Telomerase-Dependent Virotherapy Overcomes Resistance of Hepatocellular Carcinomas against Chemotherapy and Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand by Elimination of Mcl-1

Thomas Wirth, Florian Küehnel, Bettina Fleischmann-Mundt, Norman Woller, Meta Djojosubroto, Karl Lenhard Rudolph, Michael Manns, Lars Zender, and Stefan Kubicka

Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Hannover, Germany

Abstract

Hepatocellular carcinomas (HCC) are drug-resistant tumors that frequently possess high telomerase activity. It was therefore the aim of our study to investigate the potential of telomerase-dependent virotherapy in multimodal treatment of HCC. In contrast to normal liver, HCC xenografts showed high telomerase activity, resulting in tumor-restricted expression of E1A by a telomerase-dependent replicating adenovirus (hTERT-Ad). Neither tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) nor chemotherapy alone nor the combined treatment with both agents resulted in significant destruction of HCC cells. Application of hTERT-Ad at low titers was also not capable to destroy HCC cells, but telomerase-dependent virotherapy overcame the resistance of HCC against TRAIL and chemotherapy. The synergistic effects are explained by a strong down-regulation of Mcl-1 expression through hTERT-Ad that sensitizes HCC for TRAIL- and chemotherapy-mediated apoptosis. To investigate whether down-regulation of Mcl-1 alone is sufficient to explain synergistic effects observed with virotherapy, Mcl-1 expression was inhibited by RNA interference. Treatment with Mcl-1-siRNA significantly enhanced caspase-3 activity after chemotherapy and TRAIL application, confirming that down-regulation of Mcl-1 alone is responsible for the drug sensitization by hTERT-Ad. Consistent with these results, heterologous expression of Mcl-1 significantly reduced the sensitization of hTERT-Ad transduced cells against apoptosis-inducing agents. Chemotherapy did not interfere with quantitative hTERT-Ad production in HCC cells. Whereas hTERT-Ad virotherapy alone was only capable to inhibit the growth of Hep3B xenografts, virochemotherapy resulted in almost destruction of the drug-resistant HCC. In conclusion our data indicate that telomerase-dependent virotherapy is an attractive strategy to overcome the natural resistance of HCC against anticancer drugs by elimination of Mcl-1.

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Introduction

Hepatocellular carcinoma (HCC) is one of the main complications of liver cirrhosis and a serious health problem worldwide with rising incidences particularly in the western world (1). HCCs are usually treated by surgical resection or liver transplantation with curative options for the patients when the disease is caught at an early stage (2). However, ~70% of patients diagnosed are inoperable because of advanced tumor growth or liver cirrhosis. Percutaneous ethanol injection (PEI) and radiofrequency or laser thermal ablation are minimally invasive techniques providing a curative potential for patients with liver cirrhosis and small nodular-type tumors. Nonrandomized studies have shown that PEI produces prognoses similar to surgical resection.

Patients with HCCs that cannot be completely removed by surgery or ablated by minimal invasive approaches have a dismal prognosis, as HCCs belong to the group of cancers that are resistant to systemic chemotherapy. Most of the patients with HCCs die due to intrahepatic tumor growth rather than extrahepatic metastases (3), which is considered as a rationale for regional intra-arterial therapy. But even intra-arterial chemembolization provides, if at all, only a small survival advantage for patients as shown in randomized phase III studies (4, 5). Therefore, more effective approaches for the treatment of HCCs are urgently needed.

Gene therapy with viral vectors such as adenoviruses may be an attractive tool to treat HCCs. Recently, we showed that replacement of p53 by intra-arterial adenoviral gene therapy was capable to induce tumor remissions in p53 mutated HCCs in the liver of rats and improved the survival of the animals (6). However, gene therapy strategies using nonreplicating adenoviral vectors have some limitations for clinical application because of the ineffective transduction rate of cancer cells. Principally, replicating vectors have the potential to overcome the hurdle of ineffective tumor transduction by lysis of infected cancer cells and spreading of the infection throughout the whole tumor mass. Tumor-specific replicating adenoviral vectors can be generated by deletion or mutation of viral genes (7, 8), tumor-specific recombination of viruses (9), transcriptional repression of cellular genes essential for viral replication (10), and by transcriptional control of viral genes by tumor-specific promoters (11–13).

Conditionally replicating adenoviruses have been constructed for the treatment of HCCs by using AFP-promoter controlled viral gene expression (12, 14, 15). However, only in 30% of patients with HCCs the α-fetoprotein (AFP) serum level is elevated above 400 ng/mL (3) and even in tumors with high AFP serum levels the activity of the AFP promoter may be heterogeneous within the tumor tissue resulting in ineffective spreading of AFP-promoter controlled viruses. A more promising approach for the construction of tumor-specific replicating vectors seems the linkage of viral replication to frequently activated oncogenic pathways.

