Cancer Gene Therapy (2002) 9, 53–61

www.nature.com/cgt

Gene therapy with Apoptin induces regression of xenografted human hepatomas

Marjolijn M van der Eb,[1,2,a] Alexandra M Pietersen,[1,3,a] Frank M Speetjens,[2] Peter JK Kuppen,[2] Cornelis JH van de Velde,[2] Mathieu HM Noteborn,[1,3] and Rob C Hoeben[1]

Departments of [1]Molecular Cell Biology and [2]Surgery, Leiden University Medical Centre, Leiden 2333 AL, The Netherlands; and [3]Leadd BV, Leiden, The Netherlands.

The chicken anemia virus–derived Apoptin protein shows remarkable specificity; namely, it induces apoptosis in tumor cells, but not in normal diploid cells. We have exploited the *Apoptin* gene for use in cancer gene therapy. Here we demonstrate that adenovirus-mediated intratumoral transfer and expression of the *Apoptin* gene results in regression or complete remission of human hepatomas grown as xenografts in immune-deficient mice, and significantly increases their survival long term. Early after intratumoral injection, Apoptin could be detected in significant quantities by Western blot analyses and immunohistochemistry. Furthermore, cell death and disruption of the tumor integrity were apparent in the transduced regions. This experimental gene therapeutic strategy constitutes a unique example of specific antitumor activity using a virus-derived gene with broad-spectrum applicability.
Cancer Gene Therapy (2002) **9,** 53–61 DOI: 10.1038/sj/cgt/7700397

Keywords: adenovirus vector; animal model; apoptosis; cancer; tumor-specific therapy

Current anticancer therapies are limited by toxicity to normal tissue and the occurrence of therapy-resistant tumor cells. The Apoptin protein, encoded by the chicken anemia virus (CAV), shows unexpected specificity and potency toward human tumor cells. In young chickens, CAV infection leads to a depletion of the thymus caused by extensive apoptosis.[1,2] In transfection studies, Apoptin was shown to induce apoptosis in a large panel of transformed and malignant cells of avian, murine, and human origin including carcinomas, sarcomas, melanoma, lymphoma, and leukemia.[3,4]

Remarkably, Apoptin did not induce apoptosis in "normal," diploid cells.[4] However, transformation of diploid human fibroblasts or keratinocytes by expression of SV40 large T antigen rendered these cells sensitive to Apoptin-induced apoptosis.[5] The apoptosis-inducing activity correlates with the subcellular localization of the protein. Specifically, in normal cells, Apoptin resides predominantly in the cytoplasm, whereas in transformed cells, it localizes to the nucleus.[3]

Intriguingly, although Apoptin-induced apoptosis involves caspase-3, it bypasses most of the upstream components of the apoptotic pathway, making it resistant to mutations in this pathway.[6] Moreover, Apoptin-induced apoptosis is not affected by loss of functional p53, or by overexpression of either Bcl-2 or Bcr-abl — conditions that often frustrate conventional therapies such as chemotherapy and radiation. Indeed, it is even stimulated by Bcl-2 overexpression.[7] Thus, in addition to its intrinsic specificity, which allows selective action against transformed cells, Apoptin may also work in cases when chemotherapy and radiation have failed. Although the mechanism by which Apoptin distinguishes malignant cells from their "normal" primary counterparts has not yet been fully elucidated, it is attractive to exploit these abilities for cancer gene therapy.

To examine the antitumor effect of Apoptin, we generated a replication-deficient adenovirus containing the *Apoptin* gene (AdMLPvp3, further referred to as AdMLP.Apoptin). We showed that adenoviral expression of Apoptin both *in vitro* and *in vivo* did not change its specificity; namely, it still induced apoptosis in tumor cells and had no deleterious effects on normal cells. The first, short-term pilot experiment, which comprised a single intratumoral injection of AdMLP.Apoptin, the adenovirus vector that carries the *Apoptin* gene in subcutaneous human hepatoma in nude mice, showed a significant decrease in tumor growth.[8] Because we noticed that a single intratumoral injection only reaches a fraction of the tumor cells, we designed a regimen using multiple injections to determine whether AdMLP.Apoptin can cause actual tumor regression. A parallel experiment was carried out to follow the effects of AdMLP.Apoptin at the cellular level.

In this study, we show the induction of apoptosis by AdMLP.Apoptin in human hepatoma (HepG2) tumors similar to that observed for CAV in transformed chicken cells.[9] Furthermore, multiple injections of the adenovirus

Received August 29, 2001.

Address correspondence and reprint requests to: Dr Mathieu HM Noteborn, Leadd, Wassenaarseweg 72, Leiden 2333 AL, The Netherlands. E-mail: noteborn@leadd.nl

[a]Both authors contributed equally to this paper and should both be considered to be "first author."

vector with the *Apoptin* gene resulted in partial or complete regression of the established tumors in the majority of the recipient mice. Our data show for the first time that treatment with Apoptin significantly improves long-term survival of tumor-bearing animals.

Materials and methods

Cells and cell culture

Human hepatoma cells (HepG2) (obtained from ATCC, Manassas, Virginia), mycoplasm-free and negative in mouse antibody production test, were grown in Dulbecco's modified Eagle's medium CO_2 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum at 37°C/5%. The cells were harvested by trypsinization, resuspended in cold Hanks (13.6 mM NaCl, 530 μM KCl, 81 μM $MgSO_4$, 44 μM KH_2PO_4, and 34 μM Na_2HPO_4) containing 2% horse serum, and the viable cell number was determined by trypan blue exclusion and adjusted to 5×10^7 cells/mL in serum-free Hanks. Within 1 hour of harvesting, the cells were injected subcutaneously (s.c.) in the flanks of nude mice, depositing 200 μL of cell suspension per flank with a 25-gauge needle. The cells were kept on ice during the time between harvest and injection.

