A Novel *Ex vivo* Model System for Evaluation of Conditionally Replicative Adenoviruses Therapeutic Efficacy and Toxicity

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ABSTRACT

Purpose: **Current animal tumor models are inadequate for the evaluation of toxicity and efficacy of conditionally replicative adenoviruses. A novel model system is needed that will provide insight into the anticipated therapeutic index of conditionally replicative adenoviruses preclinically. We endeavored to show a novel model system, which involves *ex vivo* evaluation of conditionally replicative adenovirus toxicity and therapeutic efficacy in thin, precision-cut slices of human primary tumor and liver.**

Experimental Design: **The Krumdieck thin-slice tissue culture system was used to obtain and culture slices of tumor xenografts of ovarian cancer cell lines, human primary ovarian tumors, and human liver. We determined the viability of slices in culture over a period of 36 to 48 hours by ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxphenyl-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)]) (MTS) assay. *In vitro* Hey cells, slices of Hey xenografts, and human ovarian tumor or human liver slices were infected with 500vp/cell of either replication competent wild-type adenovirus (Ad5/3wt), conditionally replicative adenovirus (Ad5/3cox-2), or the replication deficient adenovirus (Ad5/3luc1). At 12-, 24-, and 36-hour intervals, the replication of adenoviruses in these slices was determined by quantitative reverse transcription-PCR of adenoviral E4 copy number.**

Results: **Primary tumor slices were able to maintain viability for up to 48 hours after infection with nonreplicative virus (Ad5luc1). Infection of Hey xenografts with Ad5/3cox-2 showed replication consistent with that seen in Hey cells infected in an *in vitro* setting. Primary tumor slices showed replication of both Ad5/3wt and Ad5/3cox over a 36-hour time period. Human liver slices showed replication of Ad5/3wt but a relative reduction in replication of Ad5/3cox-2 indicative of conditional replication "liver off" phenotype, thus predicting lower toxicity.**

Conclusions: **The thin-slice model system represents a stringent method of *ex vivo* evaluation of novel replicative adenoviral vectors and allows assessment of human liver replication relative to human tumor replication. This is the first study to incorporate this system for evaluation of therapeutic efficacy and replicative specificity of conditionally replicative adenoviruses. Also, the study is the first to provide a valid means for preclinical assay of potential conditionally replicative adenovirus-based hepatotoxicities, thus providing a powerful tool to determine therapeutic index for clinical translation of conditionally replicative adenoviruses.**

INTRODUCTION

Conditionally replicative adenoviruses have emerged as a novel and promising approach for a range of advanced neoplasms (1–6). In this regard, direct translation of this approach from the laboratory to human clinical trials has proceeded at an unprecedented pace (2, 7–9). Whereas these studies have highlighted the overall safety of this approach, only limited efficacy has been noted when conditionally replicative adenoviruses have been used as single modality agents (10–12). On this basis, it is clear that substantial design advancements must proceed to allow full realization of the promise of conditionally replicative adenovirus agents. Indeed, the recent development of advanced generation conditionally replicative adenoviruses, which embody enhanced infectivity (13–17), has established a rational framework for additional developmental strategies based on addressing the defined biological limitations of conditionally replicative adenovirus function.

The development of conditionally replicative adenovirus agents has exploited available murine SCID/xenograft systems to study efficacy parameters (18). In this regard, human serotype adenoviruses adapted as conditionally replicative adenoviruses undergo only limited replication in a murine host background. This fact has severely limited any understanding of toxicity related to the replicative phenotype of current conditionally replicative adenoviruses. Additionally, issues related to vector-host interaction may not be characterizable in these immunod-

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eficient test models. This limited repertoire of appropriate model systems has hampered clinical translation of candidate conditionally replicative adenovirus agents. This issue is additionally complicated by the fact that few useful surrogate endpoints indicative of conditionally replicative adenovirus efficacy have been defined in current human clinical trials.

The current model system limitations have been especially relevant in the context of designing conditionally replicative adenoviruses, which embody the capacity to achieve tumor selective replication. As ectopic adenovirus localizes largely to the liver, the practical realization of the desired goal of tumor selectivity is based on an adenovirus, which selectively replicates in tumor and not in normal liver. Thus, whereas there are a number of substrate systems to assay tumor selective replication of candidate conditionally replicative adenoviruses, a stringent system to validate lack of replication within the liver has not been reported. On this basis, actual human translation has been required to ascertain the essential conditionally replicative adenovirus attribute of tumor selectivity. This situation has practically limited the developmental timeline of conditionally replicative adenovirus agents and has precluded direct comparison of candidate conditionally replicative adenoviruses. On this basis, it is clear there is an urgent need for stringent model systems for the analysis of conditionally replicative adenovirus agents to gain insight into their antitumor activity and potential toxicities.

