Abstract

Current therapies for adrenocortical carcinomas do not improve the life expectancy of patients. In this study, we tested whether a gene-transfer therapy based upon a suicide gene/prodrug system would be effective in an animal model of the disease. We employed E4- and EIA/B-depleted herpes simplex virus-thymidine kinase-expressing adenoviral mutants that transcomplement each other within tumor cells, hereby improving transgene delivery and efficacy by viral replication in situ. Transcomplementation of vectors increases the fraction of transduced tumor cells. This increase was accompanied by greater tumor volume reduction compared to non-transcomplementing approaches. Survival time improved with non-replicating vectors plus GCV compared to controls. However, transcomplementation/replication of vectors led to a further significant increment in anti-tumor activity and survival time (p <0.02). In treated animals, we observed a high number of apoptotic nuclei both adjacent to and distant from injection sites and sites of viral oncolysis. Ultrastructural analyses exhibited nuclear inclusion bodies characteristic of virus production in situ, and provide further evidence that this therapy induced apoptotic cell death within tumor cells. We conclude that the efficacy of suicide gene therapy is significantly amplified by viral replication and, in combination with GCV, significantly reduces tumor burden and increases survival time.

Key words
Adrenal Gland • Cancer • Gene Therapy • Adenovirus -Transcomplementing • Bicistronic

Introduction

Adrenocortical carcinomas are severe malignancies. The prognosis is poor, as metastases in lung and liver develop rapidly; 5-year survival rates range between 15 and 35% [1]. Two age peaks of higher incidence within the 1st and 4th decades of life have been reported [2,3]. Molecular, paracrine, immunologic, and hormonal features of these tumors have been studied extensively, and prognostic signs have been developed [4]. Impaired cell-cell communication between tumor and effector cells of the immune system may be related to immune surveillance due to the decreased MHC class I expression [5]. Interestingly, soluble mediators of the immune system itself have been shown to promote adrenocortical tumors by the action of aberrantly expressed receptors [6]. However, despite efforts to shed light upon the molecular and immunologic mechanisms leading to neoplastic degeneration in the adrenal gland, no effective treatments have been developed. Few chemotherapeutic options for patients...
bearing unresectable o r metastasizing g tumor s ar e available . Standard therapies have severe side effects, and evidence of their success is anecdotal [7-9].

Animal modeling g of adrenocortical carcinoma a with ne w treat -
ment modalities could accelerate t he development o f therapies for this disease, and may have broader application; however, no appropriate mode l i s currently available t o test possible t reat -
ment strategies. CE mice have been reported to develop adrenocortical hyperplasi a with h progressio n t o carcinoma af t er r ovariec tomy [10]. This is highly suggestive of an involvement of regu-
lation of hormones; instead, estrogen l replacemen t pre vent s de-
development o f transplanted carcinoma s i n m i c e [11]. However, hormone-dependent adren al l carcinoma s h ave no t bee n de-
scribed in human s with or without adrenal hyperplasia. To de-
velop a more pertinent animal mode l of this disease, we gener-
ated SW-13-derived human adrenocortical carcinoma xenografts in nude mice. The SW-13 cell line was initiated from an hormon-
ally inactive small-cell carcinom a o f the huma n adrena l corte x [12] an d h as pre viousl y bee n use d t o t est c om poun ds s wit h h ome chemotherapy capacity [13-15].

Gene-transfer therapy could improve treatments of adrenocorti-
cal carcinomas. A principal limitation o f gene therapy is ineffi-
cient gene delivery [16]. Therefore, biological amplificatio n o f anti-tumor effects against both transduced ad non-transduced d
tumor cells has been sought in the recruitment of host immune effector cells [17], production of antiangiogenic factors [18-21], or the use of suicide genes. The prototypical suicide gene, herpes simplex virus (hsv)-thymidine kinase (TK), activates a relatively non-toxic prodrug (such as Ganciclovir) to a cytotoxic compound [22,23]. In addition to direct toxicity in the transduced cell, the expression of hsv- TK causes cell death in non-transduced tumor cells via a "bystander effect," which transfers the activated pro-
drug fro m HSV-T K expressin g cell s t o adjacent , metabolically u ncoupled cells [24-27].