Note: T. Wirth and F. Küehnel contributed equally to this work.

Requests for reprints: Stefan Kubicka, Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Carl Neuberg Str. 1, 30625 Hannover, Germany. E-mail: Kubicka.Stefan@mh-hannover.de.

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Reactivation of telomerase activity is a characteristic molecular mechanism in hepatocarcinogenesis and it has been shown that hTERT is overexpressed in ~80% to 100% of human HCCs (16–18). We and others recently showed that the hTERT-promoter can be used to generate tumor-specific replicating vectors for safe and effective virotherapy of telomerase-positive cancers (19–28). The resulting adenoviral vector hTERT-Ad contained the E1A protein under the control of the hTERT promoter thus restricting its replication to telomerase-positive cells.

It was therefore the aim of this study to investigate the value of hTERT-Ad in virotherapy of HCCs and to develop effective antitumor strategies based on virotherapy in conjunction with chemotherapeutic and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In contrast to normal liver, all investigated HCCs had high telomerase activity, resulting in tumor-restricted expression of E1A by hTERT-Ad. Chemotherapy interfered neither with adenoviral replication in HCCs nor with the quantitative production of the adenoviral vector, which are important preconditions for an effective simultaneous virochemotherapy. Treatment of HCC cells with low titers of hTERT-Ad overcame the resistance of the tumor cells against chemotherapy and TRAIL by down-regulating Mcl-1 expression. Whereas hTERT-Ad virotherapy alone was only capable to inhibit the growth of HCCs, the combination of systemic chemotherapy and local hTERT-Ad virotherapy showed strong synergistic effects in vivo, resulting in significant tumor remission. Our data indicate that virochemotherapy may be an attractive strategy for the treatment of drug-resistant tumors, such as HCCs.

Materials and Methods

Cell lines, plasmids, and adenoviral vector preparation. The human cell lines Hep3B, HuH7 (both hepatoma), AGS (gastric carcinoma), HT1080 (fibrosarcoma), and the embryonal kidney cell line 293 was obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in growth medium (DMEM + Glutamax; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 100 units/ml penicillin, and 100 g/ml streptomycin (Seromed, Berlin, Germany) at 37°C in 5% CO2. CaCo cells were maintained in 20% fetal bovine serum containing medium. The Mcl-1 expression plasmid pcDNA3.15-V5-Mcl-1 was kindly provided by Prof. Dr. Roland Schmid (München, Germany). Construction and characterization of hTERT-Ad as well as adenoviral vector preparation have been described previously (22).

Telomerase extract preparation and telomerase repeat amplification protocol assay. Telomerase extract preparation and telomerase repeat amplification protocol (TRAP) assays were done with the TRAP-eze Telomerase Detection System (Chemicon, Temecula, CA) according to the manufacturer's recommendations. Briefly, a small portion (~2 mm3) of liver/tumor tissue was minced in ice-cold PBS. After washing with PBS, tissue samples were incubated in 200 μL CHAPS lysis buffer containing 200 units/ml RNase inhibitor (Sigma, St. Louis, MO) for 30 minutes on ice and centrifuged at 15,000 rpm for 20 minutes at 4°C. The resulting supernatant (200 ng) was used for the TRAP Assay. Protein concentration determination was done using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). The protein concentration determination was then done using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). The resulting supernatant was subjected to telomerase extension at 30°C for 30 minutes and then to PCR amplification in the presence of TS primer labeled with 32P-y-ATP (Amersham Biosciences, Freiburg, Germany). PCR was done with an initial denaturation (94°C, 30 seconds) followed by denaturation (94°C, 30 seconds) and annealing extension step (60°C, 30 seconds) for 27 cycles, and final incubation at 4°C. TRAP products were size fractionated on a 10% polyacrylamide gel. The gel was dried at 75°C and then exposed to Hyperfilm MP (Amersham Biosciences) at ~80°C for 12 hours.