Viruses and virus techniques

The recombinant adenovirus vector expressing *Apoptin* (Ad.MLP.vp3, further referred to as AdMLP.Apoptin) was generated as described previously.[8] Briefly, 911 helper cells were cotransfected with the linearized Ad adapter construct, pMad-vp3, and pJM17. After homologous recombination, this results in an Ad5 genome lacking the E1 and E3 regions, containing the transgene under control of the adenovirus major late promoter (MLP). Plaques were isolated and after three rounds of plaque purification transferred to PER.C6 cells. These cells contain the E1 region regulated by a heterologous promoter which, combined with matched adaptor plasmids, eliminates the generation of Replication Competent Adenovirus (RCA) by heterologous recombination.[23] All batches of AdMLP.Apoptin were tested for Apoptin expression by indirect immunofluorescence[24] and for the presence of RCA by a polymerase chain reaction assay.[8] All virus batches used passed the RCA test detecting 1 pfu of an E1-containing adenovirus amidst 10^7 pfu of an E1-deleted vector. Construction of the recombinant adenovirus vector AdCMV.LacZ under the control of the cytomegalovirus enhancer/promoter has been reported previously.[25] Recombinant viruses were propagated on PER.c6 cells and purified by double CsCl density centrifugation. Titers of the viral stocks were determined both by plaque assay on 911 cells.[26] Virus was aliquoted and stored in sucrose buffer (140 mM NaCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 20 mM $MgCl_2$, and 5% sucrose) at -80°C.

Immunoblot

After sacrifice, tumors were dissected and half of each tumor was fixed in 4% formaldehyde for immunohistochemistry and half was flash frozen in -80°C cold isopentane. Frozen samples were subsequently ground with a microdismembrator, resuspended in 1 mL of lysis buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.1% Triton, and protease inhibitors) and incubated on ice for 1 hour. The lysate was cleared by centrifugation and immunoprecipitated with protA beads (Santa Cruz Biotechnology, Santa Cruz, CA) coupled to a polyclonal rabbit antibody recognising the C-terminus of Apoptin, αVP3C. The immunoprecipitates were washed three times with cold lysis buffer and boiled for 5 minutes in Laemli sample buffer. Samples were subjected to electrophoresis on a 12.5% polyacrylamide sodium dodecyl sulfate gel, followed by transfer to Immobilon P membranes (Millipore, Bedford, MA). After blocking in milk buffer (5% milk in Tris-buffered saline with 0.2% Tween), the membranes were incubated with the mouse monoclonal antibody 111.3 against the N-terminus of Apoptin in milk buffer overnight at 4°C. The membranes were washed with Tris-buffered saline with 0.2% Tween, and proteins were detected with Horse Radish Peroxidase (HRP)–conjugated goat antimouse IgG (Sigma-Aldrich, Zwijndrecht, the Netherlands) secondary antibody, followed by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Animals

Male Balb/c nu/nu mice, aged 7–8 weeks (Harlan, Zeist, The Netherlands), were kept in filtertop cages under DII safety conditions following Dutch government guidelines. Animals were fed sterilized laboratory chow and water *ad libitum* and were kept at alternating 12-hour periods of light and darkness. All experiments were approved in advance by the Dutch Animal Welfare Committee.

Evaluation of cellular effects after a single intratumoral injection of HepG2 tumors with AdMLP.Apoptin

Mice were injected s.c. in both flanks with 1×10^7 HepG2 cells suspended in 200 μL of serum-free Hanks. The mice were checked daily for tumor growth. Once a tumor became detectable by eye, registration of tumor growth started by measuring tumor length, width, and height using microcalipers and tumor volume was calculated every other day. Calculation of tumor volume was done using the following formula: [(smallest diameter)^2(largest diameter)/2]. Three weeks after tumor cell injection, when tumors had reached an average volume of 350 mm^3, the mice were randomized for one of the three treatment groups (AdMLP.Apoptin, AdCMV.LacZ, or virus dilution buffer), and each tumor received an intratumoral injection with 5×10^9 pfu virus or control buffer in a total volume of 100 μL. Each following day, all mice were weighed and tumors were measured and examined for changes in color or morphology. At days 2, 3, 4, 5, and 6 days after intratumoral injection, a group of mice was sacrificed, comprising of three to four tumors of each treatment group. At sacrifice, blood was collected, and after removal, the tumors, liver, and spleen were weighed and stored partly in 3.7% formalin or were flash frozen in liquid nitrogen for the prospective measurements. One hour before sacrifice, each mouse was injected intraperitoneally with

50 mg/kg 5-bromo-2′-deoxy-uridine (BrdU; Boehringer Mannheim, Mannheim, Germany).

Long-term analysis of multiple intratumoral injections in HepG2 tumors

Mice were injected s.c. in the left flank with 1×10^7 HepG2 cells suspended in 200 μL of serum-free Hanks. Every other day following tumor cell injection, the mice were checked for tumor growth. Once a tumor became detectable by eye, registration of tumor growth started as described under *Evaluation of cellular effects after a single intratumoral injection of HepG2 tumors with AdMLP.Apoptin*. Calculation of tumor volume was done using the following formula: [(smallest diameter)^2(largest diameter)/2]. Height was evaluated separately. Each tumor was allowed to grow up to a minimum volume of 250 mm^3 and a minimal height of 3 mm. When a tumor met *both* these criteria, the mouse entered the experiment and was randomly designated to one of the three treatment groups (AdMLP.Apoptin, AdCMV.-LacZ, or virus dilution buffer). The same day, the first virus injection was given intratumorally, followed by four intratumoral injections on alternating days. The injections were given on alternating days to allow for resorption of the injected volume before the next injection. For each injection, 3×10^9 pfu in 50 μL of virus in virus dilution buffer or virus dilution buffer alone was injected into the tumor tissue that looked most viable (as judged by color). The tumor was measured every other day until the end-criteria were reached (>1800 mm^3 and/or >8 mm in height). At that time, the mouse was sacrificed, blood was collected, and the tumor was excised, weighed, and fixed for further examination.