Anti-tumor strategies have recently been modified by including viral replication as a means of increasing intratumoral transgene delivery [28] an d a s a n induc tio n o f d efectiv e l vira l c ytotoxicit y y
[29,30]. In anim al model s using g adenoviro ses with attenuated replication potential, increased survival has bee n demonstrated [31 ]; a further improvement was reported when partial i nfection competenc e e w a s combine d w ith h suicid e g en e expr ession n
[30]. However, adenovir a l vect or s expressin g herpes simplex virus-thymidine kinase e n m a y damage e proliferatin g n on-cancer-
ous tissues, and can cause severe liver necrosis, including death in animal models after systemic application [32,33]. Such toxiciti-
ies may be even more prominent i n system s usin g replication competent vectors expressing hsv-TK.

As a ne w therapeutic a pproach f or adrenocortical cancer , w e modified the replicating vector strategy to utilize replication-depen-
dent vector distribution within the tumor, but sought to re-
duce systemic vector spread, potentially minimizing side effects. To achieve this, we employed two replication-deficient adenovi-
ral vectors, E4-deleted l 5 d f o r [34] an d a El/A-B-deleted vect or carryin g herpes simplex virus-thymidine kinase gene cloned into a vector derived from H5.d'309 (AVC2.TK). The two separate adenoviral variant s ar e individually replication-deficient d au te r to
defects i n essent ial viral genes (in cis-) but replication-compe-
tent whe n combine d i n cell s via trans complementation. Thi s
system was tested to establish whether replication results in in-
creased vector spread with tumors following co-transduction and whether anti-tumor efficacy in adrenocortical tumor xenografts is improve d b y thi s approach. The in v i vo tumor model was characterized by radiographic and histological studies. Adeno-
oviral vectors have been injected into xenograft tumors directly to achieve efficient transduction of tumor cells by both vectors. We the n use d FACS a nalysis , immunohistochemistry, i n s itu
end-labeling, ultrastructural l analyse s t o monito r gene transfer, virus particle e production, apoptotic c ell death, and virus/trans-
gene-mediated tumor lysis. Our findings show that co-transduc-
tion o f adrenocortical tumor r e ce lls e nables s trans complementa-
tion and results i n replicatio n o f vect ors. Thi s loc al production
of vector leads to a further increase in the fraction of transduced tumor cells and enhances transgene distribution, hereby improv-
ying efficacy of suicide gene/prodrug strategy.

Materials and Methods

Cells

293 cells (CRL-1573). Yl cells (CCL-79), AtT20 cells (CCL-89), and SW-13 cells (CCL-105) (12) were obtained from ATCC (Gaithers-
burg, MD). WI62 cells were kindly provided by G. Ketner (Johns Hopkins U., Baltimore, MD). Rat 9L cells were kindly provided by E. Oldfield (NINDS, Bethesda, MD). All cell lines were grown in DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, 100U/ml penicillin and 100ug/ml streptomycin (Life Technolo-
gies, Grand Island, NY) at 37 °C in 10% CO2.

Adenoviral vectors

AVC2null is identical to AVC2.TK except for the absence of the
hsv-TK transgene, and was generated by the technique e of Gra-
ham [35]. Briefly, the adenovir a l shuttl e plasmid pAVC2 [36 ]
was introduced into pJM17 [37] by Ca-phosphate co-precipita-
tion technique e i n n 29 3 celle s [35]. Recombinant El-delete d
AVC2TK was generated by the technique of Okada [38]. Hsv-TK
function was confirmed by transduction of rat 9L cells at a multi-
pli city of infection (MOI) of 50 PFU/cell, exposure to ganciclovir,
and measurement of MOI t o F' H-thymidine incorporation. AVG L i s a n
El-deleted virus expressing green fluorescent protein under the control of the EFl-a promoter (kindly provided b y J. Wahlfors,
University o f Kuopio, Finland). The E 4 mutan t d dill014 (kindly
provided by G. Ketner, Johns Hopkins University) was grown w
WI62 cell s an d isolate d b y standard techniqu e . Virus s wer e
plaque-purified twice, amplified u sin g d t echniqu e o f standard techniqu e , an d analy ze d b y rest rictio n g en e o f proteins e K-digest e d
d viral DNA. All viral stocks were plaque-titered in triplicate by se-
rial dilution an d agar overlay on their r espective e transcomple-
menting cell lines according to standard protocols.