Western blot analysis. For protein extract preparation Hep3B and Hep3B cells were harvested and subsequently lysed with radioimmunoprecipitation assay (RIPA) buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.02% protease inhibitor cocktail (Sigma), in PBS]. For analysis of transduced HCC tumors in vivo, 1 x 106 cells were inoculated s.c. in nude mice and tumors with an approximate volume of 500 mm3 were injected with 1 x 107 viral particles of Ad-GFP hTERT-Ad, or Ad-wt, respectively. Tumors were harvested 24 hours after injection. For murine liver tissue analysis, 1 x 107 viral particles were injected i.v. into nude mice and mice livers were harvested after 24 hours. Tissue samples were homogenized in RIPA buffer using a Potter-Elvehjem homogenizer. After centrifugation the supernatant was obtained and the protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA). Proteins (10-40 μg) were separated on a 10% SDS polyacrylamide gel and electrotransferred to a HyBond N-membrane (Millipore, Eschborn, Germany). Equal loading and protein expression were determined by Coomassie staining and analysis of actin expression. Primary antibodies for protein detection were rabbit anti-Ad2E1A-135S-5 (sc-430; Santa Cruz Biotechnology, Santa Cruz, CA) for E1A, mouse anti-bcl-2 human/mouse (Calbiochem, La Jolla, CA) for Bcl-2, rabbit anti-bcl-2 XL (sc-834, Santa Cruz Biotechnology) for Bcl-2XL, rabbit anti-human/mouse TRAIL-R2/DR5 (AF 837, R&D Systems, Wiesbaden, Germany) for DR5, rabbit anti-Mcl-1 (A20-240, Stressgen, San Diego, CA) for Mcl-1, and goat anti-actin-(C-11) (sc-1615, Santa Cruz Biotechnology) for actin. As secondary antibodies, the following horseradish peroxidase-conjugated antibodies were used: donkey anti-mouse IgG (Dianova, Hamburg, Germany), donkey anti-rabbit IgG (Dianova), and mouse/human absorbed anti-goat (sc-2056, Santa Cruz Biotechnology). Antigen-antibody complexes were visualized using the enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham Biosciences).

Chemosensitivity assessment and cytostasis assays in vitro. Cells were seeded at 1 x 105 cells/cm2 density in 24-well plates. For initial analysis of sensitivity to chemotherapy, HCC cells were exposed to doxorubicin (50 to 400 ng/ml) or 5-fluorouracil (1 to 20 μg/ml) in medium. After 48 hours, crystal violet staining was done to visualize cell death. Cell layers were rinsed with PBS and fixed for 10 minutes with 10% formalin in PBS. Cells were washed with distilled water and stained for 30 minutes with 0.1% crystal violet in 10% ethanol.

For the cytostasis assays, cells were seeded at 1 x 105 cells/cm2 density in 24-well plates and infected with recombinant human/human/mouse-drug resistance proteins (MOI of 0.1 for HuH7 and 0.01 for Hep3B. After 4 days of incubation, 1 ml of chemotherapeutic agents in medium was added in low concentrations [doxorubicin, 50 ng/ml; cisplatin, 0.5 μg/ml; 5-fluorouracil, 5-FLU, 5 μg/ml]. For TRAIL experiments, either recombinant human soluble TRAIL (KillerTRAIL, AXXORA, Grünberg, Germany) in concentrations of 50 ng/ml or Ad-TRAIL was added after 4 days in nontoxic MOIs (1-10) for HuH7 and 1 for Hep3B. After addition of chemotherapeutic agents, Ad-TRAIL or the combination of both, the cells were further incubated for 2 to 4 days. Cell layers were then stained with crystal violet to visualize cytostasis.

Small interfering RNA experiments and caspase assays. For small interfering RNA (siRNA)–mediated down-regulation of Mcl-1 the following siRNA sequences were purchased from MWG Biotech (Ebersberg, Germany): 5’-GGUCGCGGGAUUCGGUAUAdTdT-T3’ as sense and 5’-AUUCCCAGAUUCCCGGACAdTdT-T3’ as antisense strand for the first siRNA duplex and sense strand 5’-GAACCCGCUAUCGCAGAdTdT-T3’ and antisense strand 5’-UCCGAGUAUCCGCGGAAUCdTdT-T3’ for the second siRNA duplex. For Western blot analysis of Mcl-1 down-regulation and caspase assays 2 x 105 cells were seeded in 6-cm dishes at a confluency of ~20%. Cells were transfected twice with 500 pmol of Mcl-1 or scrambled siRNA using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 24 hours, the cells were then harvested for Western blot analysis. Twenty-four hours after the second siRNA transfection, cells were incubated with doxorubicin (3 mL of 200 ng/ml) or infected with Ad-TRAIL (MOI 5) and harvested after a further incubation for 24 hours. For caspase assay a slightly modified Caspase-3 assay from BD
Biosciences (BD ApoAlert Caspase Fluorescent Assay Kit; Heidelberg, Germany) was used. Cells were washed once with PBS, lysed with 200 μL of lysis buffer supplied by the manufacturer, and were then subjected to five cycles of freezing and thawing. Ten microliters of native protein extracts were measured with or without caspase inhibitor. Samples were incubated for 2 hours at 37°C and fluorescence was detected after 1 hour and 2 hours using a standard fluorometer. Protein concentration in extracts was measured by Bio-Rad protein assay (Bio-Rad) and fluorescence of each sample was normalized against protein content. The results were processed by using Student’s t test, with P < 0.05 accepted as denoting statistical significance.