Histopathological analysis

Paraffin-embedded sections were prepared by routine methods and stained with hematoxylin and eosin. Additionally, sections (5 μm) were stained for Apoptin and β-galactosidase expression by a three-step immunoperoxidase staining using the αVP3C antibody (produced by EurogenTec, Seraing, Belgium) and an anti-β-galactosidase antibody (Dako, Glostrup, Denmark). Briefly, slides were deparaffinated, washed, and endogenous peroxidase activity was blocked for 20 hours with methanol/H_2O_2 0.3%. After rehydration, antigen retrieval was performed by a 10-hour incubation in boiling citrate buffer (0.01 M citric acid/0.01 M sodium citrate, pH=6.0). After cooling, slides were washed in phosphate-buffered saline (PBS) and incubated overnight with the antibodies anti-VP3C and anti-β-galactosidase in PBS/bovine serum albumin 1% (1:2000 for both). After washing, the slides were incubated with biotinylated antirabbit Ig (1:400, 30 hours). Next, after washing, the slides were incubated with biotinylated HRP/streptavidin complex (Dako), washed with PBS, and developed in di-amino carbazole (DAB). Staining of Apoptin and β-galactosidase was scored semiquantitatively by two independent observers and expressed as percentage of positive tumor cells within a tumor. DNA-incorporated BrdU was detected by a three-step immunoperoxidase staining with the anti-BrdU monoclonal antibody IU_4 (Hycult, Uden, the Netherlands).[27]

Statistics

Log rank tests were used for survival analysis. One AdMLP.Apoptin-treated mouse died 67 days after the start of treatment for no apparent reason. Because this mouse had been tumor-free for over a month, it was censored in the analysis. Based on normal probability plots, one outlier (in the LacZ group) was excluded from the log rank test. All animals (including outlier) are shown in the Kaplan-Meier plot (Fig 5). Results were considered statistically significant at $P<.01$. In all cases, the investigator responsible for treatment and measurement was "blinded" to the experimental status of the mouse.

Results

To investigate the effects of the adenovirus expressing *Apoptin* on a cellular level *in vivo*, HepG2 tumor-bearing mice were injected once intratumorally with AdMLP.Apoptin or with AdCMV.LacZ or virus dilution buffer as a control, and were sacrificed 2, 3, 4, 5, and 6 days postinjection. To ascertain the presence of Apoptin, protein extracts from the tumors 2 days postinjection were subjected to immunoprecipitation with an antibody against Apoptin and the protein was detected by Western blot analysis (Fig 1). Additionally, the Apoptin protein could be readily detected in HepG2 tumor tissue sections by immunohistochemistry using the polyclonal antibody anti-VP3C (Fig 2A,a). In contrast, no Apoptin signal was detected in the AdCMV.LacZ or buffer control (Fig 2A,b,c). All stainings were performed on serial sections to determine the areas of the tumor transduced with adenovirus.

Tumor areas expressing Apoptin exhibited aberrant morphology characterized by loss of tissue integrity, increase in interstitial space, and visible remnants of disintegrated cells. These disrupted areas showed loss of cell–cell contact and tumor cells containing hypodense cytoplasms and condensed dark nuclei (Fig 2A,d). Such morphological aberrations were not found in the β-galactosidase–positive fields of the HepG2 tumors treated

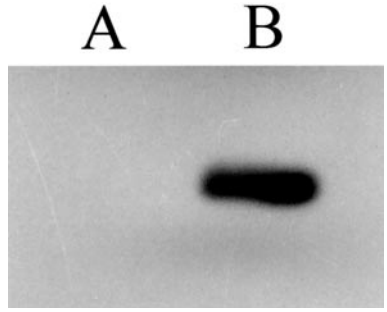
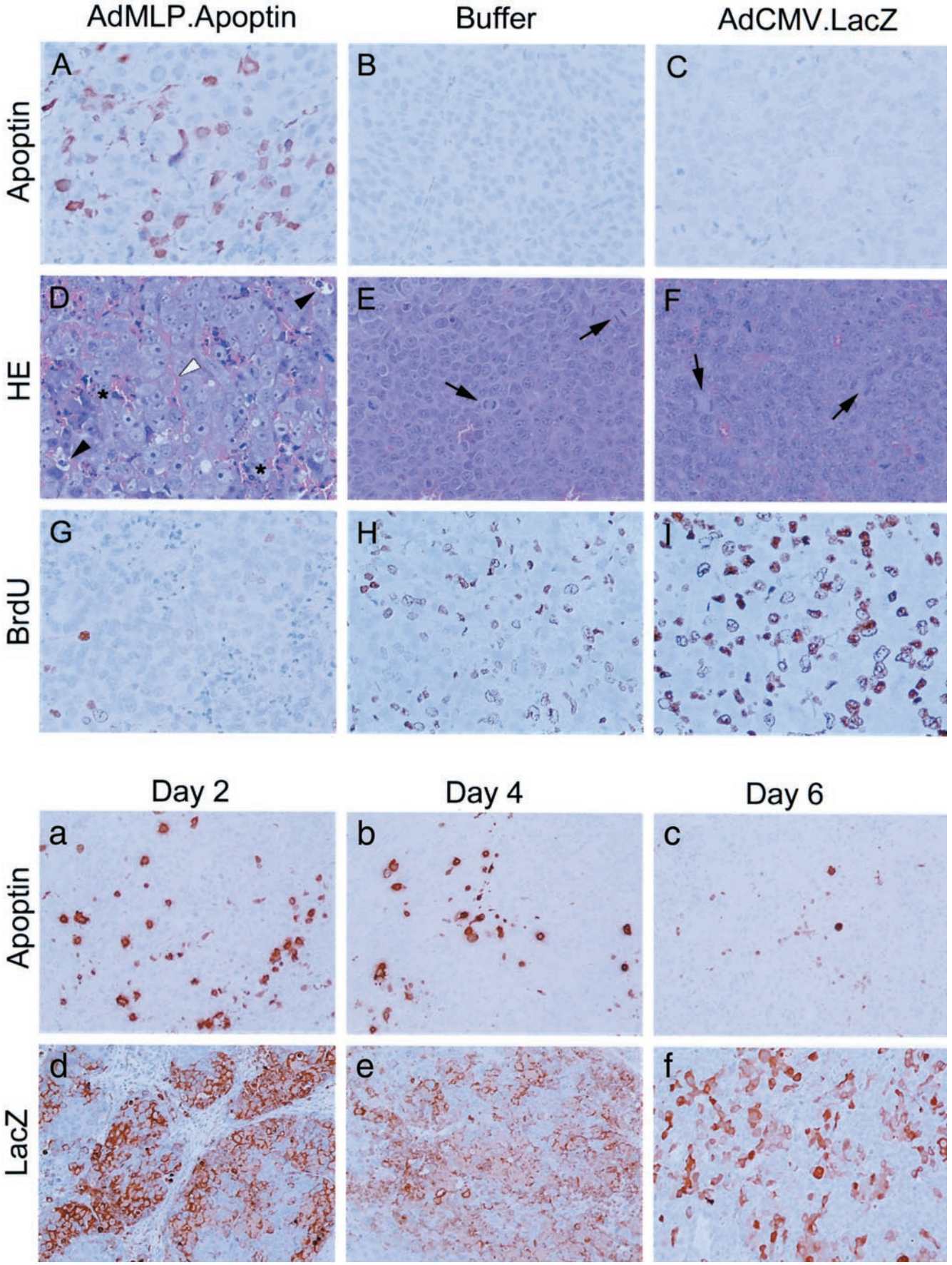


