAV-2	adenoassociated virus 2
G-CSF	granulocyte colony-stimulating factor
GCV	ganciclovir
hGFP	humanized green fluorescent protein
HSV	herpes simplex virus
MoMLV	Moloney murine leukemia virus
PBPC	peripheral blood progenitor cells
PGP	P glycoprotein
RQ-PCR	real-time quantitative PCR
TK	thymidine kinase
wtAAV	wild-type adenoassociated virus 2

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Gene therapeutic strategies may also be used to directly target sarcoma cells, e.g. by transfer of suicide genes. We found that adenoassociated virus 2 (AAV-2) vectors efficiently transduce human HS-1 and HT1080 sarcoma cells (>90%) while other tumor cell lines and primary human PBPC were less susceptible. The *thymidine kinase (TK)* suicide gene was cloned into an AAV-2 vector and a complete kill of *TK*-transduced HS-1 and HT1080 cells was observed following exposure to aciclovir or ganciclovir (GCV), while >90% of mock-transduced HS-1 cells survived at these dosages. Transplantation of those sarcoma cells to nonobese diabetic (NOD)/LtSz-severe-combined immunodeficient (scid)/scid (NOD/SCID) mice resulted in a survival of >5 months in the AAV-TK-transduced/GCV-treated group, while the mice in the mock-transduced/GCV-treated group had died after 3 weeks. These data show that soft tissue sarcomas are a particularly suitable model system for the development and clinical testing of new gene therapeutic concepts.

Introduction

A highly efficient antitumor therapy with minimal side effects to normal tissue is the goal of therapeutic research in oncology. It is the yet unfulfilled promise of tumor gene therapy to open this therapeutic window (fig. 1). Transfer of cytostatic drug resistance genes to normal hematopoietic cells can confer myeloprotection and possibly spare tumor patients the morbidity associated with an effective chemotherapy [Galski et al., 1989]. On the other hand, tumor cells can be sensitized to otherwise harmless antiviral drugs following the transfer of suicide genes [Smythe, 2000]. Sensitivity of tumor cells to chemotherapeutic drugs may be restored by the transfer of intact tumor suppressor genes [Swisher and Roth, 2000]. Viral vectors serve as shuttles to introduce therapeutic DNA into the target cells. In the following we describe the development of normal cell-protecting and tumor cell-sensitizing gene therapeutic strategies in soft tissue sarcoma. Due to its bad prognosis there is an urgent need to come up with innovative strategies for this tumor entity [Junginger et al., 2001].

Fortunately, the chemotherapeutic agents which do show activity and the inherent properties of the tumor cells render soft tissue sarcomas a model system for gene therapy in oncology.

Myeloprotective Gene Transfer for the Treatment of Sarcomas

Clinical Background and Standard Therapy of Soft Tissue Sarcoma

Soft tissue sarcomas are malignant tumors, which are derived from mesodermal or ectodermal tissue and can appear ubiquitously in the extremities, trunk, retroperitoneal space and in the head. They are quite rare making up 1% of all malignant tumors of adults. Nowadays half of the patients die, mostly due to metastases of the primary tumor which usually occur up to 5 years after diagnosis.

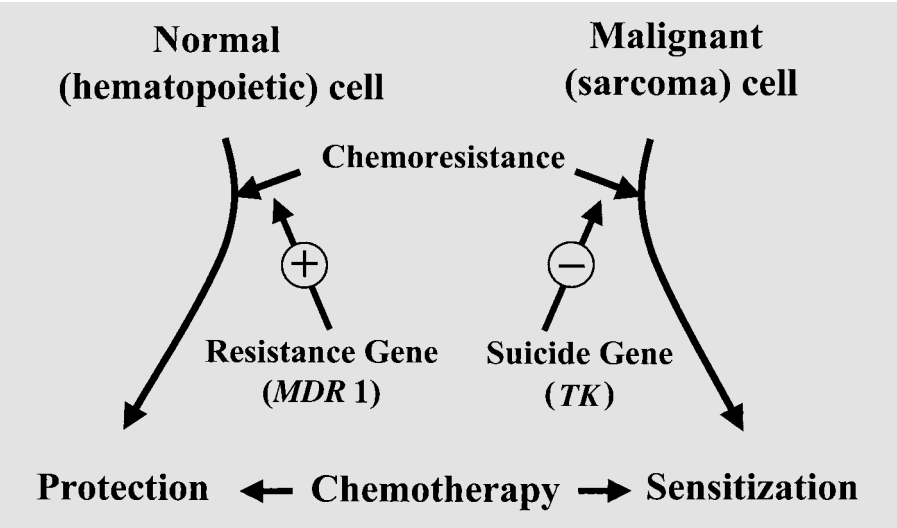

Fig. 1. Gene therapeutic concepts for the treatment of sarcoma.

While 10% of all sarcoma patients already have metastases at the time of diagnosis, 40–60% of patients with localized, high-grade soft tissue sarcoma develop metastases – mostly in the lung. Mean survival time from time of diagnosis is 8–12 months.