Toward this end, we have endeavored to develop a novel model system, that will allow us to characterize conditionally replicative adenovirus agents preclinically (38, 39). This model involves derivation of human, precision-cut tumor slices or human liver slices, which can be maintained *ex vivo* for evaluation of conditionally replicative adenovirus replication. In this regard, the Krumdieck tissue slicer is a novel instrument, which was introduced several decades ago to cut precise tissue slices (with the thickness of 8 to 10 cell layers) from an organ of interest (19). Tissue slices thus obtained are capable of maintaining their original *in vivo* structure and composition. In the case of liver slices the extracellular matrix and Kupfer cells are maintained, which are important to the normal function of the hepatocyte unit (19, 20). Similarly, the heterogeneity and complex phenotype of tumor, including the vasculature, is maintained in the tumor slices. We describe herein the use of precision-cut human liver and tumor slices to determine conditionally replicative adenovirus replication for stringent therapeutic and toxicologic evaluation.

MATERIALS AND METHODS

***In vitro* Cell Culture.** Hey cells were obtained and cultured at 37°C/5% CO_2 in a humidified environment to 80% confluency. After trypsinization, the cells were harvested and plated at 50,000 cells per well into 12-well plates and allowed to adhere overnight. The next morning, the cells were infected with Ad5/3luc1 and Ad5/3cox-2 viruses (described below) in complete media (FCS, 1% penicillin/streptomycin, and 1% L-glutamine) with 2% FCS. Media was changed after 2 hours to 10% FCS complete media, and cells were allowed to incubate in the above conditions for up to 36 hours.

Murine Xenografts. Hey ovarian carcinoma cells (graciously obtained from Timothy J. Eberlein, Harvard Medical School, Boston, MA) were cultured in complete media with 10% FCS to 80% confluency. Cells were harvested by trypsinization, centrifuged, and resuspended in a 50% solution of BD Matrigel basement membrane matrix (BD Biosciences). Athymic female nude mice (4 to 6 weeks old) were given injections with 1×10^6 cells s.c. in the flank and allowed to grow to 1 cm in size. The mice were then euthanized, the tumors excised in a sterile fashion, and the xenografts placed immediately into ice-cold University of Wisconsin solution for transport to slicing.

Human Primary Tissue Samples. Human liver samples were obtained (Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama) from seronegative donor liver that was to be transplanted into waiting recipients. Approval was obtained from the Institutional Review Board before initiation of studies on human tissue. All of the liver samples were flushed with University of Wisconsin solution (ViaSpan, Barr Laboratories, Inc., Pomona, NY) before harvesting and kept on ice in University of Wisconsin solution until slicing. Time from harvest to slicing was kept at an absolute minimum (<2 hours). Human ovarian primary tumor was obtained from epithelial ovarian carcinoma patients undergoing debulking as primary therapy–no prior chemotherapy had been given. Omental samples extensively infiltrated with tumor were used to obtain tumor samples as these were the most easily obtained and had a large volume of tumor from which to generate slices. These were handled as the liver above, with the exception that the organ was not flushed with University of Wisconsin solution before harvesting.

Krumdieck Tissue Slicer. The Krumdieck tissue slicing system (Alabama Research and Development, Munford, AL) was used in accordance with the manufacturer's instructions and previously published techniques (19, 21). An 8-mm coring device (Alabama Research and Development) was used to create an 8-mm diameter core of tissue from the organ of interest (human liver, xenograft, or primary tumor). This was then placed in the slicer filled with ice-cold culture media. Slice thickness was initially set at ~250 μm with a tissue slice thickness gauge (Alabama Research and Development), and slices were cut with the reciprocating blade at 30 rpm. These were stored in ice-cold culture media for transportation to culture to serve as a wash/equilibration solution between preservation in University of Wisconsin solution and culture media.

Tissue Slice Culture. Tissue slices were placed into six-well plates (1 slice per well) containing 2 mL of complete culture media (liver, William's Medium E with 1% antibiotics, 1% L-glutamine, and 10% FCS; Hey cells and ovarian primary tumor, RPMI with 1% antibiotics, 1% L-glutamine, and 10% FCS). The plates were then incubated at 37°C/5% CO_2 in a humidified environment under normal oxygen concentrations for up to 48 hours. A plate rocker set at 60 rpm was used to agitate slices and ensure adequate oxygenation and viability (22).

