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RESEARCH ARTICLE

An *ex vivo* human model system to evaluate specificity of replicating and non-replicating gene therapy agents

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Abstract

Background Inefficiency, aspecificity and toxicity of gene transfer vectors hamper gene therapy from showing its full potential. On this basis significant research currently focuses on developing vectors with improved infection and/or expression profiles. Screening assays with validity to the clinical context to determine improved characteristics of such agents are not readily available since this requires a close relationship to the human situation. We present a clinically relevant tissue slice technology to preclinically test improved vector characteristics.

Methods Slices were prepared from rat, mouse and human liver samples and from tumor tissue. Specificity of gene expression and replication was determined by infecting target and non-target tissue slices with transcriptionally retargeted adenoviruses and oncolytic viruses.

Results Using rat liver slices, we demonstrate efficient knob-mediated adenoviral infectivity. A favorable tumor-on/liver-off profile, resembling *in vitro* and mouse *in vivo* data, was shown for a tumor-specific transcriptionally retargeted adenovirus by infecting slices prepared from tumor or liver tissue. Similar liver-off data were found for mouse, rat and human samples (over 3-log lower activity of the tumor-specific promoter compared to cytomegalovirus (CMV)). More importantly, we show that this technology when applied to human livers is a powerful tool to determine aspecific replication of oncolytic viruses in liver tissue. A 2- to 6-log reduction in viral replication was observed for a tumor-specific oncolytic virus compared to the wild-type adenovirus.

Conclusions The precision-cut tissue slice technology is a powerful method to test specificity and efficiency of gene transfer as well as of viral replication using human tissue.

Keywords toxicity; retargeting; carcinoma; virotherapy; oncolysis

Introduction

Despite the promise of gene therapy as a powerful approach to treat or even cure a broad spectrum of diseases, only few clinical successes have been reported to date [1]. Moreover, unfortunate adverse events in clinical gene therapy trials have had a negative influence on public opinion regarding gene therapy. So far, clinical trials have been performed mainly using retroviruses or adenoviruses as the transgene carriers. Specifically, adenoviruses exhibit

a marked hepatotropism which forms the basis of the toxicities associated with this agent. This has become a major issue since the death of Jesse Gelsinger, an 18-year-old boy who died in an adenoviral gene therapy trial designed to treat the liver disease ornityl transcarbamylase deficiency [2]. This liver tropism of the vector dictates major vectorological improvements to untarget the liver. Current (adenoviral) gene therapy research is dedicated to identifying ways to circumvent liver toxicity by targeting of the vectors to the desired tissue.

In addition, as a distinct approach to circumvent inefficiency of current vectors in cancer therapy, viruses are being constructed to selectively replicate in tumor cells. Viral replication causes cell lysis, followed by release of virus progeny within the tumor mass, which allows infection and lysis of neighboring tumor cells. As this cycle theoretically continues until no more tumor cells are present, this approach provides a potential powerful way to treat cancers, currently tested in clinical trials. To restrict replication to tumor cells, tumor/tissue-specific promoters are used. However, leaky expression has been observed [3] and the strict absence of replication in non-tumor cells, especially liver cells, needs to be proven. Studies on tumor-specific replication are hampered by the fact that human adenoviruses do only replicate in human cells and not in animal cells. Therefore, endeavored animal studies are informative only on oncolytic efficiency, not on specificity of replication.

To evaluate the efficiency and specificity of retargeted (oncolytic) viruses, infection of precision-cut slices prepared from target and non-target (human) tissue represents a new approach in gene therapy research. Precision-cut liver slices are about 10-cell layer thick organ sections (1×10^6 cells, 10–15 mg, 200–250 μm thickness and 8 mm diameter) and can be prepared from theoretically all organs derived from all species. We have experience with mouse, rat and human slices prepared from liver, lung, kidney, colon and intestine [4]. All cell types present in the *in vivo* situation are represented in the slices and the original architecture and matrix environment is conserved. This makes tissue slices a powerful tool in toxicity assays, liver inflammation studies [5,6] and in drug-targeting studies [7] and has been approved by the FDA as a model in drug metabolism studies. In this study, we show that (human) tissue slices provide relevant information on efficiency and specificity of novel generation gene therapy agents.