Mcl-1 complementation experiments. The day before transfection a total of 7 × 10^5 HuH 7 cells per well were seeded in 6-well plates and grown to subconfluence. Transfection was done with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. Four micrograms DNA per well (1 μg pcDNA3.1/V5-Mcl-1, 0.5 μg pCMV-GFP, and 2.7 μg pBlueScript or 1.3 μg pCMV-GFP and 2.7 μg pBlueScript for corresponding controls) were transfected at a DNA:LipofectAMINE ratio of 1:2.2. The transfection was carried out for 4 hours and complexes were removed by PBS washing and medium exchange. The cells were then treated with the following agents if indicated in the figures: 1 hour after transfection (medium exchange) the cells were infected with hTERT-Ad at an MOI of 1. Twelve hours later, doxorubicin was added at a final concentration of 200 μg/mL and further 8 hours later Ad-Trail was added at an MOI of 0.3. This time schedule was chosen to synchronize optimal caspase-3 activity induced by Ad-Trail or doxorubicin with physiologic down-regulation of Mcl-1 caused by hTERT-Ad. Cells were harvested in parallel 48 hours after transfection for the preparation of cellular extracts. Mcl-1 and actin Western blots and caspase-3 activity measurements were done according to the methods described above. Protein content was determined using the Bio-Rad protein assay.

Quantitative analysis of viral replication. For measurement of viral replication in tumor cells and supernatants, 2 × 10^5 cells were seeded in 6-well plates and infected with an MOI of 5. To examine the effect of chemotheraphy on viral replication, one group was additionally supplied with 100 ng of doxorubicin. At the time points indicated in the figures, 1 mL of the supernatant was removed and stored at −20°C. Infected cells were harvested by scraping off in the remaining medium, centrifuged at 500 × g for 5 minutes and the supernatant was discarded. The cell pellet was then washed twice with PBS and resuspended in PBS. After four cycles of freezing and thawing, 100 μL of cell lysate or the corresponding supernatant were analyzed for viral particles using the TCID$_50$ method. For this purpose, 293 cells were seeded in 96-well plates (10^4 cells per well). The next day, eight harvested cells. For this purpose, organs from mice infected i.v. with 1 μL per well). After 14 days of incubation, the observable

Virotherapy Overcomes Drug Resistance of HCC

Results

Specific expression of E1A in telomerase-positive hepatocellular carcinomas by hTERT-Ad. To investigate the activity of telomerase in normal liver and in HCCs, we did TRAP assays in specimen of liver tissues and in tumor tissue derived from HCC cell lines. The TRAP assays showed high telomerase activity in all HCC cell lines investigated (data not shown) as well as in their s.c. implanted tumor equivalents in vivo (Fig. 1). In contrast, mouse genome templates as control were determined using the primers Intr2-s (5′-CCAGATGTACTCTGCGTTGTC-3′) and Exon3-as (5′-GGTTATTCCTCCAGATGGTCC-3′), directed against the murine locus of caspase-8 (intron 2/exon 3) resulting in a 320-bp fragment. PCR was done using the HotStarTaq Master Mix (Quagen, Hilden, Germany), 50 ng DNA and murine genomic primers at a concentration of 400 nmol/L each. The duplex PCR reaction was started as follows: 15 minutes at 95°C; 30 seconds at 94°C, 30 seconds at 57°C; 1 minutes at 72°C × 3. The reaction was interrupted, fiber primers were added (same concentration) and further 27 cycles were done. For single PCR against adenoviral fiber DNA, 30 cycles with the conditions mentioned above were done. Products of both PCRs were resolved on a 1.5% agarose gel.

Animal experiments and in vivo monitoring of viral replication. Pathogen-free male NMRI nu/nu mice (age 6-8 weeks) were obtained from the Animal Research Institute of the Medizinische Hochschule Hannover. All animal experiments were done according to the German legal requirements. Tumor xenografts were established by s.c. injection of 1 × 10^7 cells into both flanks of mice. For infection experiments, adenoviruses were prepared, purified, and titered as described above. Before infection the virus was dialyzed twice against a solution containing 10 mmol/L Tris (pH 8.0), 1 mmol/L MgCl$_2$ and 140 mmol/L NaCl at 4°C. Tumor nodules were grown to a size of − 400 mm$^3$ before infection. For combination therapy, mice received 10 μg of doxorubicin i.v. twice a week. Infection with hTERT-Ad and Ad-GFP, respectively, was done on day 0, 7, and 14 with 1 × 10^5 pfu in 200 μL for each injection. Additional infection with Ad-Trail (1 × 10^5 viral particles) was done on days 3, 11, and 17. For statistical tumor growth assessment, infected tumors were measured in size twice a week using a digital caliper. Tumor volume was calculated using the following equation: V(tumor) = length × width^2 / 2. For each group six animals (n = 12) were assessed. The difference in mean tumor volume between treatment groups was compared for statistical significance using the unpaired, two-tailed t test with P < 0.05 accepted as denoting statistical significance.