In vitro transduction and FACS analyses

For in vitro transduction, SW-13 cells, AtT20- and Yl-cells were
plated in 6-well plates at a density of 300 000 cells per well. Cells were transduced with MOI s of 1 to 100 PFU/cell. After 80 h. cells were trypsinized, harveste d b y centrifugatio n a nd resuspended d
in ice-cold HBSS containing 5% FCS and 0.1 M EDTA (Sigma, St.
Louis, MO). Cells were subjected to FACS analysis using a Becton Dickinson FACSort analyzer and 10000 ungated events were col-
lected for analysis.
Mice and tumor generation, treatment groups

6-8 week-old female nude mice were obtained from DCT (Frederick, MD). Mice were injected subcutaneously into the right flank with 10 x 10^6 cells in 200 pi HBSS (Biofluids, Gaithersburg, MD) containing 20% Matrigel (Collaborative Biomedical Products, Waltham, MA). Weekly monitoring of tumor size was carried out by measurement diameters in three dimensions at right angles with calipers [volume = 1 x wxh]. In vivo transduction was performed when tumors grew to a mean volume of 300 mm^3.

In vivo transduction was performed when tumors grew to a mean volume of 300 mm^3. Treated tumors were injected with 2 x 10^9 PFU of adenoviral vector in a volume of 100 pi using 25 g needles. In treatment groups, GCV (Roche Bioscience, Palo Alto, CA) was injected intraperitoneally twice daily for 5 days at a dose of 100 mg/kg/d. Animals were injected with 1 x 10^9 PFU AVC2.TK plus 1 x 10^9 PFU AV.d/0/4, groups [TK/d/0/4/GCV] (n = 8) and [TK/d/0/4/GCV] (n = 7) or 2 x 10^9 PFU AVC2.TK, group [TK/GCV] (n = 9) to compare transcomplementing vs. replication-defective vector approaches of therapy. Ganciclovir was injected intraperitoneally after 80 h in group [TK/d/0/4/GCV] and additionally after 30 h in group [TK/d/0/4/GCV] to compare early and late blocking of viral replication. Two control groups [TK/d/0/4/GCV] (n = 9) and [TK] (n = 8) were transduced as groups [TK/d/0/4/GCV] and [TK/GCV], but did not receive GCV. [d1014] (n = 9) was transduced with 2 x 10^9 PFU AV.d/0/4 alone. [GCV] (n = 9) was only treated with GCV and (untreated) (n = 9) received no treatment. Animals were euthanized when tumor size exceeded 1500 mm^3. All animal experiments were conducted in accordance with NIH guidelines for the care and treatment of laboratory animals.

Planar imaging/positron emission tomography

The accumulation of F-18 fluorodeoxyglucose (FDG) in the implanted adrenal tumors was imaged tomographically in several mice by planar imaging/positron emission tomograph (PiPET), a device developed specifically for imaging small animals. FDG was administered by tail vein injection (150 microcuries / 0.1 ml). At 30 minutes post-injection, the animal was fixed to a vertical rotation device between the two PiPET detectors and rotated continuously for 30 minutes during acquisition of all possible coincidence lines connecting the two detectors. Two sequential data collections were performed with the first collection being used to visualize the subcostal region of the animal and the second, which visualized the more rostral areas of the animals. Mice were euthanized and reconstructed to form a continuous tomographic representation of the distribution of FDG in the whole animal.