Materials and methods

Cells and tissues

To determine promoter activity *in vitro*, target cells (EGP2-positive colon carcinoma cells (LS174T) and tyrosinase-positive melanoma cells (SK-Mel28)) and non-target cells (pancreatic cancer cells (Panc-1) and lung cancer cells (H460)) were cultured in recommended media and infected with adenoviruses in infection medium containing 2% fetal calf serum (FCS). To establish promoter activity in target cells *in vivo*, tumors were induced in flanks of BALB/c nu/nu mice by injecting 2×10^7 LS174T cells subcutaneously. At 6–8 mm in diameter, the tumors were intratumorally injected with 10^8 plaque-forming units (pfu) adenovirus. After 2 days, tumors were removed, snap-frozen and stored at −80 °C for luciferase analysis. Absence of promoter activity in non-target cells was assessed by removing livers 2 days after intravenous administration of 10^9 pfu AdGL3CMV or AdGL3EGP2. Livers were snap-frozen and stored at −80 °C until further luciferase activity measurements.

Non-treated tumors and livers were also removed from mice for immediate slice preparation followed by *ex vivo* infection. In addition, rat livers were removed from adult male Wistar rats (250 g) for slice preparation and *ex vivo* infection. All experiments were performed after approval by the local ethical committee for animal experiments. Human liver samples (HLS) were obtained from the Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation of the University Medical Center Groningen, after obtaining approval of the local medical committee and informed consent. Samples were stored in University of Wisconsin organ preservation solution (UW, Du Pont Critical Care, Waukegan, IL, USA) at 4 °C until slicing.

Slice preparation

Slices were prepared as described previously [8]. In short, cores of 8 mm were drilled from the tissues (5 mm for tumor and mouse liver), which subsequently were sliced using a Krumdieck slicer (Alabama R&D, Munfort, AL, USA) for optimal and reproducible preparation of liver slices. The thickness of the slices was adjusted to 200–250 μm (10–14 mg). The slicing procedure itself was performed in ice-cold physiological Krebs buffer assuring minimal trauma of the tissue [5]. Slices were washed and preincubated at 37 °C for 1 h in Williams' medium E supplemented with D-glucose (25 mM) and gentamycin (50 μg/ml), saturated with 95% O_2/5%CO_2 before experiments were started.

Slices were infected by adding virus to 3.2 ml of fresh oxygenated WME medium in 6-well plates. Slices were further incubated in 95% O_2/5% CO_2 for 24, 48, or 168 h at 37 °C under continuous shaking. For replicative viruses, medium and slices were snap-frozen and stored at −80 °C until further analysis.

Viruses

Initial experiments were performed using an adenovirus with two expression cassettes (CMV-green fluorescent protein and CMV-luciferase; AdTL) [9]. The infection pathway was investigated by preincubating slices with recombinant knob protein of adenovirus type

5 (10 mg/ml) for 10 min at room temperature before adding virus (50 pfu/cell) to block cell entry. Alternatively, virus was preincubated with the single chain antibody S11 [10] directed against the viral knob (10 μg/10^9 viral particles (vp)) to block infection.

Other viruses used in this study have been transcriptionally retargeted in order to improve on specificity and efficiency for tumor cells. AdGL3EGP2 contains a fragment of the epithelial glycoprotein 2 (EGP2) promoter [11] to restrict luciferase expression to carcinoma cells, AdGL3CMV is the positive control with the cytomegalovirus (CMV) promoter controlling the expression of luciferase. Both viruses were added at 50 pfu/cell. AdTyrE1 [12] is a replication-competent adenovirus with the melanoma-specific tyrosinase promoter restricting the expression of E1A (and therefore the replication) to melanoma cells. Adwt is the wild-type adenovirus serotype 5 which is capable of replicating upon infection in all human cells, while AdCMVLuc represents a replication-incompetent virus. Replication-competent and -defective viruses were added to the human slices in the wells at a final amount of 1 vp/cell.