Only few chemotherapeutic drugs induce response in more than 15% of patients, including doxorubicin, ifosfamide, epirubicin, dacarbacin (DTIC), dactinomycin and methotrexate [Brennan et al., 1997]. Unfortunately, other cytostatics have not shown any activity in the treatment of soft tissue sarcoma in adults [Verweij et al., 2000]. After the introduction of doxorubicin in 1970 this drug has become a standard therapy for the treatment of soft tissue sarcoma in adults. A randomized study of the European Organization for Research and Treatment of Cancer (EORTC) did not show any advantage of combinations of cytostatics such as cyclophosphamide, vincristine, doxorubicin and DTIC (CYVADIC) compared to monotherapy with doxorubicin alone [Santoro et al., 1995]. After introduction of ifosfamide for treatment of sarcomas a new substance has become available, which shows similar or even somewhat higher response rates than doxorubicin [Bramwell et al., 1987; Elias et al., 1990]. However, there has been no randomized comparison of these two substances for the treatment of sarcoma. The combination of doxorubicin with ifosfamide was readministered in different therapy schemes and dose-dependent responses between 7 and 67% in uni- and multicenter studies were observed. So far no definite superiority of a combined therapy compared to a monotherapy could be shown. In contrast to a study of the EORTC, in which the combination of doxorubicin and ifosfamide showed no advantage to monotherapy with doxorubicin only, a study of the Eastern Cooperative Oncology Group (ECOG) showed significantly higher responses to the combined chemotherapy, however, at the price of increased myelosuppression. A longer disease-free mean survival time after combined chemotherapy has not yet been observed [Edmonson et al., 1993]. Similarly, overall survival was not affected in metastatic soft tissue sarcoma patients. Multimodal therapy approaches have contributed to improving the prognosis of metastatic soft tissue sarcoma. The resectability of metastasis is a significant and independent prognostic factor with regard to prolonged survival [Billingsley et al., 1999]. For that reason a surgical removal of the tumor is important. High necrosis rates of resected tumors are considered to be advantageous for prognosis [Wiklund et al., 1997].

Improvement of responses in metastatic soft tissue sarcoma patients treated with chemotherapy can only be reached by optimizing current combination regimens, or by introduction of new substances. After having shown dose-dependent response rates for doxorubicin [O'Bryan et al., 1977; Borden et al., 1987] and ifosfamide [Cerny et al., 1992; Patel et al., 1992], these substances seemed to be promising for administration in higher doses. Ifosfamide is used in current clinical trials treating patients with soft tissue sarcoma as single drug or together with anthracyclin at a dose of 5–12 g/m^2 and more per cycle [Reichardt et al., 1998; Le Cesne et al., 2000; Nielsen et al., 2000]. Due to the overlapping toxicity of high-dose ifosfamide and anthracyclin therapy, the dose-limiting myelosuppression is the major concern. The option to use colony-stimulating factors (CSF), for example the granulocyte (G)-CSF, has permitted dose intensification of cytostatic drugs in many tumor entities including soft tissue sarcoma [Reichardt et al., 1998]. G-CSF leads to a quick regeneration of neutrophil granulocytes after chemotherapy. For fast regeneration of thrombocytes there are no effective growth factors available at the moment. Bone marrow aplasia at higher doses of chemotherapy cannot be avoided by the administration of cytokines [Le Cesne et al., 2000].

This problem can possibly be solved by a transfer of the *multidrug resistance* 1 (*MDR*1) gene into hematopoietic progenitor cells. This would allow the administration of a higher dose intensity of *MDR*1-associated cytostatics with lower morbidity and mortality, which could lead to higher response and survival rates in patients with metastatic soft tissue sarcoma.

Background of Myeloprotective Gene Transfer Including Risk Estimation Studies

Human cells contain two *MDR* genes, *MDR*1 and *MDR*2. The *MDR*1 gene encodes a 170-kD ATP-binding transmembrane protein (P glycoprotein, PGP) which transports lipophilic cytostatics out of the cell, such as doxorubicin, etoposide, paclitaxel (taxol) and vincristine. Regulation of the expression of other genes by *MDR*1 has not yet been observed. Cells which express a sufficient amount of PGP are resistant against toxic effects of these drugs. Transgenic mice which expressed the human *MDR*1 gene were resistant against the myelosuppressive effect of doxorubicin in vivo [Galski et al., 1989]. Beyond that, nontransgenic mice, to which bone marrow of *MDR*1-transgenic mice had been transplanted, were myeloprotected after administration of doxorubicin.

For gene transfer in primary hematopoietic cells retroviral vectors are frequently used, because they allow a stable integration and expression of the vector [Brenner et

al., 1993]. Potential risks of retroviral gene transfer are contamination of the graft with replication-competent retroviruses, insertional mutagenesis, recombination with endogenous human retroviral sequences and the transfer of additional genetic material. Due to high safety standards in clinical trials no such side effects have been reported yet. The success of clinical retroviral gene transfer in hematopoietic cells of patients has been limited over the last years due to low gene transfer efficiency to long-term repopulating hematopoietic stem cells [Hanania et al., 1996; Devereux et al., 1998; Hesdorffer et al., 1998].