Tissue Slice Viability Assay. Tissue and liver viability in culture have previously been shown with this system. However, we sought to determine whether viral infection would alter the viability of slices. Therefore, nonreplicative Ad5.luc1 (at concentrations of 150, 300, 500, and 1,000 vp/cell) was used to assess viral infection on slice viability. Primary human ovarian tissue slices were eval-

uated for viability in culture 48 hours after infection with an MTS Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, slices were cultured for 48 hours in conditions described above. MTS substrate was added to slice media in a ratio of 20 μL of MTS for every 100 μL of culture media, and slices were incubated for a period of 3 hours at 37°C. SKOV3ip1 ovarian cancer cells were cultured in 10% complete media (DMEM/F12) until 80% confluency, trypsinized, counted, and replated in dilutions of 100, 500, 1,000, 1×10^4, 1×10^5, and 1×10^6 cells per well in a six-well plate. MTS was added to these wells and incubated alongside the slices. After incubation, 100 μL from each well was analyzed in triplicate on a colorimetric plate reader at 490 nm. SKOV3ip1 cell readings were used to generate a viability curve based on the assumption (from the manufacturer) that absorbance at 490 nm was linear with cell number; this allowed us to estimate the viable cell number for each slice.

Viral Infection. Ad5/3luc1 (23), a replication-deficient adenovirus with Ad-5 knob replaced by Ad-3 knob (for enhanced infectivity of tumors), was chosen as a control virus for replicative experiments. Ad5/3wt (14), a wild-type replicative adenovirus with Ad-3 knob substitution, was chosen as a transcriptional control, whereas Ad5/3cox-2 (24) was chosen for liver off transcriptional regulation by cox-2 promoter. All viral infections were done with 500 viral particles/cell in 2% FCS complete culture media (William's Medium E for liver; RPMI for tumor). Cell number for tissue slices was estimated at 1×10^6 cells per slice based on a 10-cell thick slice (~250 μm) and 8-mm slice diameter. Infections were allowed to proceed overnight, and on the subsequent day the media was removed and replaced with 10% FCS complete culture media. Slices were removed from culture at 12-hour intervals and snap frozen for later RNA extraction.

Quantitative Reverse Transcription-PCR. Tissue slices, infected and frozen as described previously, were thawed and placed in RLT buffer (RNEasy RNA extraction kit, Qiagen, Valencia, CA) with β-mercaptoethanol (Sigma, St. Louis, MO). Slices were homogenized immediately with an ultrasonic sonicator (Fisher Scientific Model 100) at a setting of 15 watts for 10 seconds. The homogenate was centrifuged, and the supernatant was removed to separate Eppendorf tubes for subsequent RNA purification with the Qiagen, RNEasy RNA purification kit according to the manufacturer's directions. For *in vitro* cell culture, cells were trypsinized in their respective wells before removal and homogenization with 20-guage syringe/needle according to the manufacturer's directions (Qiagen, RNEasy RNA purification kit). Purified RNA was eluted in 30 μL of DNase/RNase free water and stored at −80°C until analysis. Reverse transcription-PCR for the E4 region of adenovirus was done as previously described (25) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) human housekeeping gene as an internal control. This was used both to control for slice size as well as viable cell number, as GAPDH RNA copies would be expected to be degraded by endogenous RNAses after cellular death. Results are presented as E4 copies/ng of total RNA.

RESULTS

Demonstration of Slice Viability in Culture. Previous studies have determined viability of tissue slices *in vitro*; however, we wished to determine whether viral infection itself would affect slice viability. Therefore, primary human ovarian tumor slices from omental metastases were obtained and infected with a nonreplicative Ad5.luc1 virus. MTS cell viability assays were done after 48 hours of culture and showed that slices were able to maintain viability for up to 48 hours with 3.9×10^5–4.6×10^5 viable cells/slice for a range of used viral titers from 150 vp/cell to 1,000 vp/cell (data not shown). Thus, our culture system was capable of maintaining viability of primary tumor slices in the presence of virus for the desired time period required for evaluation of conditionally replicative adenoviruses.