Transgene measurements

Green fluorescent protein (GFP) expression was detected in slices using a fluorescent microscope (Axiovert 25, Carl Zeiss BV, Weesp, The Netherlands) and pictures were taken using a digital camera. Luciferase activity was measured after snap-freezing the slices in liquid nitrogen and crunching the slices in cell culture lysis buffer (Promega, Madison, WI, USA). Luciferase substrate was added as recommended by the manufacturer (Promega) and light emission was measured using a luminometer (Packard, Groningen, The Netherlands). Cell survival was determined for efficiency of oncolysis by adding the tetrazolium compound MTS (Promega) to the cells 16 days after infection with replication-competent viruses at 1 vp/cell. Mitochondrial conversion of the substrate by living cells was measured spectrophotometrically 2 h after addition of MTS as recommended by the manufacturer.

E1-specific quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR specific for the E1 gene was performed with the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Molecular Biochemicals). PCR was performed as described by the manufacturer. Total DNA was isolated out of slices using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. The primers jzp1 and jzp2 [13] were used as forward and reverse primers. The primers amplify a 219 bp (base pairs) fragment of the E1A gene. Primers were obtained from TibMolbiol (Berlin, Germany). Plasmid DNA (1×10^1 to 1×10^7 copies as a 10-fold dilution series) containing the E1A and E1B genes was used as an external standard to generate a calibration curve for quantitation of test samples.

Quantification of viral yield

Medium samples taken after 7 days were used to determine viral yield using an adaptation of the limiting dilution assay. In short, 293 human embryonic kidney cells were plated at 10 000 cells/well in a 96-well plate. The next day, 100 μl of 10-fold dilutions of the sampled media were added to the wells in 5- or 10-fold. Cytopathic effect was monitored microscopically every 2–3 days and scored at day 14. Plaque-forming units were calculated according to standard procedures.

Results

Efficient receptor-mediated adenoviral infection of liver slices

To investigate whether the precision-cut tissue slice technology could be adapted as a representative *ex vivo* model system for preclinical screening on efficiency and specificity of adenoviral agents, precision-cut slices were prepared from rat liver tissue. *Ex vivo* infection of rat liver slices with adenoviruses transferring transgenes encoding GFP and luciferase (AdTL) was efficient as shown by fluorescent microscopy (Figure 1a), but seemed restricted to the outer layers of the liver slice, as shown by a cross-section of an infected slice (Figure 1b). To investigate whether the transgene expression was due to active receptor-mediated infection, slices were preincubated with recombinant adenovirus knob protein. Indeed, the blockage of the native adenovirus receptor, coxackie-adenovirus receptor (CAR), resulted in a 2-log decrease in infection efficiency (Figures 1c and 1e). Similarly, infection of the virus complexed with an antibody directed against the knob of the virus efficiently prevented adenoviral infection: again a 2-log lower luciferase activity was measured for complexed adenoviruses compared to unmodified adenoviruses (Figures 1d and 1e). Quantification of gene expression by luciferase readings are shown in Figure 1e. Overall, the tissue slice system reflects efficient knob-mediated infection of liver cells by adenoviruses.

Tumor-on/liver-off profile of a transcriptional retargeted adenovirus

To prevent unwanted effects due to infection of non-target cells like hepatocytes in the case of cancer therapy, transcriptional retargeting approaches have exploited tissue- or tumor-specific promoters to restrict the expression of a transgene to the target cells.