Recently, some modifications have led to improved gene transfer and to higher gene expression in stem cells, for example the use of modified vectors [Baum et al., 1995], of highly expressed retrovirus receptors [MacNeill et al., 1999], the colocalization of stem cells and vector particles by centrifugation or the transduction in the presence of fibronectin [Kotani et al., 1994; Moritz et al., 1994; Hanenberg et al., 1996] and the use of newly discovered cytokines, which are able to activate early stem cells [Cavazzana-Calvo et al., 2000; Schilz et al., 2000].

In a clinical trial patients suffering from metastatic mammary carcinoma were transplanted with *MDR*1-transduced blood progenitor cells. In 0.01–9% of circulating granulocytes the *MDR*1 transgene could be detected [Cowan et al., 1999]. However, in 2 of 3 patients there was a decrease of the *MDR*1 transgene after paclitaxel chemotherapy, likely due to the low expression of the Moloney murine leukemia virus (MoMLV) vector used here. After including more patients and after comparing the frequency of the *MDR*1 transgene with a control transgene (NeoR) a selective expansion of *MDR*1-transduced hematopoietic cells could be shown after paclitaxel chemotherapy even with the MoMLV vector [Moscow et al., 1999].

In a trial including patients with germ cell tumors the *MDR*1 transduction was done with an MoMLV vector on the fibronectin fragment CH-296 and in the presence of the cytokine Flt3 ligand. One year after transplantation more than 10% of the blood progenitor cells contained the *MDR*1 transgene in 2 patients [Abonour et al., 2000]. Despite splicing of the mRNA of the *MDR*1 transgene in some of the progenitor cells, which leads to lower gene expression, cytostatic-drug-resistant colonies demonstrating functionally active *MDR*1-transduced progenitor cells could be detected in the bone marrow of patients for the first time. For myeloprotection this was, however, not sufficient. On the other hand, the hematopoiesis of the patients who received *MDR*1-transduced blood progenitor cells was regular. This is encouraging considering data in mouse systems which describe the development of a myeloproliferative syndrome after *MDR*1 gene transfer [Bunting et al., 1998, 2000]. Perhaps these preclinical observations are based on using a different *MDR*1-cDNA (valine-185-glycine isoform) and clinically uncommon transduction conditions, which could have led to multiple integrations and therefore to a higher risk of insertional mutagenesis. The same group found in a primate model no pathological changes in hematopoiesis even under an extreme stimulation of proliferation [Sellers et al., 2001]. A pathological hematopoiesis has not been observed yet in any of the six clinical *MDR*1 gene transfer trials [Hanania et al., 1996; Devereux et al., 1998; Hesdorffer et al., 1998; Cowan et al., 1999; Moscow et al., 1999; Abonour et al., 2000] nor in transgenic mice which overexpressed MDR-1 in bone marrow [Mickisch et al., 1991].

For new protocols replication-incompetent retroviral vectors should be used which have been selected for high expression in primitive hematopoietic stem cells. The vectors contain, for example, regulatory sequences of spleen-focus-forming virus (SFFV) and murine embryonic stem cell virus (MESV). Compared to MoMLV-*MDR*1 vectors, these SF1m vectors are significantly more strongly expressed in early hematopoietic cells, resulting in 3-fold increased resistance to paclitaxel [Eckert et al., 1996]. In a murine stem cell transplantation model the provirus remained stably integrated in repopulating blood cells for more than 1 year [PCR analyses, sensitivity one transduced cell in 10^5 nontransduced cells; Baum, pers. commun.]. After modification of the vector and the *MDR*1-cDNA (SF91m3 vector), expression could be further increased [Knipper et al., 2001]. Possible rearrangements or recombination events with endogenous or other viral sequences in patient cells are minimized by the lack of overlapping sequences.

In a number of studies we transduced human modified peripheral blood progenitor cells (PBPC) from tumor patients or normal donors with retroviral vectors containing the human *MDR*1 gene and transplanted these cells to immune-deficient mice (fig. 2a).

In experiments with the SF1m vector we found gene transfer rates and expression in repopulating human hematopoietic cells equivalent to more than 500 *MDR*1-expressing granulocytes/μl blood, while normally PGP is not expressed by granulocytes (fig. 2b) [Schiedlmeier et al., 2000; Schilz et al., 2000]. With a provirus-specific real time quantitative PCR (RQ-PCR) a gene transfer rate of 9.4–12.3% was obtained in human marrow-repopulating hematopoietic cells. Statistically one vector integration had occurred per stem cell. 59% of the cells transduced