For immunohistochemical analysis, tumor nodules were harvested at day 20, embedded in TissueTec (Sakura, Zoeterwonde, the Netherlands) and shock-frozen in liquid nitrogen. Slices (7 μm) were prepared from cryopreserved tumor specimen and fixed in 4% paraformaldehyde in PBS. For histologic investigation, the slices were stained with H&E according to standard procedures. Viral replication was detected by treating the slices with a goat anti-hexon antibody (20-AG92, Fitzgerald, Concord, MA), diluted 1:100 in 20% FCS/PBS. As secondary antibody, a Cy3-conjugated rabbit anti-goat antibody (C-2821, Sigma) in a dilution of 1:100 was used and positive cells were detected by fluorescence microscopy.
normal human liver was negative for telomerase activity thus providing obligatory preconditions required for telomerase-dependent tumor virotherapy. Murine liver showed detectable levels of telomerase activity but substantially lower than the human HCC cells examined. We therefore investigated the expression of E1A by hTERT-Ad in murine liver and s.c. growing HCCs 24 hours after viral transduction. In accordance with the telomerase activity findings, infection studies of hTERT-Ad in vivo revealed effective, tumor-restricted expression of E1A without E1A expression in liver tissue (Fig. 2). Despite the low telomerase activity observed in murine liver, indicating a low transcriptional activity of the mTERT promoter, regulation of E1A expression by the hTERT promoter in hTERT-Ad did not result in detectable E1A expression in murine liver.

Telomerase-dependent virotherapy overcomes resistance of hepatocellular carcinomas against tumor necrosis factor-related apoptosis-inducing ligand and chemotherapy. HCCs are resistant to chemotherapy- and TRAIL-mediated apoptosis. Recently, it has been shown that chemotherapy agents augment TRAIL-induced apoptosis in human HCC cell lines (29), suggesting multimodal strategies for the treatment of HCCs. Therefore, we investigated the influence of hTERT-Ad-virotherapy on chemotherapy- and TRAIL-mediated apoptosis of HCC cells. First we assessed the susceptibility of Hep7 and Hep3B cells to chemotherapy by incubating confluent cell layers with increasing concentrations of doxorubicin or 5-FU for 48 hours. In contrast to fibrosarcoma (HT 1080) and gastric carcinoma (AGS) cell lines, HCC cells were nearly resistant to chemotherapy (Fig. 3A).

Next we incubated Hep3B and Hep7 HCC cells with low titers of an E1-deleted adenoviral vector (Ad-GFP) or with replicating adenoviruses (Ad-wt, hTERT-Ad) and the cells were subsequently treated with different chemotherapeutic agents as indicated in Fig. 3B. Adenoviruses applied at a MOI of 0.01 to 0.1 were not capable to destroy the cell layer of HCC cells, as visualized by crystal violet staining. In contrast to application of the E1-deleted Ad-GFP, both Ad-wt and hTERT-Ad were capable to overcome the resistance of Hep3B and Hep7 cells to doxorubicin, cisplatin, and 5-fluorouracil and resulted in almost complete disruption of the cell layers (Fig. 3B). Similar results were obtained when HCC cell lines were exposed to TRAIL to induce apoptosis. Only the E1-expressing adenoviruses Ad-wt and hTERT-Ad strongly sensitized Hep3B and Hep7 cells to apoptosis, induced by either Ad-TRAIL (Fig. 3C) or recombinant TRAIL (50 ng/mL, data not shown). In contrast, infection with Ad-GFP before TRAIL treatment did not result in significant cell apoptosis. Additionally, antitumor efficacy could be even further enhanced by employing triple combination therapy including replicative virus, chemotherapy, and TRAIL administration. Interestingly, triple therapy without replication-competent vectors had only little effects on tumor cells confirming the essential contribution of adenoviral E1 expression for strong synergistic effects with apoptosis-inducing agents.