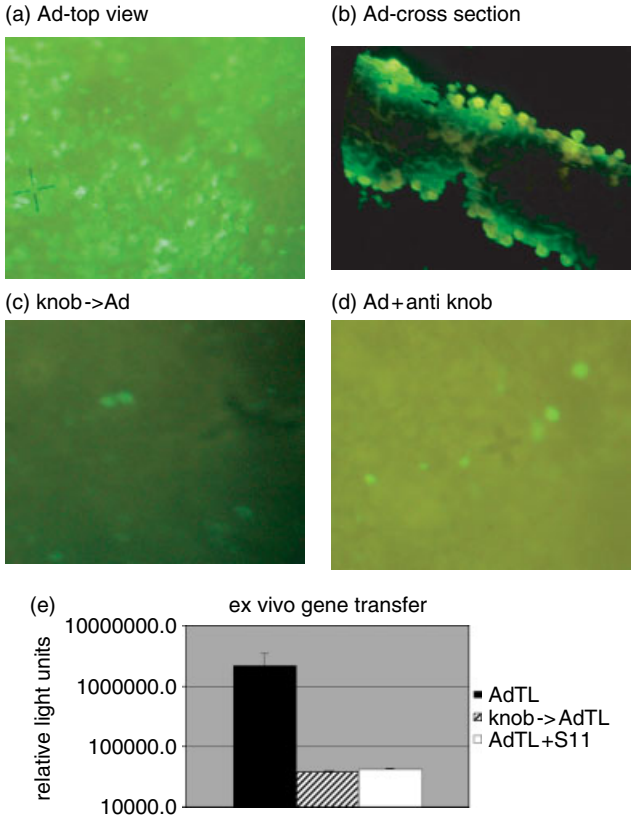

Figure 1. Efficient adenoviral gene transfer in slices as visualized by green fluorescent protein (GFP) expression in liver slices infected with (a) unconjugated adenovirus expressing GFP and luciferase (AdTL), (b) unconjugated adenovirus expressing GFP and luciferase (shown as a cross-section), (c) unconjugated adenovirus after blocking the adenoviral receptor CAR on cells with recombinant knob, and (d) adenovirus conjugated to the antibody S11 blocking the knob of the adenovirus. (e) Luciferase readings

Several tumor-specific promoters have been identified to successfully restrict transgene expression to tumor cells and some have been translated to the clinic. In this study, we were interested in using the slice technology as a preclinical model to study tumor-on/liver-off profiles of tumor-specific promoters. We constructed an adenovirus containing the epithelial glycoprotein 2 promoter [11] to restrict luciferase expression to carcinoma cells (AdGL3EGP2). The tumor-on/liver-off profile of this tumor-specific virus was determined by infecting slices prepared from target tumor tissue and from non-target mouse liver tissue. Slices prepared from a EGP2-positive tumor grown subcutaneously in nude mice showed a 1-log decrease in luciferase activity after *ex vivo* infection with AdGL3EGP2 compared to AdGL3CMV ($8.7 \pm 5.5\%$). In contrast, slices prepared from mouse liver showed a 4-log lower luciferase activity when infected *ex vivo* with AdGL3EGP2 compared to AdGL3CMV (Figure 2a). Thus, although the EGP2 promoter is less active then the CMV promoter in target tissue, in non-target liver tissue the EGP2 promoter activity was almost absent, showing its power as a tumor-on/liver-off promoter for cancer gene therapy.

To validate these *ex vivo* data on the tumor-on/liver-off profile of AdGL3EGP2, infections were also performed *in vitro* and/or *in vivo*. In accordance with the *ex vivo* data, high levels of luciferase activity were measured in tumor tissue after intratumoral injection with AdGL3EGP2 (*in vivo*) (10% compared to AdGL3CMV) and in tumor cells grown *in vitro* (40% compared to AdGL3CMV) (Figure 2b). In contrast to tumor tissue, again low luciferase activity was measured in homogenates prepared from liver tissue after *in vivo* intravenous administration (over 3-log lower luciferase activity compared to AdGL3CMV) (Figure 2b). As described previously, EGP2 promoter activity has been found absent in mature primary hepatocytes (*in vitro*) and in HepG2 hepatocarcinoma cells [14]. For AdGL3EGP2, we confirmed a relative promoter activity of less then 1% compared to AdGL3BCMV in the HepG2 cells ($1.1 \times 10^7 \pm 1.6 \times 10^6$ vs. $5.7 \times 10^4 \pm 8.1 \times 10^3$). These data show the power of tumor-specific promoters to untarget the liver. More importantly, these results indicate that *ex vivo* tissue slices are predictive of the *in vivo* situation and that slices are powerful tools to determine liver-off profiles of transcriptionally retargeted viruses.

Filling the gap between laboratory animals and the clinical setting is of utmost importance. We therefore extrapolated this technology and also performed experiments on liver specimens originating from other species (Figure 2c). Infection of rat and human liver slices again resulted in an over 3-log lower luciferase activity for slices infected with adenoviruses containing the EGP2 promoter restricting luciferase expression (AdGL3EGP2) compared to those containing the constitutive CMV promoter (Figure 2c). Apart from this similarity between rodent and human results, the data demonstrate the feasibility of using clinically relevant human material to test improved adenoviral vectors on specificity of gene expression.