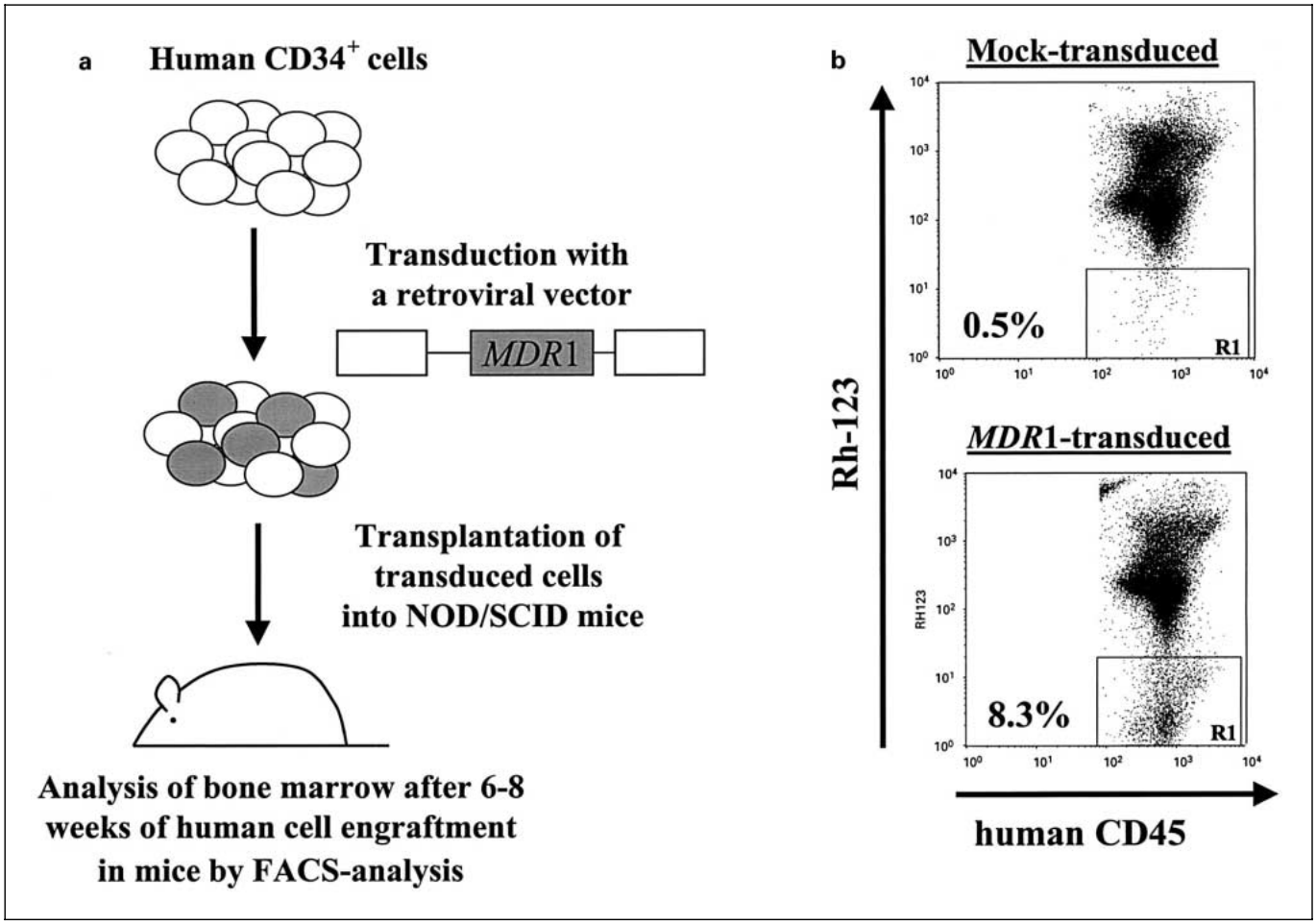

Fig. 2. a Experimental design of myeloprotective gene transfer into hematopoietic stem cells. Mobilized peripheral blood CD34+ cells are transduced with a retroviral vector containing the human multidrug resistance-1 (*MDR*1) gene. Engraftment of transduced human cells is assayed following transplantation to immune-deficient mice (nonobese diabetic severe combined immunodeficient strain, NOD/SCID). b Following gene transfer engrafting human leukocytes transduced with the human *MDR*1 gene are able to exclude the fluorescent dye rhodamine-123 (Rh-123) while mock-transduced cells do not exclude Rh-123. Rh-123 exclusion correlates with resistance to chemotherapeutic drugs [Schiedlmeier et al., 2002].

with the optimized vector SF91m3 expressed the transgene. Gene integration and expression strongly correlated. Chimeric mice were given a sublethal dose of paclitaxel. The number of human cells in mice transplanted with *MDR*1-transduced cells decreased significantly less than in mice transplanted with mock-transduced cells ($p < 0.05$), allowing to draw the conclusion that *MDR*1-transduced cells were chemoprotected. Compared to untreated mice that had received *MDR*1-transduced cells, a significant 1.4- to 1.8-fold increase in the number of gene-marked ($p > 0.05$ to $p > 0.01$) or PGP-expressing ($p > 0.01$) human cells in transduced paclitaxel-treated mice could be shown [Schiedlmeier et al., 2002]. Due to our positive preclinical data with the new SF91m3 vector we consider that in an *MDR*1 gene therapy trial it will now be possible to show a clinically relevant myeloprotection – in contrast to earlier *MDR*1 gene therapy trials using first generation vectors. The myeloprotection experiments described above were performed in compliance with the Good Manufacturing Practice suitable for clinical gene transfer trials [Schilz et al., 2000]. In pharmacological and toxicological studies no indication for possible harmful side effects of this gene transfer were observed. Furthermore, there were no indications for transduction of other than the vector-exposed cells (vector spreading).