Electron microscopy

Small tissue pieces of adrenal tumor were fixed in 4% paraformaldehyde - 1% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.3, for 3 h, postfixed in 2% OsO4 in 0.1 mol/l cacodylate, pH 7.3, dehydrated in ethanol, and embedded in epoxy resin. 70 nm sections were stained with uranyl acetate and lead citrate and examined at 80 kV under a Phillips electron microscope 301 (Philips, Rahway, NJ).

In situ labeling of DNA fragments

The staining of apoptotic nuclei was achieved by non-radioactive in situ end-labeling. The free 3'-ends of cellular DNA served as template and were elongated and labeled with digoxigenin-marks deoxyUTP. The digoxigenin was subsequently immunodetected with anti-DIG peroxidase, visualized with 3-amino-9-ethylcarbazole (AEC) and counterstained with methylene green. In additional experiments, immunodetection and visualization were carried out with anti-digoxigenin-rhodamine conjugate according to the supplier's protocol (ApopTag®-Kit, Intergen Company, Purchase, NY).

Statistical analyses

All in vitro experiments were performed in quadruplicate. Statistical analysis was performed using the Mann-Whitney test for evaluation of tumor volume experiments and the Kaplan-Meier method for survival curves. Results were considered statistically significant where p < 0.05.

Fig. 1 Vector maps of AVC2.TK and AV.d1014. AVC2.TK has a deletion in early region 1, in which the CMV promoter and herpes simplex type I thymidine kinase have been inserted. AV.d1014 has a deletion in early region 4 with only open reading frame left (F-orf4). Both deletions render the adenoviral vectors replication incompetent, however, if El and E4 products will be expressed in a single cell from both vectors, replication can occur due to transcomplementation. Horizontal black bars in the figure depict early region 1 or 4 by which gene products have to be supplemented to the second vector.
Fig. 2 Human adrenocortical xenograft tumors in nude mice. A Female nude mice were injected subcutaneously into the right flank with $10^6$ SW-13 cells. Prominent tumor nodules formed intra- and/or subcutaneously and after 6-7 weeks metastases occurred. B Whole-body volume reprojection image of an adrenal tumor-bearing mouse after injection of $^{18}$F-FDG. Primary tumor (T) and metastasis (M) are visible. Two data collections were required to visualize the whole animal (H = heart, BR = brain, HA = Harderian glands, B = bladder).

Fig. 3 Transduction of tumor cells in vitro. Cells plated in 6-well plates at a density of $3 \times 10^5$ cells per well were transduced with AV.CL at MOI of 1 to 100 PFU/cell, harvested after 80 h and subjected to FACS analysis. Maximum transduction for human adrenocortical SW-13 and mouse pituitary AtT20-cells was achieved at MOIs of 30 PFU/cell. At higher MOIs no further increase in transduction rate could be detected and virus-dependent cytotoxicity increased (data not shown).

Fig. 4 In vivo transduction of human adrenocortical cell xenografts. A Tumors were transduced in vivo with either $2 \times 10^9$ PFU AVC2-null, $1 \times 10^{10}$ PFU AV.GLp1, $1 \times 10^9$ PFU AV.CL plus $1 \times 10^9$ PFU H5.d1014 and transgene expression was assessed by FACS analysis. Tumors transduced with transcomplementing vectors exhibited an up to 4-fold higher rate of transduction, median transduction efficiency was 9.8% with non-transcomplementing viruses and 36.7% for r transcomplementing virus transduced tumors ($p<0.05$). B Transmission electron micrographs demonstrating nuclear viral particles in situ. Transcomplementing transduced tumors were examined for newly assembled viral particles 9-6 h after transduction. De novo assembling of viral particle in the nucleus is a characteristic feature of virus production and indicative for replication. The figure depicts characteristic adenoviral particles with circular electron dense structure and a halo as nuclear inclusions in a SW-13 tumor cell.