Tumor-on/liver-off profile of conditional replicative adenoviruses

On the basis of the promising findings on transcriptional retargeting, we evaluated the utility of the tissue slice system to study replicating viral agents. Conditionally replicative adenoviruses have been successfully constructed to increase the efficiency of cancer gene therapy. Previously, we constructed a melanoma-specific tyrosinase-controlled replicative adenovirus (AdTyrE1) [12]. Cell survival assays using cell lines demonstrated equal efficient cell lysis after infection with AdTyrE1 compared to wild-type adenovirus (Adwt) of target melanoma cells (SK-Mel-28), while replication of this virus in non-target cells (H460, Panc1) was strongly impaired (Figure 3a). Next, we sought to demonstrate the attenuation of replication in non-target human liver. Infection of slices with wild-type adenoviruses resulted in high E1 DNA copy number. Infection of the human liver slices with AdTyrE1 resulted in a 2- and 4-log lower E1 copy

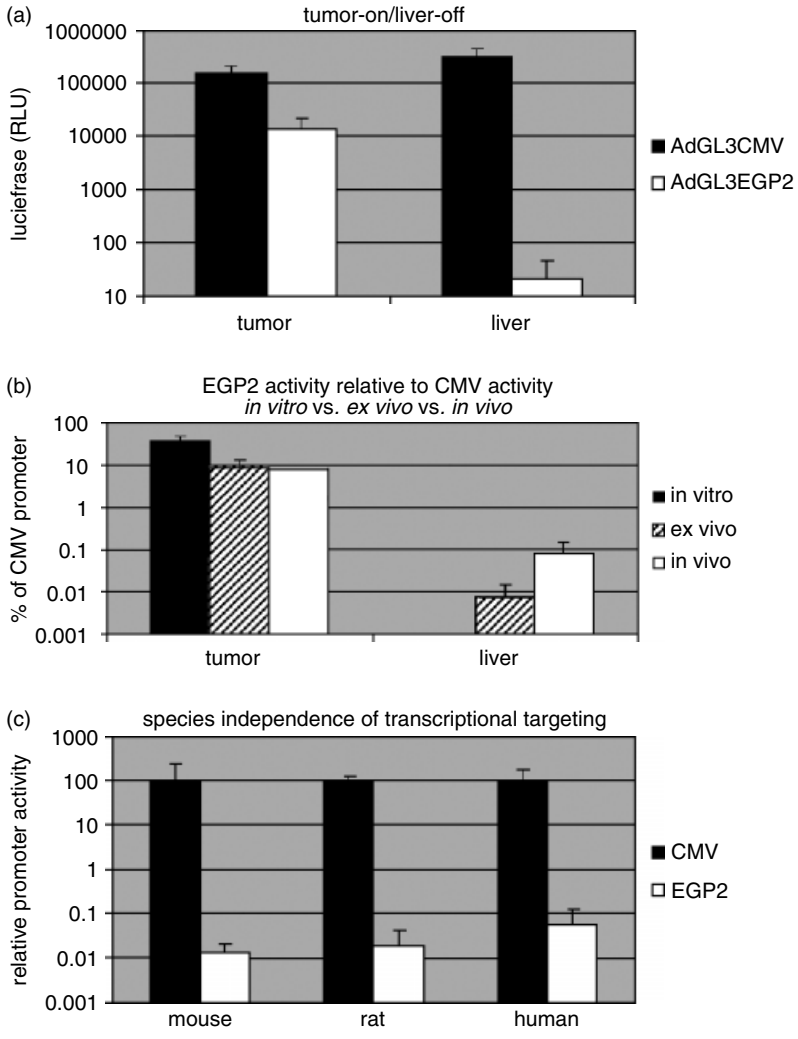

Figure 2. Tumor-on/liver-off profiles of transcriptionally retargeted viruses can be evaluated using tissue slices. (a) *Ex vivo* evaluation of tumor specificity of EGP2: slices prepared from an *in vivo* grown EGP2-positive tumor showed high luciferase activities after infection with AdGL3EGP2 and AdGL3CMV. In contrast, slices prepared from livers removed from mice showed strong attenuation of EGP2 promoter activity. (b) *In vivo* vs. *ex vivo* vs. *in vitro* evaluation of EGP2 promoter activity: *in vivo* mouse studies demonstrate a favorable tumor-on (carcinoma *in vivo*) liver-off (liver *in vivo*) profile for transgene expression by AdGL3EGP2 compared to AdGL3CMV (open bars), which is in accordance with data derived on cultured cells (carcinoma *in vitro*) and tissue slices (liver and tumor *ex vivo*). (c) Evaluation