Figure 1 Apoptin expression after injection of AdMLP.Apoptin in HepG2 tumors. Two days after intratumoral injection of buffer **(lane A)** or AdMLP.Apoptin **(lane B)**, HepG2 tumors were lysed and the protein fractions were subjected to immunoprecipitation with a polyclonal rabbit antibody specific for the C-terminus of Apoptin. After blotting, the proteins were immunoprobed with mAb 111.3, which is directed against the N-terminus of Apoptin.


AdMLP.Apoptin
Buffer
AdCMV.LacZ
Apoptin
HE
BrdU
A
B
C
D
E
F
G
H
I
Day 2
Day 4
Day 6
Apoptin
LacZ
a
b
c
d
e
f

with AdCMV.LacZ (Fig 2A,e,f). These data demonstrate an Apoptin-induced antitumor effect at a cellular level *in vivo*.

To determine the viability of the infected areas in the tumors, the mice were administered BrdU before sacrifice. The number of cells containing actively replicating DNA was found to be reduced in most of the disrupted areas of the Apoptin-treated tumors (Fig 2A,g–i), but not in the β-galactosidase–positive areas (β-galactosidase staining not shown). This suggests that Apoptin-transduced areas are repressed in their outgrowth.

Over time, the number of Apoptin-positive cells decreased within the HepG2 tumors. Two days post-injection, several areas of the tumors showed up to 40% Apoptin-positive cells, as determined by immunohistochemistry (Fig 2B,a). On subsequent days, however, the number of Apoptin-positive cells decreased to less than 1% (Fig 2B,b,c). Between days 2 and 6, the Apoptin pattern distinctly changed: from a mixture of cells with homogeneously distributed nuclear and some cytoplasmic staining, to exclusively condensed nuclear staining. After 6 days, the Apoptin-positive cells had almost completely disappeared and only sporadic fragments of positive cells were found (Fig 2B,c). β-Galactosidase–expressing cells in tumors injected with AdCMV.LacZ, however, were still abundant after 6 days. In contrast to Apoptin-positive cells, virus-infected cells expressing β-galactosidase appeared histologically normal (Fig 2B,d–f), indicating a toxic effect of Apoptin, but not β-galactosidase, on the HepG2 cells.

Light microscopic evaluation of individual Apoptin-positive cells at high magnification revealed a typical pattern of the Apoptin distribution within the nuclei. Two days after infection, the distribution was faint and finely granular. Later, the granules increased in size, and gradually accumulated into "doughnut"-shaped structures (Fig 3A). Strikingly, these distribution patterns of Apoptin within the tumor cell nuclei closely resembled those observed for CAV-infected transformed chicken lymphoblastoid cells (Fig 3B).[9] Apparently, the effects of Apoptin are very similar when delivered by an adenovirus or by its natural vector, the CAV.

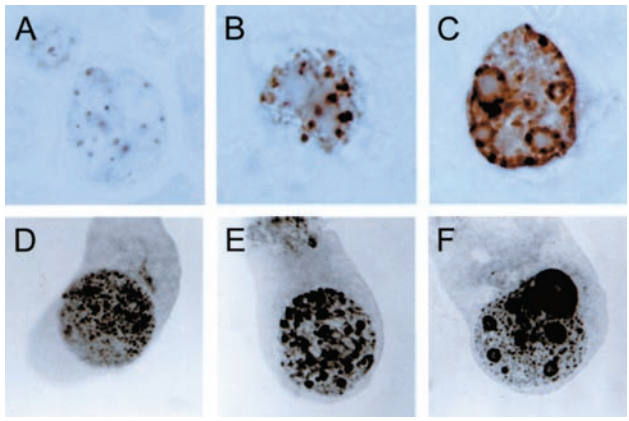


Figure 3 Immunoperoxidase staining of Apoptin. **A–C:** Staining on paraffin sections of xenografted human HepG2 tumors infected with adenovirus expressing the Apoptin protein with Apoptin-specific antibody 111.3. The tissue sections were fixed and stained 2 days after infection; three representative images are shown (original magnification: ×1250). Typically, early after infection, the distribution was faint and finely granular **(A)**. Later, the granules increased in size **(B)**, and gradually accumulated into "doughnut"-shaped structures **(C)**. Strikingly, these distribution patterns of Apoptin within the tumor cell nuclei closely resembled those observed for CAV-infected transformed chicken lymphoblastoid cells.[9] **D–F:** For comparison purpose, indirect immunoperoxidase staining of CAV-infected chicken lymphoblastoid T cells is shown from Noteborn et al.[9] In this experiment, the Apoptin-specific antibody 85.1 was used and the cells were fixed and stained 76 hours after infection.