Results

Characterization of tumor model

SW-13-derived tumor nodules were established in the flanks of nude mice by subcutaneous and/or intradermal injection of $10^6$ cells. The adrenocortical tumor model used in this study was a progressive and lethal disease with no evidence of spontaneous regression. Animal with ulcerating tumors or tumors greater than 1500 mm$^3$ were euthanized, and are referred to in the assessment of the survival rate as “succumbed to tumor burden”. Tumors reached an average volume of 300 mm$^3$ by 5 weeks after inoculation. By 7 to 9 weeks, metastases could be detected by visual examination (Fig. 2 A) or gross dissection and metastatic tumor deposits could be readily localized by PET scan with
F-FDG (Fig. 2B). Histological studies revealed multilobulated tumors with an adrenal glandular texture (Fig. 6). They developed a pseudo-capsule and were highly vascularized. Histology of the primary and metastatic tumor deposits demonstrated characteristic signs of their common origin; both demonstrated compact cells with pleomorphic nuclei and an abundance of mitochondria with tubulovesicular structure and ample smooth endoplasmic reticulum typical of adrenocortical cells. The tumor cells did not express gap junctions by ultrastructural analysis (see Figs. 4B and 7) or immunohistochemistry (data not shown).

In vitro transduction with AVGL
SW-13 cells were transduced in vitro with AVGL at MOIs of 1 to 30 PFU/cell. Transduction efficiency was compared to AV.C2.TK plus GCV treatment (after 29 d; p > 0.001). Transcomplementing transduction with viral replication retarded by administration of GCV at 30 h post-transduction demonstrated a noticeable but statistically insignificant improvement compared to AV2.C2.TK plus GCV treatment (after 29 d; p > 0.001).

In vitro transduction with AVGL
SW-13 cells were transduced in vitro with AVGL at MOIs of 1 to 30 PFU/cell. Transduction efficiency was compared to AV.C2.TK plus GCV treatment (after 29 d; p > 0.001). Transcomplementing transduction with viral replication retarded by administration of GCV at 30 h post-transduction demonstrated a noticeable but statistically insignificant improvement compared to AV2.C2.TK plus GCV treatment (after 29 d; p > 0.001).

Fig. 5 Treatment of human adenocortical SW-13 xenograft tumors in nude mice. Tumor size was measured (volume = l x w x h) and transduced with 2 x 10^6 PFU of either trans-complementing or non-complementing adenoviral vectors. Ganciclovir (100 mg/kg/d) was given in divided doses twice daily for 5 days by intraperitoneal injection. Contro l animals receive d either virus alone or without GCV or GCV alone. A The additive effects of TK, transcomplementation and GCV administration markedly improved survival compared to control (p < 0.02). Median survival time was 50 d (p = 0.07) for TK/GCV, and 68.5 d (p = 0.002) for animals transduced with transcomplementing vectors but without ganciclovir (transcomp). B Tumor size was monitored weekly. Growth inhibition and tumor volume reduction in animals receiving transcomplementing vectors plus GCV at 80 h [transc/GCV/80] post-transduction was superior to all other approaches, including non-replicating AV.C2.TK plus GCV treatment (after 29 d; p > 0.001). Transcomplementing transduction with viral replication retarded by administration of GCV at 30 h post-transduction demonstrated a noticeable but statistically insignificant improvement compared to AV2.C2.TK plus GCV treatment (after 29 d; p > 0.001).

In vitro transduction with AVGL
SW-13 cells were transduced in vitro with AVGL at MOIs of 1 to 30 PFU/cell. Transduction efficiency was compared to AV.C2.TK plus GCV treatment (after 29 d; p > 0.001). Transcomplementing transduction with viral replication retarded by administration of GCV at 30 h post-transduction demonstrated a noticeable but statistically insignificant improvement compared to AV2.C2.TK plus GCV treatment (after 29 d; p > 0.001).