of species independency of promoter activity. The liver-off profile for the tumor-specific EGP2 promoter compared to the CMV promoter is similar for mouse, rat and human liver slices. (Luciferase readings in (b) and (c) are depicted relative to data derived after infection with adenoviruses transferring luciferase under the control of the CMV promoter (AdGL3CMV))

number after 7 days compared to infection with Adwt, showing inefficient DNA replication of the tumor-specific virus in liver tissue (Figure 3b). Human liver slices had been determined previously to be viable for up to 7 days in culture (Dr A. Vickers, Allergan Inc., Irvine, CA, USA, personal communication).

Apart from measuring viral DNA replication by determination of viral E1 gene copy number in the tissue, actual virus amplification and subsequent cell lysis were assessed by measuring functional virus released in the medium after 7 days of incubation. Again, viral yield was 2- to 6-log lower for the melanoma-specific replication-competent virus compared to wild-type viruses for slices from the same liver (Figure 3b), demonstrating a liver-off profile for this oncolytic virus. Thus this study presents a model system to screen conditionally replicative viral agents on specificity and provides an important step forward for the promising field of virotherapy.

Discussion

In the present study, we demonstrated that (human) tissue slices can be used to screen improved adenoviral gene therapy agents for specificity and efficiency. In addition, we showed that the data are in accordance with both *in vitro* and *in vivo* studies. More importantly, we show attenuation of replication of a tumor-specific conditionally replicating adenovirus in a clinically relevant human model. This system will allow a rigorous analysis of replicative specificity in advance of human trials [15].