To further evaluate the potential of *Apoptin* gene therapy, we designed an experiment to study the long-term effects

Figure 2 A: Effects of *Apoptin* gene therapy on HepG2 tumors at the cellular level. Tumor-bearing mice were treated with a single intratumoral injection of 5×10^9 pfu AdMLP.Apoptin, AdCMV.LacZ, or were injected with virus dilution buffer. At days 2, 3, 4, 5, and 6 after treatment, four mice per treatment group were sacrificed and tumors were processed for immunohistological analyses. Depicted are paraffin sections of tumors fixed 2 days after treatment. **a–c:** Sections were incubated with an Apoptin-specific antibody and labeled with peroxidase-coupled second antibody. Cells expressing Apoptin are visualized by DAB signal. The Apoptin protein was readily and exclusively detected in AdMLP.Apoptin-treated animals **(a)**. In buffer **(b)** and AdCMV.LacZ-treated animals **(c)**, no Apoptin was detected. **d–f:** Hematoxylin and eosin staining of sequential sections of **(a)**, **(b)**, and **(c)**. **d:** At the tumor sites infected with AdMLP.Apoptin, tumor cell integrity was disrupted with decreased cell–cell contact (▷), containing enlarged hypodense cells with dark shrunken nuclei. Scattered throughout these areas, fragments of disintegrated cells (*) and apoptotic cells (▶) were observed. In contrast, tumors treated with buffer **(e)** and AdCMV.LacZ **(f)** showed a uniform and regular structure. Additionally, frequent mitotic figures were present (→). **g–i:** Mice were injected with BrdU before sacrifice to determine the number of DNA-replicating cells in the HepG2 tumors. **g:** Anti-BrdU staining in areas of Apoptin expression (sequential sections) revealed predominantly low incorporation of label. In contrast, areas expressing β-galactosidase **(h)** or injected with buffer **(i)** showed persistent BrdU incorporation. **B:** Transgene expression over time after recombinant adenovirus injection: sections of HepG2 tumors growing in nude mice. The animals were sacrificed 2, 4, or 6 days after a single virus injection (AdMLP.Apoptin or AdCMV.LacZ). **Upper panel:** Apoptin staining as described under **(A)**. **Lower panel:** β-Galactosidase expression in AdCMV.LacZ-treated tumors was determined by labeling with a specific antibody, visualized by DAB oxidation. **a–c:** High expression of Apoptin is detected in tumors 2 days after intratumoral AdMLP.Apoptin injection. After 4 days, the number of Apoptin-positive cells decreases and after 6 days, only sporadic Apoptin expression is found. Furthermore, the structure of Apoptin staining in the cells also changes over time. Early after virus infection, the tumor cells show both cytoplasmic and nuclear-localized Apoptin **(a)**. Later, mostly nuclei are stained positive **(b,c)**. Six days after injection, predominantly fragments of stained tumor cells and few intact stained tumor cells are detected **(c)**. **d–f:** The number of β-galactosidase–expressing cells in AdCMV.LacZ-injected tumors remains constant over time. Two days after infection, a high number of positive cells was detected **(d)**. In contrast to the Apoptin expression in Apoptin-treated animals, β-galactosidase is still present in a high percentage of cells 4 **(e)** and 6 **(f)** days after virus injection.

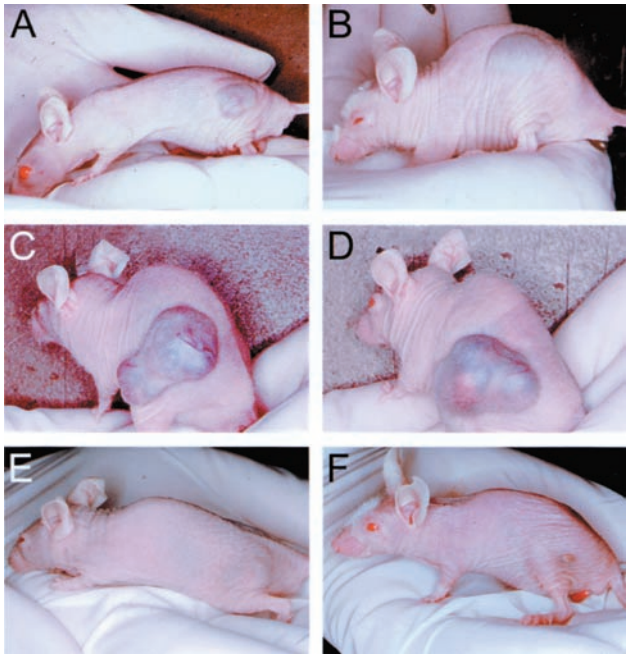


Figure 4 Human hepatomas (HepG2) in nude mice before and after Apoptin gene therapy treatment. Subcutaneously grown HepG2 tumors were injected five times on alternating days with 3×10^9 pfu AdMLP.Apoptin, 3×10^9 pfu AdCMV.LacZ, or buffer. Hereafter, tumor growth was measured regularly. **A,B:** Examples of HepG2 tumors at the start of treatment. The criteria to start virus treatment were met at this point: a tumor volume > 250 mm^3 and tumor height of 3.0 mm. **C,D:** Examples of HepG2 tumors treated with LacZ or buffer at the point when the criteria were met to end the experiment. The end-criteria were: a tumor volume of > 1800 mm^3 and a tumor height of 9 mm. **E,F:** Tumor regression upon AdMLP.Apoptin treatment. Examples of partial and complete remission **(E,F)** of HepG2 tumors achieved after Apoptin gene therapy.

and survival benefit of treating tumor-bearing mice with AdMLP.Apoptin. Based on previous observations that the percentage of transduced tumor cells is limited after one intratumoral injection, we attempted to achieve overall transduction of the tumor by using an improved treatment protocol. This protocol consisted of five intratumoral injections/tumor every other day, each injection comprising 3×10^9 pfu AdMLP.Apoptin, 3×10^9 pfu AdCMV.LacZ, or sucrose buffer. At the time of treatment, the tumors had a volume of minimally 250 mm^3 and a tumor height of at least 3 mm (Fig 4A and B). Each tumor was measured four times