Fig. 6 Free 3'-ends of DNA of apoptotic nuclei were labeled with digoxigenin-marked deoxyUTp, subsequently immunodetecte d with anti-DIG-peroxidase and visualized with AEC. Fig. 6A shows an AV.GL transduced SW-13 tumor with single apoptotic nuclei and cell clusters with brown stained nuclei throughout the specimen. In Fig. 6B apoptotic foci were frequently found adjacent to the site of injection and to areas within the tumor showing viral transduction as demonstrated by fluorescence induction of the transgene (bar = 50 Lim).
In vivo transduction and transcomplementation

Tumors (n = 10) were transduced in vivo with either 2 × 10^8 PFU AVC2.null, 1 × 10^9 PFU AV.GL plus 1 × 10^9 PFU AVC2.null, or 1 × 10^9 PFU AV.GL plus 1 × 10^9 PFU AV.d1014. Tumors were harvested, digested with triple enzyme solution [39], and transgene expression was assessed by FACS analyses 72 and 96 h after injection. Tumors transduced with transcomplementing vectors exhibited up to 4 times the rates of transduction. Median transduction efficiency was 9.8% with non-transcomplementing viruses and 36.7% for tumors transduced with vectors that transcomplement (p < 0.05; Fig. 4A). The percentage of transduced cells remained constant after three days, suggesting that a steady state between viral de novo transduction and cell loss via viral cell lysis is reached in this time frame.

**Treatment of adrenocortical cell tumors**

Tumors with an average volume of 300 mm³ at the time of treatment were injected with 2 × 10^8 PFU of adenoviral vector. The effect of the treatment was monitored by weekly measurements of tumor size and assessment of survival.

A suppression in the growth of SW-13 derived tumors could be observed in all animals transduced with vectors expressing hsv-TK and treated with GCV [groups [TK/d1014/GCV₉₀], [TK/d1014/GCV₃₀] and [TK/GCV]] within 15 days after onset of treatment. Shrinkage of the tumors in group [TK/d1014/GCV₉₀] continued up to 30 days after GCV treatment (Fig. 5A). Interestingly, there was a gradation of effectiveness in growth inhibition and volume shrinkage. The gradation was dependent on time allowed for completion of virus replication and ganciclovir administration; growth inhibition after transcomplementing transduction with GCV treatment 80 h post-transduction was superior to transcomplementing transduction and GCV treatment 30 h post-transduction as well as tumors transduced with AVC2.TK plus GCV treatment and significant after 29 d (p < 0.001; Fig. 5A). The tumors grew continuously in the untransduced control groups [GCV] and [untreated] and in the AVC2.TK and AV.d1014 transduced control groups [TK] and [d1014].

Survival was poor in the control groups with an average survival rate below 37% after 4 weeks and below 25% after 6 weeks, with a median survival time of 29 d. Median survival in single groups was: [GCV] = 22 d, [untreated] = 22 d, and [d1014] = 29 d. In contrast, mice with transduced tumors and GCV treatment had a notably prolonged survival when compared to controls. The median survival time was 50 d (p = 0.07) for AVC2.TK with GCV treatment (group [TK/GCV]), and 60.5 d (p = 0.002) for animals transduced with transcomplementing vectors but without GCV (group [TK/d1014]). Importantly, administration of GCV to transcomplementing transduced animals further improved survival significantly (Fig. 5B, p < 0.02), showing additive effects of TK, transcomplementing and GCV administration. There was no statistical difference between transcomplementing transduced animals with early (after 30 h) or delayed (after 80 h) administration of GCV groups [TK/d1014/GCV₉₀] and [TK/d1014/GCV₃₀]. The survival was 100% in the transcomplementing transduced group with GCV treatment 80 h after transduction after 6 weeks and declined minimally to 85% at the termination of the experiments, providing an estimated median survival time of 1.2 years by regression analyses.

**Analyses of cell death in adrenocortical-small cell carcinoma after transduction**

Tumors were examined 96 hours after transduction to explore the mechanism of tumor-cell death induced by this therapy. The cleavage of nuclear DNA with a characteristic pattern of fragmentation is a hallmark of programmed cell death. The free 3'-ends of these fragments are available for labeling and thus allow detection of apoptotic nuclei within a tissue specimen. In trans-
duced SW-13 tumors, we observed single apoptotic nuclei as well as groups and cell clusters with stained nuclei throughout the specimens (Fig. 6A). Clusters were frequently found adjacent to sites of injection and virus replication (Fig. 6B).