Rationale for a Clinical MDR1 Gene Transfer Trial in Sarcoma Patients

In solid tumor patients a high number of PBPC can be mobilized following chemotherapy. In our own collective (n = 61), patients with solid tumors who until then had been chemotherapy-naive received G-CSF-supported chemotherapy and subsequent progenitor cell harvests, and good to very good PBPC yields could be achieved in the majority of them. In maximally two leukaphereses 40% of patients mobilized $\geq 15 \times 10^6$ $CD34^+$ cells/kg body weight; 30% <15–7.5 $\times 10^6$ $CD34^+$ cells/kg body weight; 23% <7.5–3.0 $\times 10^6$ $CD34^+$ cells/kg body weight; 7% <3 $\times 10^6$ $CD34^+$ cells/kg body weight. This high PBPC yield allows for further processing by CD34 selection in order to obtain the target cells for retroviral gene transfer. Sarcoma is an attractive target for the clinical testing of *MDR*1 gene therapy as ifosfamide which has been used for PBPC mobilization by many groups [Buzzi et al., 1999] is one of the few drugs active in this tumor. This drug has also been used for conditioning therapy before transplantation in advanced soft tissue sarcoma [Blay et al., 2000] as a combination of ifosfamide 12 g/m^2, etoposide 800 mg/m^2 and cisplatin 200 mg/m^2. The hematotoxicity of this protocol is mainly caused by ifosfamide and etoposide. The dose-limiting toxicity of cisplatin is mostly nephrotoxic; etoposide was applied here in low doses for transplantation trials [compare for example with Kleiner et al., 1997, 2,400 mg/m^2 etoposide]. Interestingly, data from allogeneic transplantation show that even after nonmyeloablative conditioning engraftment is possible [Maris et al., 2001]. On the other hand, doxorubicin, the gold standard for the treatment of sarcoma, is detoxified via PGP and could be administered after transplantation of *MDR*1-transduced PBPC. However, cytostatic drugs of other substance classes (taxotere, actinomycin D, etoposide) could also be used [Patel, 2000; Hensley et al., 2001]. In a clinical setting the *MDR*1 gene transduction needs to be monitored by RQ-PCR for the *MDR*1 provirus. *MDR*1 gene expression should be determined by rhodamine efflux assays in granulocytes which usually do not express the *MDR*1 gene [Fruehauf et al., 1996]. The German *MDR*1 Gene Therapy Study Group was founded in 1999 to address these issues.

Suicide Gene Transfer in Sarcoma Cells

A second possibility to employ gene therapy in the treatment of sarcomas would be to directly target the tumor cells and induce apoptosis. A suicide gene therapy approach, which selectively targets only the sarcoma and not the hematopoietic cells, would thus be a major improvement in the treatment of sarcomas. The *thymidine kinase (TK)* suicide gene was chosen in combination with the prodrugs aciclovir and ganciclovir (GCV) by many groups. The *TK* gene originates from the herpes simplex virus (HSV) and the resulting gene product is able to phosphorylate several substrates and their derivates. In the case of GCV, the *HSV-TK* gene metabolizes the nucleoside analogue GCV into a phosphorylated compound, then normal cellular kinases further phosphorylate the GCV monophosphate to GCV diphosphate and GCV triphosphate. GCV triphosphate competes with deoxyguanosine triphosphate for DNA polymerase and, as a result, DNA synthesis is impaired producing a chain termination and causing cell death in the dividing cells [Cheng et al., 1983].

Many groups have been using the *TK* gene in several different vector systems, for example adeno- [Boviatsis et al., 1994; Hasenburg et al., 2001; Sung et al., 2001], adenoassociated [Su et al., 1996; Kanazawa et al., 2001; Nguyen et al., 2001] and retroviral [Culver et al., 1992; Kruse et al., 2000; Carrio et al., 2001] systems and were able to efficiently kill the infected cells after supplementing the medium with the prodrug GCV. Most interestingly, it seems that in in vivo experiments the combination TK/GCV mediates beside the direct and indirect toxicity (bystander effect) also an immunotoxic effect, the mechanism of which has not yet been fully elucidated [Kruse et al., 2000; Okada et al., 2001]. Both the bystander and the immunotoxic effect will be helpful, especially when not all tumor cells have been infected and will greatly enhance the efficiency of the system.