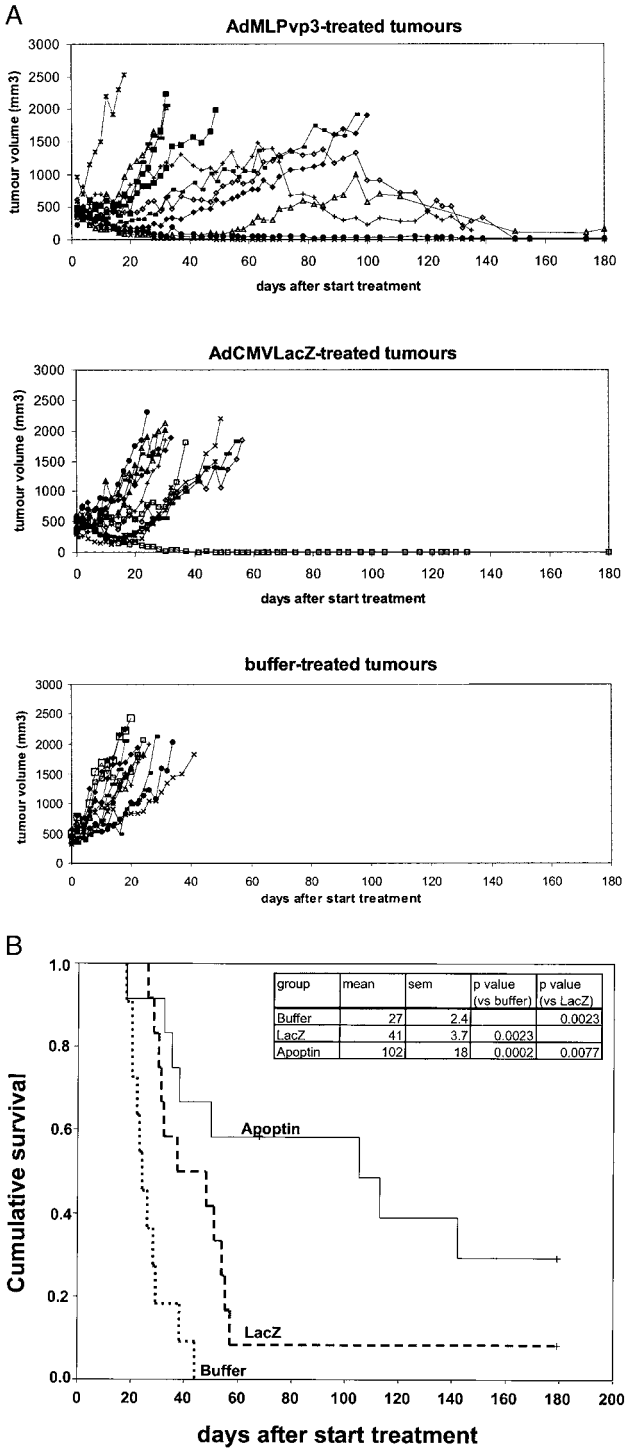


Figure 5 Long-term effect of *Apoptin* gene therapy *in vivo*. **A:** Tumor growth kinetics after treatment. Nude mice (nu/nu) were implanted subcutaneously with HepG2 cells and tumor growth was determined every other day. Each individual mouse was monitored until the criteria were met to enter the experiment. At that point, each mouse was randomly divided for multiple injections either with AdMLP.Apoptin **(a)**, AdCMV.LacZ **(b)**, or buffer **(c)**. In the following weeks, each tumor was measured until its size reached the end-criteria at which point the mouse was sacrificed. **B:** Survival analysis. Mice treated with AdMLP.Apoptin survived longer than the mice in the other two groups ($P<.01$). One hundred days after the beginning of the treatment, still 60% of the animals treated with AdMLP.Apoptin (straight line) was alive, whereas at that point, 90% of AdCMV.LacZ (dashed grey line) and 100% of buffer-treated (dotted black line) mice had met the end-criteria and were sacrificed by the experimentator. **Insert:** Mean survival after *Apoptin* gene therapy. Tumor-bearing mice treated with buffer had a mean survival of 27 days (SEM=2.4). The mean survival for AdCMV.LacZ was 41 days (SEM=3.7). The mean survival of Apoptin-treated animals was almost twice as long compared to LacZ and three times longer than the buffer-treated mice.

weekly. The virus-injected tumors showed a pale appearance during the 10-day treatment period. To optimise the treatment, the inoculation was administered to the areas of the tumor that appeared most viable, as judged by the dark red color of nontreated HepG2 tumors. After the treatment period, the AdCMV.LacZ-treated tumors gradually regained this dark red color, whereas Apoptin-treated tumors remained pale.

The growth kinetics of the tumors following treatment is shown in Figure 5A. During treatment, the tumors in both virus groups were delayed in growth, but soon after the end of treatment, the LacZ-treated tumors resumed growing (Fig 5A,b), whereas most of the Apoptin-treated tumors did not (Fig 5A,a). The majority of the mice treated with AdMLP.Apoptin showed partial or complete response to treatment (Fig 4E and F). However, there were also several nonresponders to the Apoptin treatment, which had to be sacrificed early in the experiment along with animals from the buffer- and AdCMV.LacZ-treated groups (Fig 5A,a–c). Animals were sacrificed when their tumor had reached a minimal size of 1800 mm^3 and a height of at least 8 mm (Fig 4C and D).

Between 8 and 28 days after intratumoral injection, all buffer-injected animals had to be sacrificed. After 60 days, only 1 of 12 of the AdCMV.LacZ-treated mice survived, whereas at that point, 7/12 of Apoptin-treated animals were still alive (Fig 5B). To comply with the standard criteria for normal distribution, one outlier from the LacZ-treated group was excluded from statistical analysis. This results in a mean survival of LacZ-treated mice of 41 days, compared to a mean survival of buffer-treated mice of 27 days (Fig 5B). Tumor-bearing mice treated with AdMLP.Apoptin, however, survived much longer than the mice in both other groups (mean survival 102 days; $P<.01$). Six months after treatment, 30% of Apoptin-treated animals was completely tumor-free. These results demonstrate that Ad-Apoptin can confer significant survival benefits and tumor reduction when used *in vivo*.