hTERT-Ad down-regulates Mcl-1 expression and sensitizes hepatocellular carcinoma cells for chemotherapy and tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis. To explore the molecular mechanism involved in hTERT-Ad-mediated sensitizing for apoptosis, we investigated the expression of members of the Bcl-2 family and TRAIL-DR5 receptor in HCC cells after adenoviral transduction. Whereas Bcl-2, Bcl-xl, and DR5 remained constant after viral infection, Mcl-1 was strongly down-regulated 48 hours after transduction with replication-competent adenoviral vectors (Fig. 4A). In contrast, infection with the replication-deficient vector Ad-GFP did not influence the expression of Mcl-1 indicating that adenoviral infection itself is not sufficient to decrease Mcl-1 expression levels. These results show that the inhibitory effect on Mcl-1 expression is mediated specifically by replicative viruses, probably due to protein expression from the adenoviral E1 region. Consequently, Mcl-1 down-regulation may therefore represent a potential cause for synergistic effects of virochemotherapy as described above. We therefore inhibited Mcl-1 expression by specific siRNA to clarify the relationship between Mcl-1 expression levels and HCC cell responses to chemotherapy- or TRAIL-mediated apoptosis. As shown in Fig. 4B, effective down-regulation of Mcl-1 in hepatoma cells was accomplished following transfection of Mcl-1 siRNA compared with untransfected cells or cells transfected with
scrambled siRNA. Subsequent caspase-3 assays showed that siRNA-mediated inhibition of Mcl-1 resulted in significantly enhanced susceptibility of HCC cells to chemotherapy- or TRAIL-mediated apoptosis (Fig. 4C). Elimination of Mcl-1 proved to be sufficient to overcome the tumor cells' resistance to chemotherapeutic agents and TRAIL. To further confirm the importance of Mcl-1 in susceptibility to apoptosis, complementation experiments were done by heterologous overexpression of Mcl-1. Transfection of an Mcl-1 expression plasmid before hTERT-Ad infection sufficiently restored Mcl-1 protein levels as detected by Western blot analysis (Fig. 4D). Measurement of caspase-3 activity after application of doxorubicin or TRAIL revealed significant reduction of caspase activity when Mcl-1 expression was restored (Fig. 4E). These observations confirmed our hypothesis that the interference of virotherapy with Mcl-1 levels in infected cells represents an important molecular mechanism underlying the synergistic therapeutic effects of virotherapy combined with chemotherapy and/or TRAIL.

Chemotherapy does neither interfere with adenoviral replication nor with net release of vectors in hepatocellular carcinomas. The outcome of virotherapy depends on a complex balance between viral replication and cytotoxicity (30). If infected tumor cells die prematurely with regard to viral progeny, this can impair the therapeutic effect of hTERT-Ad in HCCs, as an optimized progress of production, release, and spread of viral vectors may be prevented. We therefore assessed the effect of chemotherapy on intracellular virus production and release of viral particles in HCC cells. Interestingly, although hTERT-Ad strongly
sensitized HCC cells for chemotherapy-mediated apoptosis, therapeutic concentrations of doxorubicin did not significantly interfere with the course of adenoviral replication within infected Hep3B (Fig. 5) and Huh7 cells (data not shown). In addition, we revealed that the net release of viral particles by infected tumor cells is unchanged in the presence of therapeutic concentrations of doxorubicin, indicating that chemotherapy would not impair hTERT-Ad virotherapy.

Telomerase-dependent virotherapy acts strongly synergistic with systemic chemotherapy in vivo without significant side effects to the normal liver. To evaluate the therapeutic potential of virotherapy in HCC using hTERT-Ad in conjunction with...
chemotherapy and TRAIL, Hep3B-derived s.c. tumor xenografts were established on nude mice. Tumor xenografts were infected thrice with Ad-GFP or hTERT-Ad (1 × 10⁹ pfu), respectively. Subgroups of mice were subjected to either additional administrations of Ad-TRAIL (1 × 10⁹ pfu) thrice in the course of the experiment, or i.v. injections with doxorubicin twice a week, or a combination of these two treatments, respectively. In agreement with the results obtained from cell culture experiments, treatment of HCC with Ad-GFP in combination with either Ad-TRAIL or doxorubicin resulted only in negligible effects on tumor growth (Fig. 6A). Even in the group of animals receiving the combined treatment of Ad-GFP, Ad-TRAIL, and doxorubicin, only a slight retardation of tumor growth could be observed. In contrast, virotherapy by hTERT-Ad alone or the combination of hTERT-Ad with Ad-TRAIL significantly inhibited tumor growth. However, synergistic effects of Ad-TRAIL on hTERT-Ad virotherapy could not be confirmed in these in vivo experiments, which may be explained by the heterogeneous Ad-TRAIL distribution within the tumor nodules and the lack of TRAIL activity in some tumor areas. When systemic chemotherapy was combined with virotherapy, strong synergistic effects could be observed in vivo resulting in significant remission of tumors. Again, the coinjection of Ad-TRAIL to the group receiving virotherapy only resulted in minor effects on tumor growth curves confirming that the combined approach of virotherapy and Ad-TRAIL was not an effective therapeutic option in vivo.

Because a rapidly growing HCC xenograft model in nude mice was used in our in vivo experiments, ineffective removal of necrotic debris may lead to underestimation of the veritable therapeutic efficacy of virotherapy. Therefore, H&E-stained cryosections were done from tumor xenografts when the experiment has been terminated at day 20. As expected from the study of tumor growth curves, cryosections obtained from the groups receiving Ad-GFP with either Ad-TRAIL, doxorubicin, or combined treatment revealed solid tumor architecture without evidence of significant intratumoral necrosis (Fig. 6B). Tumors treated with hTERT-Ad alone or with the combination of hTERT-Ad and Ad-TRAIL showed large necrotic areas distributed throughout the tumor mass. However, in animals receiving the combination of systemic chemotherapy and hTERT-Ad, the therapeutic effect was strongly enhanced resulting in nearly complete destruction of the tumor xenografts.