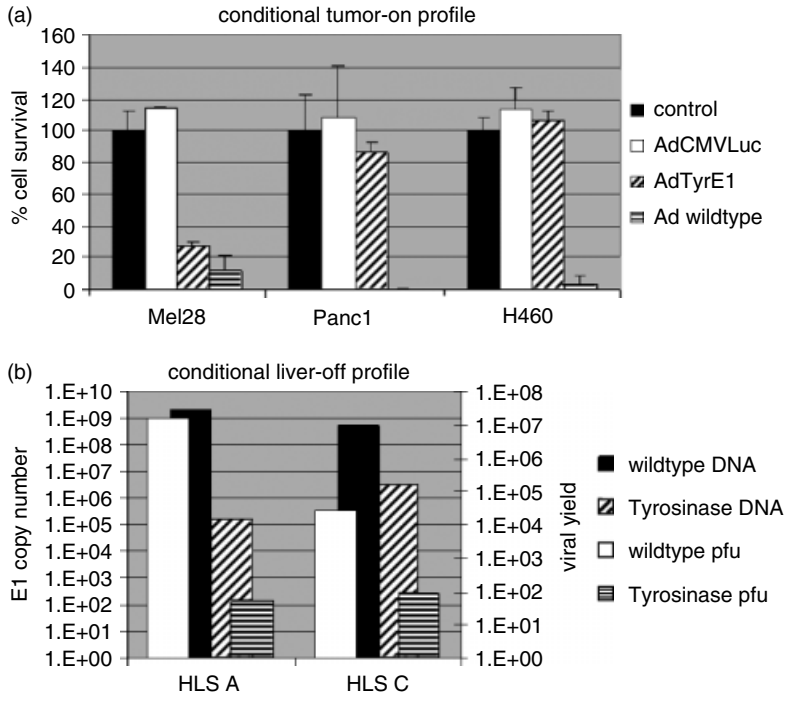

Figure 3. Testing of the tumor-on/liver-off profile of replication of a melanoma-specific replication-competent adenovirus. (a) Different cell lines (SK-Mel 28, tyrosinase positive; Panc1, tyrosinase negative; H460, tyrosinase negative) were infected with a non-replicating adenovirus (AdCMVLuc), a melanoma-specific replicating virus (AdTyrE1) and Ad wild-type as a positive control at 1 vp/cell. After 16 days, cell survival was measured by the MTS assay. (b) Slices of two primary human liver samples (HLS A and HLS C) were infected with 1 vp/cell and E1 copy number (black and white bars, left axis) or viral production (lined bars, right axis) was determined after 7 days of infection by E1-specific real-time PCR or limiting dilution assays

Clinical success of gene therapy is greatly hampered by inefficient and aspecific gene transfer. Major efforts therefore are devoted to improve specificity through retargeting of the gene transfer vectors (reviewed in [16]). Systems to evaluate such retargeted vectors include panels of (human) cell lines and animal studies. These systems, however, only poorly resemble the human context. In that respect, three-dimensional spheroids grown from patient-derived tumor material will prove useful [17], but this technology has not yet been shown feasible for liver tissue. To study preclinical safety, expensive non-human primates could be used as models to resemble the human context as closely as possible [18,19], but these are not easily available to all laboratories.

The lack of suitable preclinical screening systems is even more evident in virotherapy research where conditional replicative viruses are explored to increase efficiency for cancer therapy [20]. Specificity of replication is mainly determined in cell lines or in primary derived patient material. Animal models are only suitable to determine efficacy of oncolysis, and are not informative with respect to liver-off profile as human adenoviruses do not replicate in rodent cells. Cotton rats, which have been considered semipermissive hosts for human adenoviruses, also do not provide a suitable model system to study toxicity of replicative agents as no significant replication can be detected after intravenous administration [21]. Studies on the replication profile of species-specific replicative adenoviruses in which the respective species are used for screening of aspecific (liver) replication are indeed informative [22], but not readily applicable to all vectors in preclinical development. In this respect, it is interesting to note that the human adenovirus has been recently described to be capable of replicating in dog cells thereby potentially providing a clinically relevant, albeit virus-consuming and expensive, system [23].

The need for relevant, robust toxicity assays to determine improved safety of retargeted vectors is obvious, but single cell cultures and laborious small animal studies still are the gold standard. Especially for conditionally replicative oncolytic viruses, which show great promise in increasing efficiency of cancer gene therapy, no appropriate, small size (human) model system is available to determine tumor specificity of replication. As an alternative toxicity assay, the use of primary human hepatocytes has been suggested, but the lack of Kupffer cells (important in clearance of viral particles) and other non-parenchymal cells is a main disadvantage of this system. The precision-cut tissue slice technology is well characterized for toxicity assays, liver inflammation studies [5,6] and in drug-targeting studies [7]. Here we apply the technology in gene therapy research as an easy and reproducible screening assay providing insight in infection and expression profiles of newly developed (adenoviral) gene therapy agents.

Although this technology fills the gap between *in vitro* cell line studies and *in vivo* (animal) experiments, tissue slices cannot completely predict the human *in vivo*

situation. For tissue slices, infection takes place in all cells at the cutting edge, and not via the blood stream as in the *in vivo* situation. Therefore, saturation of Kupffer cells (which normally capture adenoviruses from the bloodstream) is not required before infection of hepatocytes can take place. However, this differential virus distribution between *in vivo* and *ex vivo* does not influence the relative promoter activity since the Kupffer cells mainly destroy the virus before transgene expression can occur. Because the different viruses are not modified at the capsid level, no difference in relative Kupffer cell uptake is expected.

As hepatocytes are the majority of all cells in the liver, liver slices (retaining the viability and normal functioning of hepatocytes) are extremely useful for testing transcriptionally retargeted (replication-competent) viruses. This technology will be of high utility in the design and functional analysis of retargeted (oncolytic) agents. In conclusion, we are confident that this technology will be a powerful addition to the preclinical screening of improved gene therapy agents.

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