To further examine this prominence of apoptosis, we investigated transduced and non-transduced tumors by electron microscopy. Ultrastructural analyses showed signs of apoptosis such as condensation of chromatin and cytoplasm, but also vacuolization of the nucleus and cytoplasm. Cytosolic organelles such as mitochondria remained morphologically unaffected, indicating a grossly intact metabolism in these cells. Most often, slight condensation of chromatin and fragmentation of nuclei with blebbing and a vacuolated structure were observed, especially when the nucleus contained inclusion bodies as a sign of viral replication in the transcomplementing transduced groups (Figs. 7A and B).

Discussion

In this study, we tested virally-mediated suicide gene therapy in a human xenograft model of adrenocortical carcinoma in nude mice. Transfer of the suicide gene hsv-TK with in situ transcomplementation of vectors and the subsequent administration of the antiviral drug GCV was a powerful tool against SW-13 derived adrenocortical tumors. The anti-tumor effects of viral cytotoxicity and transgene-mediated tumor cell ablation yielded more effective inhibition of tumor growth and significantly increased the survival rate in this model.

In the presented study, we used two separate viral species with either E4 (H5.d11014) or E1A/B deletions (AV2C.TK; Fig. 1) that are replication deficient in cis- but replication-competent in co-transduced cells. Transcomplementation in one cell creates a permissive environment for amplification and local spread of both vectors. The selection of H5.d11014 to transcomplement E1 defects of AV2C.TK was based on its unique properties related to the solely expression of E4orf4 of the E4 gene products. The E4orf4 protein itself is cytotoxic when expressed in multiple transformed cell lines [40]. Induction of a p53-independent pathway is implicated in the apoptotic death caused by E4orf4 expression in the cell lines that were tested [41]. The ability of d11014 to kill transduced tumor cells beyond the focus of transcomplementation enabled replication may greatly increase its utility as the vector that supplies the E1-transcomplementing function. Although we have not seen statistically significant anti-tumor activity of d11014 when used as a monovalent vector, no amplification or spread of the virus is possible in the absence of E4-transcomplementation, and toxicity induced in 5–10% of the cells within injected tumors is undetectable by measuring tumor volumes. However, in situ end-labeling of nuclei reveals increased intratumoral apoptosis at sites both adjacent and distant to areas of viral replication following tumor transduction (Fig. 6). Transgene expression, direct viral cytotoxicity or stimulation of normal apoptotic pathways that appear to be otherwise diminished might have contributed to this phenomenon [49].

In our model, tumor lysis by transcomplementation alone increases survival time to 60 days, consistent with recent reports on beneficial oncolytic effects through replicating adenoviral vectors without suicide gene [42]. The expression of both E1 and E4 gene products are essential for viral replication; there are wide areas of tumor tissue subjected to cell lysis when the transcomplementing approach is used (Fig. 6). In addition, ultrastructural analyses demonstrate nuclei with many inclusion bodies and altered morphologies in treated tissues, evidence of viral replication and associated cytopathic effects.

Viral replication in the transcomplementing system improves transgene distribution. Ninety-six hours after vector injection, there is a cell transduction rate 4 times above that seen in transductions using non-transcomplementing vectors. In this way, superior transgene spread relative to replication-deficient vectors in vivo was achieved as visualized through AV.GL-expression (Fig. 4). The efficacy of the hsv-TK transgene and/or E4orf4 increased in a manner dependent upon viral replication in our system and, therefore, significantly improves estimated median survival in an additive manner when combined with GCV. Replication-attenuated adenoviral vectors have been tested in systemic and local application strategies for biological amplification of anti-tumor effects [30,43], and additive benefits after GCV treatment have been reported [44]. It is noteworthy, however, that replication-attenuated vectors with E1B deletions may require dysfunctional p53 mechanisms for viral replication to occur [29]. As such, this approach may not be suitable for treatment of approximately two-thirds of adrenocortical carcinomas, which possess intact p53.