A clinical trial [Stockhammer et al., 1997] on in vivo transduction of high-grade gliomas with the *HSV-TK* gene by injection of a retroviral packaging cell line followed by GCV treatment of patients showed a poor response rate. This may be due to a low expression rate in vivo. A more recent clinical study showed a significant increase in survival time when adenoviral vectors containing the *HSV-TK* gene were used instead of retroviral vectors [$p < 0.012$, mean survival of patients 15 and 7.4 months, respectively; control group 8.3 months; Sandmair et al., 2000]. The extent of *HSV-TK* gene expression seems to predict the therapeutic response [Jacobs et al., 2001]. Adenoviral vectors can be cytopathogenic to surrounding tissues as opposed to adenoassociated virus vectors [Okada et al., 1996].

Therefore, we are using the small parvovirus adenoassociated virus 2 (AAV-2) for our suicide gene therapy studies in sarcoma. AAV-2 has as natural host cells of the

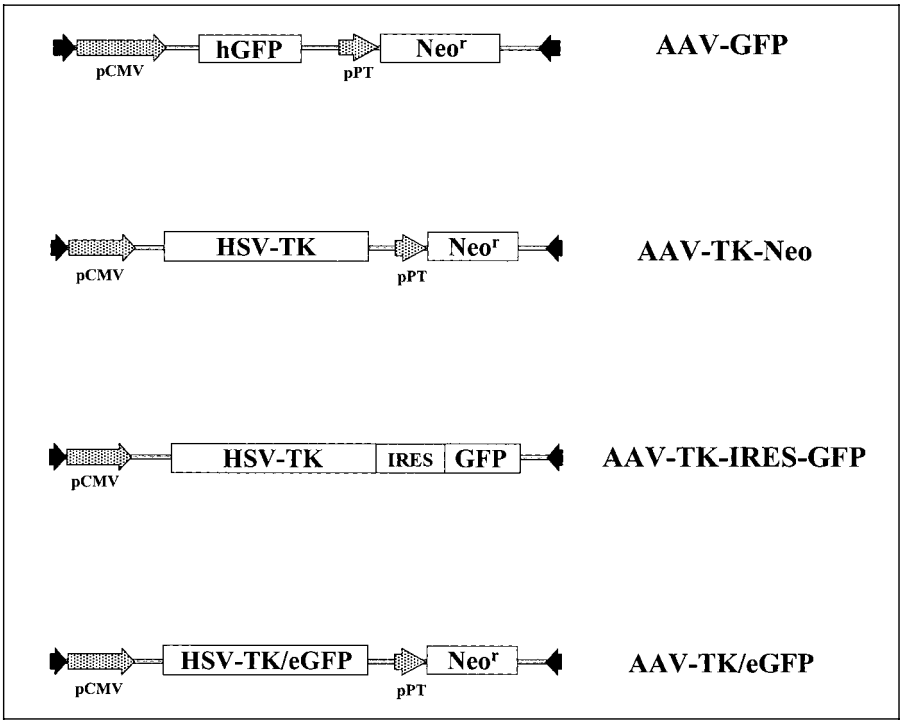

Fig. 3. rAAV-2 vectors used for transduction of sarcomas. These vectors contain the marker genes *green fluorescent protein (GFP), enhanced GFP (eGFP), humanized GFP hGFP* and/or the *neomycin resistance (Neo^r)* gene. Suicide gene vectors also contain the *HSV-TK* gene. The expression of these genes is under control of the cytomegalovirus immediate early promoter/enhancer (pCMV) or the promotor of the polioma enhancer/thymidine kinase (pPT).

respiratory tract [Dreizin et al., 1977] as well as cervical tissue [Bantel-Schaal and zur Hausen, 1984; Walz et al., 1998]. AAV-2 has until now not been associated with any disease. Another major advantage of AAV-2 is its ability to also infect nondividing cells, unlike mouse oncoretrovirus-based vector systems. Wild-type AAV (wtAAV) was for the first time observed as a contaminant found in samples from children with an adenoviral infection [Blacklow et al., 1971]. This observation can be readily explained knowing that wtAAV is unable to efficiently replicate without the help of a helper virus, normally adeno- or herpesvirus and these are therefore required for the efficient production of AAV-2 particles [Hoggan et al., 1966]. Instead of using the standard procedure which needs infection of wild-type adenovirus, we used a plasmid containing the required adenoviral and AAV-2 genes [Grimm et al., 1998]. Also, the high stability of the AAV-2 particle allows the use of purification and concentration of vector stocks. Novel methods [Clark et al., 1995; Tamayose et al., 1996; Grimm et al., 1998; Zolotukhin et al., 1999] result in highly purified and concentrated high-titer AAV-2 batches. The availability of helper plasmids and purification methods rule out contamination of AAV-2 stocks with helper virus, their often immunogenic proteins and other contaminants, thereby greatly enhancing the acceptability of AAV-2 in clinical and preclinical gene therapy settings compared to previously used methods [Toolan, 1968]. One still unaddressed major problem – not only for AAV – is the lack of standardization in vector titration [Salvetti et al., 1998; Grimm et al., 1999; Summerford and Samulski, 1999; Veldwijk et al., 1999], thereby complicating the comparison of transduction results between different groups and thus impeding the widespread application of rAAV-based gene therapy. The recently developed fluorescence-based RQ-PCR assay [Livak et al., 1995; Gibson et al., 1996; Heid et al., 1996] allows exact quantification of DNA sequences, and since AAV-2 is a DNA virus, this method can be expected to be particularly suitable for quantifying rAAV-2 genomes in viral stocks. We developed a novel assay for the titration of rAAV-2 stocks based on the TaqMan RQ-PCR method [Veldwijk et al., 2002] which satisfies even the strictest standards for sensitivity and reproducibility such as those required in clinical laboratory assays [Rümke, 1977; Barnett et al., 2000; Chen et al., 2000]. This highly standardized RQ-PCR titration assay offers us as well as others an easy, fast, safe, highly sensitive, specific and reproducible high-throughput tool for titration of rAAV-2 stocks with and without selectable marker genes and will thus facilitate the further development, optimization and application of AAV-2-based gene therapy.