Because the last intratumoral injection of hTERT-Ad into the tumor xenografts was done 7 days before the experiment was terminated, we were interested in finding out whether viral replication was still persisting at the end of the experiment. To visualize ongoing viral replication, expression of adenoviral hexon protein was investigated in immunostained cryosections. In contrast to groups of animals receiving Ad-GFP, hexon staining in the tumors of mice receiving hTERT-Ad revealed active viral replication even 7 days after the last injection. Replication could be particularly detected in areas separating intact tumor tissue from necrosis suggesting that the viral spreading was still proceeding (Fig. 6C).

Finally, for assessment of liver toxicity in simultaneous virochemotherapy, serum transaminase activity was measured at the end of the in vivo experiment for each of the eight groups examined. For both AST and ALT serum activity, no significant difference was seen between the groups receiving tumor therapy and an untreated control group (Fig. 7A). As hTERT-Ad replication takes place in telomerase-positive cells, potential side effects in stem cells and cells of germ line origin have to be investigated. For this purpose, tissue samples from liver, spleen, testis, epididymis, small intestine, and bone marrow were screened for adenoviral infection with PCR. To simulate systemic infection after intratumoral virus injection, the viral dosis that was usually injected into the tumor was directly injected i.v. As shown in Fig. 7B in comparison with a genomic template, high infection rates could only be seen in liver. When a highly sensitive single PCR against adenoviral DNA was used, liver and spleen displayed detectable viral infection. Murine bone marrow revealed low viral infection, whereas testis, epididymis, and small intestine were negative for viral DNA. To rule out long-term stem cell toxicity, blood samples were examined at the end of the experiments for their content of hemoglobin, leukocytes, and platelets. As shown in Fig. 7C, neither combination treatment of hTERT-Ad, chemotherapy and TRAIL nor the repeated i.v. injection of hTERT-Ad resulted in significant alterations of the hemograms.
Discussion

In our study, we investigated the potential of telomerase-dependent virotherapy in multimodal treatment of HCCs. Telomerase activation is one of the critical steps in malignant transformation and therefore an attractive target for selective cancer therapy. Although telomerase is a complex composed of a catalytic subunit (hTERT) and an RNA component (hTERC), telomerase activity is mainly transcriptionally controlled by the hTERT promoter (31). It has been shown that telomerase is strongly activated in the majority of HCCs and the degree of telomerase activation correlates with histologic dedifferentiation of the tumors (17), providing a precondition for effective telomerase-dependent virotherapy. In contrast to HCCs, in the liver of patients with chronic hepatitis or liver cirrhosis telomerase activity is only weakly activated in 8% and 24% of the cases and immunohistochemical investigations showed very low numbers of hTERT-positive hepatocytes (<0.5%) within these livers (17). Reconstitution of telomerase activity in those hepatocytes may be a consequence of liver regeneration in chronic liver injury. However, it seems unlikely that these cells are able to contribute significantly to an unwanted toxicity of hTERT-Ad, as they are evenly distributed as isolated single cells within the liver and therefore cannot facilitate effective viral spreading of hTERT-Ad. In contrast to normal liver tissue, the accumulation of telomerase-positive tumor cells in HCCs allows effective viral replication and spreading of hTERT-Ad within the tumor tissue, resulting in significant inhibition of tumor growth in our animal model.

Preclinical and clinical results suggest potentially synergistic interactions of replicating adenoviruses with chemotherapy, in particular using platinum-containing chemotherapy (32, 33). In addition, it has been shown that systemic chemotherapy in combination with intratumoral injection of an E1B-55k-deleted virus, dl1520 (ONYX-015), is well tolerated by patients with head and neck cancer (33) and also by patients with colorectal liver metastases after intravascular virus administration (34, 35). We recently showed that tumor-restricted replication of hTERT-Ad is synergistic with both systemic and local chemotherapy, resulting in significant tumor remission in vivo.

Figure 6. hTERT-Ad virotherapy shows strong synergistic effects in combination with chemotherapy in vivo. A, s.c. Hep3B tumor xenografts on nude mice were infected intratumorally as indicated with nonreplicative (Ad-GFP) or replicative (hTERT-Ad) adenoviral vectors. A subgroup of tumors were treated with TRAIL (intratumoral injection of Ad-TRAIL), systemic administration of doxorubicin or a combination of both agents. Observation of the tumor growth demonstrated strong synergistic effect of virotherapy and chemotherapy resulting in significant (*) tumor remission compared with virotherapy alone. B, histologic examination of the explanted Hep3B tumors with H&E-staining at the end of the experiment showed vast destruction of tumor tissue after virochemotherapy. Representative parts of tumor histologies of the different therapy groups at 100× magnification. C, hexon staining of harvested tumors was done at the end of the experiment (day 20). Replication of hTERT-Ad in border areas between necrosis and healthy tumor tissue was visualized suggesting that viral replication was still proceeding at the end of the experiment.
more effective and more specific compared with ONYX-015 (22), providing a rationale for the multimodal therapy of HCC patients with hTERT-Ad and systemic chemotherapy or TRAIL therapy without expecting cumulative toxicities.