Gap junctions enable distribution (local bystander effect) of toxic GCV metabolites into neighboring cells, leading to wider cell ablation, but many human malignancies form few if any gap junctions [45,48]. In fact, the original tumor from which SW-13 was derived was reported not to express gap junctions; in our ultrastructural and immunohistochemical analyses, gap junctions could not be observed. This is consistent with recent reports on decreased numbers of intercellular junctions in adrenocortical tumors in vivo [47]. Although gap-junction expression can be improved through several compounds [48], a substantial benefit may be achieved by the spread of the suicide gene(s) rather than toxic metabolites, using our transcomplementing system in tumors lacking significant gap junctions. Further on, healthy untransformed cells are more likely to become affected by the bystander effect since they adequately express gap junctions. However, there is no information available concerning an inadvertent bystander effect in healthy cells, and the impact of this probably toxic side effect has yet to be determined.

In our nude mouse model, efficacy is dependent upon the additive effects of direct viral cytotoxicity and TK/GCV induced cell death in a fashion independent of normal immune effectors. Nevertheless, tumor lysis induced by specific gene transfer therapies has been suggested for improving the host’s immune response in some models [17] and allows speculation of further increments in efficacy in immunocompetent organisms. Interestingly, communication between T-lymphocytes and adrenocortical cells has been reported [50]. Normal adrenocortical cells express MHC class II molecules, but this is diminished in adrenocortical tumors [5], and this disruption may be implicated in the escape of adrenocortical tumors from immune surveillance. Indeed, we have found that upregulation of MHC class I molecules occurs during apoptosis in rat glioma cells expressing hsv-TK after GCV administration and that this is associated with im-
proved anti-tumor responses which can retard progression of, or ablate, distant tumor deposits (W.J.R., personal observation). Up-regulation of MHC molecule expression induced by the therapy we describe may then improve host anti-tumor immunity in models where intact immune functions are present. Virus-mediated cell lysis will cause a release of mediators, which then activates the immune system and surrounding infected cells and – eventually – the viral cycle as well.

Inadvertent dissemination or systemic application of vectors expressing hsv-TK and even inactivated virus particles lacking transgene expression may cause severe liver damage, inflammation and necrosis, and death in experimental animals after exposure to GCV has been reported [32,33]. Similarly, liver toxicity through non-replicating vector administration has been seen in primates and humans [51]. However, in the model investigated, release of virus from transduced tumors is accompanied by a dilutional effect that stochastically diminishes the likelihood of generating the co-transduced conditions necessary for viral replication. Furthermore, H5.d11014 expresses only the orf4 peptide of early region 4 and, therefore, cells infected in vitro with H5.d11014 demonstrate only minimal viral DNA replication, which is unique among E4 mutants [34,52]. When cells are infected in the absence of E4orf6 or E4orf3, E4orf4 downregulates the activity of E1A proteins. This leads to reduced expression of viral genes dependent upon E1A R289 phosphorylation and causing the observed replication deficient phenotype [53–55]. Separate studies will be necessary to establish what dose-limiting toxicities exist in this model and if modifications can lessen any such toxicity. In addition, a restricted opportunity for the generation and release of pseudo-wild type revertants must be any such toxicity. In addition, a restricted opportunity for the generation and release of pseudo-wild type revertants must be

In summary, our findings demonstrate that this novel transcomplementing system of adenoviral vectors significantly enhances hsv-TK/GCV suicide gene therapy for cancer. Blockade of tumor growth improves and survival time is greatly prolonged. The generation and release of pseudo-wild type revertants must be

References


Abbreviations

GCV: Ganciclovir; hsv-TK: herpes simplex virus type one thymidine kinase; M01: multiplicities of infection; MHC: major histocompatibility complex.