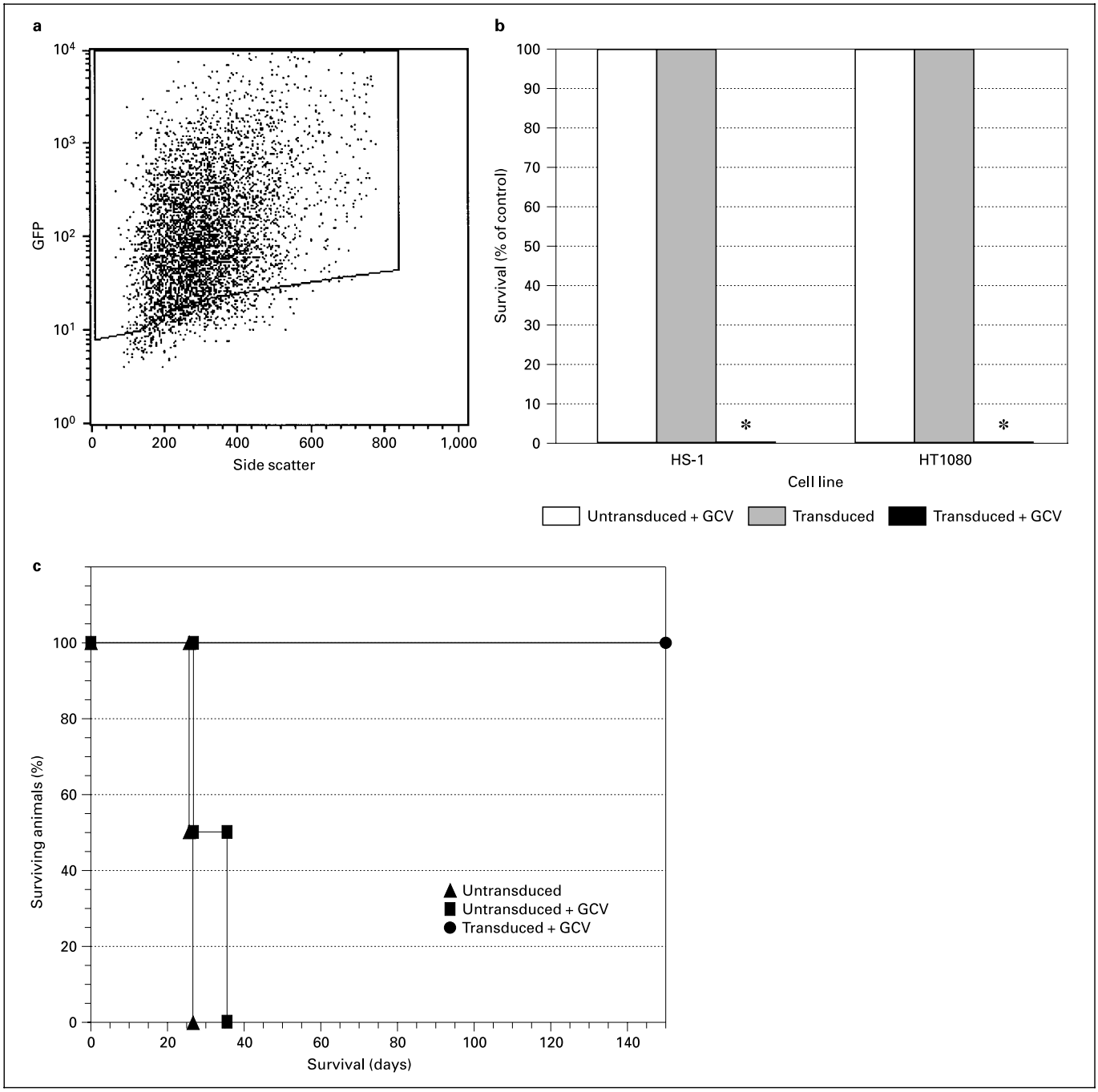

Fig. 4. a Direct demonstration of marker gene expression *(GFP)* following transduction of sarcoma cells with the rAAV-TK/eGFP vector. Untransduced and transduced HS-1 cells are compared. Results of fluorescent activated cell scanning (FACS) are displayed. b Cell kill assay of HS-1 and HT1080 cells after a single-dose transduction with rAAV-TK/eGFP (100 particles/cell). Cells were cultured for 14 days in 2.5 µg/ml GCV-containing medium (refreshed every 3rd day). Viability and cell count were analyzed using the MTT assay and light microscopy. In this setting no sarcoma cells in the group transduced with the rAAV-2 suicide gene vector survived treatment with GCV. * = Microscopic observation. On the other hand, GCV did not produce any significant toxicity in untransduced sarcoma cells. The rAAV-2 suicide gene vector alone was not toxic to the sarcoma cells as shown in experiments without GCV. These results clearly show the effectivity of a suicide gene therapy of sarcomas. c Kaplan-Meier survival plot of mice transplanted with ex vivo transduced (rAAV-TK/eGFP) or untransduced sarcoma cells (HS-1) after treatment with or without GCV for 14 days in culture. Whereas mice transplanted with untransduced and GCV-treated or untreated sarcoma cells died within 40 days, the mice receiving transduced and GCV-treated cells survived over 150 days (end of experiment).

Now, with a suitable vector, production and titration system at our disposal, we started to screen for solid tumors that were highly susceptible for our rAAV-2 vector (fig. 3) containing the *hGFP* gene (fig. 4a) [Veldwijk et al., 1999, 2000]. Among a series of primary cells and eight solid tumor cell lines infected with rAAV-2 stocks, we observed the highest infection rates (functional titer: 200 IU/cell) in soft tissue sarcoma cells (HS-1; mean: 96% hGFP+ cells) and in breast cancer cells (T47D; mean: 83%, MCF-7; mean: 85%). Other cell lines (1 ovarian tumor, 1 germ cell tumor, 1 osteosarcoma and 2 small cell lung cancer) were less permissive (3–12%). $CD34^+$ PBPC showed the lowest transduction rates (mean 4%). It appeared that the sarcoma cells were the most suitable candidates for further development of the rAAV-2 tumor suicide gene therapy approach.

To test whether the observed high susceptibility of the sarcoma line HS-1 for rAAV-2 could be reproduced, five other sarcoma cell lines were infected with the vector. For all investigated lines a high susceptibility was observed. This confirmed our initial finding that sarcoma cells are ideal targets for rAAV-2-based vectors.