In our study, neither TRAIL or chemotherapy alone nor the combined treatment with both agents resulted in significant destruction of HCC cells. However, telomerase-dependent virotherapy proved to be highly effective in a synergistic manner when applied with chemotherapy and TRAIL and consequently overcame therapeutic resistance of HCC cells. The synergistic effects are explained by an effective down-regulation of Mcl-1 expression through hTERT-Ad that sensitizes HCC cells for TRAIL- and chemotherapy-mediated apoptosis. Recently, it has been shown that E1A expression provokes a DNA damage response that results in Mcl-1 elimination (36). In accordance with these observations in our study, only E1A-expressing adenoviruses but not adenoviral infection alone were capable to down-regulate Mcl-1 expression in HCC cells. These results emphasize the importance of telomerase-controlled E1A expression in hTERT-Ad for targeted therapy of hepatocellular carcinomas. Inhibition of Mcl-1 expression by RNA interference led to an elevated caspase-3 activity in Hep3B cells, whereas the changes of caspase-3 activity were not significant in HuH7 cells. However, siRNA-mediated down-regulation of Mcl-1 significantly enhanced caspase-3 activity after application of chemotheraphy and TRAIL in both HCC cell lines, confirming that down-regulation of Mcl-1 alone is sufficient to explain synergistic effects observed with virotherapy. In agreement with these results, complementation of Mcl-1 significantly reduced caspase-3 activity in hTERT-Ad-transduced cells when exposed to subsequent apoptosis-inducing agents. Because it has recently been shown that Mcl-1 also mediates resistance to TRAIL or chemotherapy in other cancers, such as cholangiocarcinomas (37) and melanomas (38), virotherapy may be a valuable tool in other drug-resistant tumors to improve the outcome of the patients.

Because enhancement of adenoviral replication in tumor cells by chemotheraphy may also explain the synergistic effects of virochemotherapy, we investigated the effect of doxorubicin on viral replication. In contrast to previous observations in other tumor cells (39), chemotherapy neither improved viral replication nor led to an improved release of viral particles by infected hepatoma cells. Therefore, we found no evidence in our experiments that progeny and distribution of the virotherapeutic vector was directly enhanced by chemotherapy.

In accordance with the results observed in cell culture experiments, telomerase-dependent virotherapy acted also highly synergistic with systemic chemotherapy in drug-resistant HCCs in vivo and resulted in significant tumor remission and vast tumor destruction. In contrast, we observed no significant synergistic effects when virotherapy was applied together with local injection of Ad-TRAIL into the tumors. Although hTERT-Ad supports replication and spreading of E1-deleted Ad-TRAIL in HCCs by providing E1 proteins in trans, heterogenous Ad-TRAIL distribution within the tumor nodules may account for the discrepancy of in vitro and in vivo results. In contrast to TRAIL administration, systemic chemotherapy can also inhibit proliferation of nonmalignant cells within the tumor nodule, such as fibroblasts and...
endothelial cells. Because nonmalignant cells within the HCCs impair replication and spreading of hTERT-Ad, unselective inhibition of cell growth by chemotherapy may additionally contribute to synergistic effects of virotherapy in vivo.

Multimodal strategies seem necessary to successfully treat drug-resistant tumors such as HCCs. In our study, we showed that telomerase-dependent virotherapy overcomes resistance of HCCs against TRAIL and chemotherapy by down-regulation of Mcl-1 expression. In a clinical situation, hTERT-Ad virotherapy can be done analogous to our animal experiments by needle injection into tumor tissues. However, intra-arterial delivery of hTERT-Ad seems done analogous to our animal experiments by needle injection into resistant tumors such as HCCs. In our study, we showed that degree of initial transduction could be a sufficient base level for effective intratumoral replication and spreading of hTERT-Ad. The applicability of hTERT-Ad, especially in multimodal antitumor strategies, requires besides evidence for enhanced efficacy convincing proof of clinical safety. The unaltered levels of transimnase activity as well as the lack of germ cell infection and stem cell toxicity even after systemic infection with high doses of hTERT-Ad suggest that even with combined virotherapy detrimental side effects in vivo can be excluded. Taking into account that local application of viral vectors results in considerably less systemic viral infection, substantial damage to telomerase-positive host cells is very unlikely. Consequently, virotherapy should be an attractive strategy to improve the therapeutic results of regional chemotherapy or chemoembolization of HCCs.

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Thomas Wirth, Florian Kühnel, Bettina Fleischmann-Mundt, et al.