Discussion

Here, we describe an unconventional approach for cancer gene therapy that is based on the tumor-specific activity of Apoptin. We initially observed that adenoviral transfer of Apoptin into subcutaneous HepG2 tumors in nude mice had a negative effect on tumor growth.[8] To further investigate the therapeutic benefit of AdMLP.Apoptin for these tumors, we now treated well-established tumors with multiple injections over a period of 10 days. With this approach, we were able to achieve complete regression of tumors treated with AdMLP.Apoptin, thus providing proof of principle that Apoptin can be used as an anticancer agent.

Regarded in more detail, the Apoptin-treated tumors can be divided into three distinct groups: those with a complete response, those with a significant delay in tumor growth, and those with tumor growth kinetics similar to tumors treated with AdCMV.LacZ. Because no replicating virus is produced, any tumor cell that escapes viral infection during the course of the five treatments will continue to proliferate, provided that there is not too much surrounding damage. Thus, differences in response to Apoptin treatment are probably an effect of adenoviral dispersion throughout the tumor. Indeed, the characteristic architecture of HepG2 tumors, with their partitioned lobular structure, does not allow an even distribution throughout the tumor after a single injection.[8] Thus, it is plausible that complete regression occurred only in tumors in which all lobes received sufficient adenovirus. The partially responding tumors were most likely only transduced in certain areas; although substantial tumor cell death and disruption of tissue integrity contributed to a delayed outgrowth of these HepG2 tumors, there was not enough viral dispersion to completely eliminate the tumor. In the case of the nonresponders, probably only a minor percentage of tumor cells was infected, resulting in a rapid outgrowth of the nontransduced cells. Nevertheless, this *in vivo* experiment shows a significant survival benefit for tumor-bearing mice treated with AdMLP.Apoptin, provided that a substantial percentage of tumor cells can be transduced with Apoptin.

Investigation of the antitumor effect of Apoptin at the cellular level shows that a single AdMLP.Apoptin injection causes substantial damage to HepG2 tumors. In contrast, control-treated tumors showed normal morphology and high proliferation, as determined by BrdU labeling. The areas of AdMLP.Apoptin-treated tumors containing Apoptin-positive cells, however, showed extensive aberrant morphology and a substantial decrease in proliferating cells, already detectable at 2 days after injection. The extent of damage to the tumor architecture surpassed that which would be expected based on the number of positive cells that was detected. This observation likely indicates a deleterious effect of dying Apoptin-positive cells on surrounding, noninfected cells. Thus, in addition to specific tumor cell killing by Apoptin, it is possible that a critical threshold of apoptosis in tissue can lead to a bystander effect on nontransduced cells, thereby increasing the overall efficacy.

There are several pieces of evidence suggesting that AdMLP.Apoptin induced apoptosis in the treated tumors. Firstly, Apoptin accumulates in HepG2 tumors in similar structures that arise when apoptosis is induced by CAV in chicken lymphoblastoid cells. Secondly, Apoptin induces rapid apoptosis in HepG2 cells *in vitro*.[8] Finally, in contrast to LacZ-transduced tumors, Apoptin-positive cells are nearly depleted 6 days after injection. Taken together, these findings strongly suggest that the damage observed in these HepG2 tumors is a consequence of Apoptin-induced apoptosis. Although hematoxylin and eosin staining showed a trend toward increased apoptosis in tumors treated with AdMLP.Apoptin, examination of DNA fragmentation by TUNEL staining could not confirm this observation (data not shown). This difficulty in detecting apoptosis *in vivo* has been described for several *in vivo* apoptosis systems; for instance, the complete involution of islets of Langerhans in *myc*-transgenic mice[10] or the disappearance of the prostate in castrated rats.[11] In these cases, the apoptotic process combined with phagocytosis occurs in such a short time span[12–14] that only occasional apoptotic cells are detected. Nevertheless, the entire tissue eventually disappears.

Gene therapy with *Apoptin* offers unique advantages over current approaches for cancer therapy. For example, resistance to cancer therapies is often caused by the inactivation of apoptotic pathways, which has occurred in the majority of tumors.[15–17] Gene therapeutic approaches based on restoring part of the apoptotic pathway, for instance, transduction of *p53*, *p16*, *bax*, and *bcl-x*, are expected to be successful in a limited subset of tumors, depending on the particular mutations in their apoptotic machinery. The fact, that Apoptin does not need a functional p53 pathway, is not hindered by common blockage of apoptosis by Bcl-2 or Bcr-abl expression, and apparently acts downstream of most decision factors, suggests that it will be applicable to a wide range of tumors. *In vitro*, Apoptin induces apoptosis in every cancer cell type of the extensive panel that has been tested.[18]

In addition to therapy resistance, toxicity to normal tissues often hampers cancer therapies such as chemotherapy and radiation. Moreover, most conventional gene therapeutic approaches suffer from insufficient tumor cell specificity, as illustrated by reports on toxic effects in healthy tissues due to the use of the herpes simplex virus thymidine kinase.[19,20] Attempts to increase selectivity further include the use of tumor-specific promoters (e.g., CEA and AFP) or targeted vectors. Even if the desired specificity does not abolish the necessary potency, these strategies, again, are likely to be applicable to only a subset of tumor types. In marked contrast, extensive *in vitro* data show that Apoptin has unparalleled specificity; despite its potency toward tumor cells, no normal cell type tested thus far has shown sensitivity to Apoptin.[3,4,18] Consistent with this, during the *in vivo* experiments described here, we did not observe any toxic effects of AdMLP.Apoptin treatment, corroborating the data of a more extensive toxicity study after intravenous injection of AdMLP.Apoptin in healthy rats.[8]

The combination of potency and specificity residing in a single molecule provides new possibilities for cancer therapy. So far, the therapeutic use of Apoptin seems to be predominantly limited by factors in common with most gene therapy strategies; namely, the ability to transduce all target cells. New delivery methods are being developed to increase efficacy of spread throughout solid tumors and throughout the body. The *Apoptin* gene is exquisitely suited for inclusion in technologies such as conditionally replicative viruses[21,22] or nonviral transduction methods due to its potency and small size. However, these new delivery strategies still have to be validated. At this time, the marked antitumor effect and survival benefits of Ad-Apoptin as we have shown in this paper already warrant its evaluation for human clinical trials in the near future.