In order to utilize rAAV-2 for suicide gene therapy purposes, a total of three novel *TK*-containing vectors were cloned (fig. 4). Since some of the new vectors contained no marker gene *(GFP)* and, therefore, did not allow fluorescence-based titration, our new quantitative TaqMan RQ-PCR-based titration method allowed the accurate determination of titers.

In initial in vitro cell kill experiments using two sarcoma cell lines (HS-1, HT1080), we were able to show a complete eradication of AAV-TK/eGFP-transduced tumor cells following exposure to aciclovir or GCV while at this dose level >95% of mock-transduced tumor cells survived (fig. 4a, b).

Xenotransplantation tumor models for these human sarcoma cell lines were established. In proof of principle experiments, mice that were transplanted with AAV-TK-transduced and GCV-exposed tumor cells survived >5 months while in the nontransduced group all mice had died 1 month after inoculation (fig. 4c).

In currently ongoing experiments we are completing the in vitro experiments for all 6 sarcoma cell lines using our latest rAAV-TK/GFP vector containing a thymidine kinase/enhanced GFP fusion protein allowing both cell kill and tracking of infected tumor cells. Furthermore, the xenotransplantation model is optimized for all 6 cell lines for both the intraperitoneal and subcutaneous transplantation routes and optimal doses of the prodrugs are titrated in the NOD/SCID mice. In vitro and in vivo experiments using human primary sarcoma material are continuing. The main goal of this study was to show a significant increase in survival or even cure mice that were transplanted with rAAV-TK-transduced sarcoma cells and then treated in vivo with the prodrug compared to the mock-transduced control mice.

When the suicide gene therapy protocol will have been optimized, it is also conceivable to use rAAV-2 vectors to confer intact tumor suppressor genes such as p53 to deficient tumor cells. The loss of some tumor suppressor genes confers resistance to chemotherapy and corrective gene therapy may allow to reestablish chemosensitivity as it is currently being tested for adenoviral vectors and head and neck tumors [Merritt et al., 2001]. In sarcomas the loss of tumor suppressor genes has also been described [Letson and Muro-Cacho, 2001; Maitra et al., 2001].

The project as shown here holds promise for a future clinical application of rAAV-based suicide gene therapy for sarcomas.

Perspectives

The work described demonstrates that exciting new treatment strategies are being developed for soft tissue sarcoma, a tumor entity which currently has a dismal prognosis despite all conventional therapy efforts.

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