Acknowledgments

The authors gratefully thank S Le Cessie (Department of Medical Statistics, Leiden University) for statistical analysis, and Dr. PM Voorhoeve and Dr. JL Rohn for critical review of the manuscript and stimulating discussions. This work was supported by a research Grant from the Netherlands Ministry of Economic Affairs.

References

1. Jeurissen SH, Wagenaar F, Pol JM, van der Eb AJ, Noteborn MHM. Chicken anemia virus causes apoptosis of thymocytes after *in vivo* infection and of cell lines after *in vitro* infection. *J Virol*. 1992;66:7383–7388.
2. Van der Eb AJ, Todd D, Noteborn MHM. Genus Gyrovirus (Circoviridae). In: Tidona CA, Darai G, eds. *The Springer Index of Viruses*. Heidelberg, Germany: Springer-Verlag. In press.
3. Noteborn MHM, van der Eb AJ. Apoptin induces apoptosis in transformed cells specifically: Potentials for an antitumor therapy. *Biog Amines*. 1999;15:73–91.
4. Danen-van Oorschot AAAM, Fischer DF, Grimbergen JM, et al. Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci USA*. 1997;94:5843–5847.
5. Noteborn MHM, Zhang YH, van der Eb AJ. Apoptin specifically causes apoptosis in tumor cells and after UV-treatment in untransformed cells from cancer-prone individuals: a review. *Mutat Res*. 1998;400:447–455.
6. Danen-van Oorschot AAAM, van der Eb AJ, Noteborn MHM. The CAV-derived protein apoptin requires activation of caspases for induction of apoptosis in human tumor cells. *J Virol*. 2000;74:7072–7078.
7. Danen-van Oorschot AAAM, Zhang Y, Erkeland SJ, Fischerr DF, van der Eb AJ, Noteborn MHM. The effect of Bcl-2 on Apoptin in 'normal' *vs.* transformed human cells. *Leukemia*. 1999;13:S75–S77.
8. Pietersen AM, van der Eb MM, Rademaker HJ, et al. Specific tumor-cell killing with adenovirus vectors containing the apoptin gene. *Gene Ther.* 1999;6:882–892.
9. Noteborn MHM, Todd D, Verschueren CAJ, et al. A single chicken anemia virus protein induces apoptosis. *J Virol*. 1994;68:346–351.
10. Pelengaris S, Rudolph B, Littlewood T. Action of Myc *in vivo* — proliferation and apoptosis. *Curr Opin Genet Dev.* 2000; 1:100–105.
11. Hu Z, Ito T, Yuri K, Xie C, Ozawa H, Kawata M. *In vivo* time course of morphological changes and DNA degradation during the degeneration of castration-induced apoptotic prostate cells. *Cell Tissue Res*. 1998;1:153–160.
12. Negoescu A, Labat-Moleur F, Defaye G, et al. Contribution of apoptosis to the phenotypic changes of adrenocortical cells in primary culture. *Mol Cell Endocrinol*. 1995;110: 175–184.
13. Arends MJ, McGregor AH, Wyllie AH. Apoptosis is inversely related to necrosis and determines net growth in tumors bearing constitutively expressed *myc*, *ras*, and *HPV* oncogenes. *Am J Pathol*. 1994;144:1045–1057.
14. Qazilbash MH, Xiao X, Seth P, Cowan KH, Walsh CE. Cancer gene therapy using a novel adeno-associated virus vector expressing human wild-type *p53*. *Gene Ther.* 1997;4: 675–682.
15. Wallace-Brodeur RR, Lowe SW. Clinical implications of *p53* mutations. *Cell Mol Life Sci*. 1999;1:64–75.
16. Reed JC. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematol Oncol Clin North Am*. 1995;2: 451–473.
17. Hickman JA, Potten CS, Merritt AJ, Fisher TC. Apoptosis and cancer chemotherapy. *Philos Trans R Soc London, Ser B: Biol Sci*. 1994;1313:319–325.
18. Noteborn MHM, Danen-van Oorschot AAAM, van der Eb AJ. The apoptin gene of chicken anemia virus in the induction of apoptosis in human tumorigenic cells and in gene therapy of cancer. *Gene Ther Mol Biol*. 1998;1:399–406.
19. Van der Eb MM, Cramer SJ, Vergouwe Y, et al. Severe hepatic dysfunction after adenovirus-mediated transfer of the herpes

simplex virus thymidine kinase gene and ganciclovir administration. *Gene Ther.* 1998;4:451–458.
20. Brand K, Arnold W, Bartels T, et al. Liver-associated toxicity of the HSV-tk/GCV approach and adenoviral vectors. *Cancer Gene Ther.* 1997;1:9–16.
21. Bischoff JR, Kirn DH, Williams A, et al. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science*. 1996;274:373–376.
22. Doronin K, Toth K, Kuppuswamy M, Ward P, Tollefson AE, Wold WS. Tumor-specific, replication-competent adenovirus vectors overexpressing the adenovirus death protein. *J Virol*. 2000;74:6147–6155.
23. Fallaux FJ, Bout A, van der Velde I, et al. New helper cells and matched early region 1–deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther.* 1998;9:1909–1917.
24. Van den Heuvel SJ, van Laar T, Kast WM, Melief CJ, Zantema A, van der Eb AJ. Association between the cellular p53 and the adenovirus 5 E1B-55kd proteins reduces the oncogenicity of Ad-transformed cells. *EMBO J.* 1990;8:2621–2629.
25. Herz J, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci USA*. 1993;7:2812–2816.
26. Fallaux FJ, Kranenburg O, Cramer SJ, et al. Characterization of 911: a new helper cell line for the titration and propagation of early region 1–deleted adenoviral vectors. *Hum Gene Ther.* 1996;7:215–222.
27. Gratzner HG. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science*. 1982;4571:474–475.