Demonstration of Slice and Tissue Culture System for Productive Adenoviral Infection. We wished to then show consistency of viral replication between *in vitro* cell lines and slices of the same cell line grown as a xenograft. In addition, we wanted to show that we could measure viral replication in the slices and that our measured viral quantities represented actual replication and not residual virions from the initial infection. The human ovarian cancer cell line (Hey) was cultured as described above and infected with 500vp/cell of Ad5/3luc1, Ad5/3wt, and Ad5/3cox-2 viruses. These viruses have Ad-3 serotype knob incorporated into Ad-5 to achieve enhanced infectivity. Ad5/3 luc1 is replication-incompetent based on E1A/B deletion. Ad5/3cox-2 is a conditionally replication Ad with adenoviral essential genes E1A/B under the control of cox-2 promoter. Xenografts of Hey cells were isolated and sliced as described above and infected with 500 vp/cell of the same viruses, and E4 copy number was analyzed. As shown in Fig. 1, xenograft tissue slices maintained fidelity of replication of Ad5/3cox-2, whereas Ad5/3luc1 remained at low levels consistent with its nonreplicative phenotype. This shows that E4 copy number is indicative of viral replication in our assay, as well as the fact that the slices could be cultured and infected with results comparable with *in vitro* data.

Demonstration of Adenoviral Replication in Primary Tumor Slices. Next, we wished to show that primary tumor could be sliced with subsequent culture and to show that when cox-2 was induced, primary tumor was capable of supporting replication of both Ad5/3wt and Ad5/3cox-2. As shown in Fig. 2, primary tumor slices were capable of *ex vivo* culture and support active adenoviral replication, as represented by the time-dependent increase in adenoviral E4 copy number. Both Ad5/3wt and Ad5/3cox-2 replicated well in primary tumor consistent with previous studies (14), showing transductional capability of Ad5/3 knob substitution. Twelve-hour postinfection values of Ad5/3wt E4 copy number were 5.8×10^3, 0.99×10^3, and 0.13×10^3 copies/ng RNA and rose to 3.6×10^5, 11.4×10^5, and 1.9×10^5 copies/ng RNA after 36 hours in patients 1, 2, and 3, respectively. Twelve-hour postinfection values of Ad5/3cox-2 E4 copy number were 2.1×10^3, 0.79×10^3, and 0.42×10^3 copies/ng RNA and rose to 0.40×10^5, 8.79×10^5, and 0.43×10^5 copies/ng RNA after 36 hours in patients 1, 2, and 3, respectively.

Demonstration of Transcriptional Control Liver Off Phenotype of Conditionally Replicative Adenovirus Replication in Human Liver Slices. Finally, we wished to show the feasibility of slicing and culture of human liver and subsequent transcriptional control of wild-type virus relative to the liver off

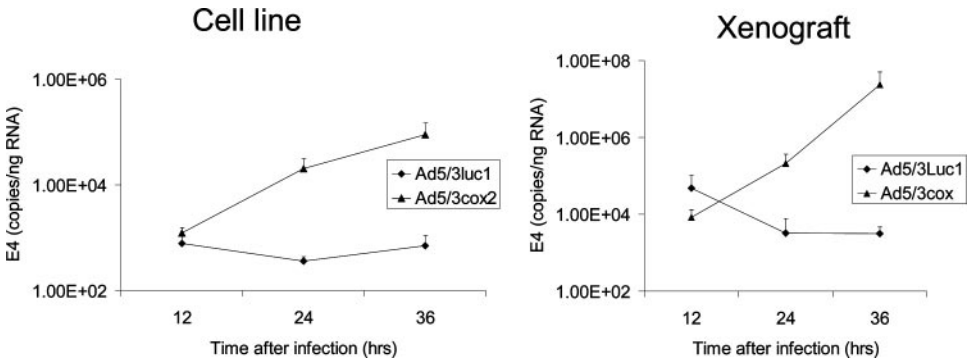

Fig. 1 Evaluation of adenoviral replication in Hey ovarian cancer cell line compared with Hey tumor xenograft-derived slices *in vitro*. Hey cells were infected in 12-well plates with 500vp/cell of Ad5/3luc1(replication-deficient) and Ad5/3cox-2(replication competent). Hey xenografts from athymic mice were harvested and sliced with Krumdieck thin-slice tissue slicer and infected similarly and evaluated for viral replication at 12-hour intervals by quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR for adenoviral E4 copy number was obtained, and results are displayed in number of copies/ng RNA as determined by GAPDH expression. All data points of triplicate slices or wells; *bars*, ±SD.

cox-2 controlled virus. As shown in Fig. 3, liver slices were able to show transcriptional tissue selectivity of adenoviral modifications. Ad5/3wt replicated quite well, consistent with known hepatotropism of wild-type adenovirus, whereas the conditionally replicative adenovirus Ad5/3cox-2, under transcriptional control of the cox-2 promoter, showed impaired replication confirming the liver off phenotype of cox-2 promoter. Ad5/3wt E4 copy number at 36-hour postinfection was an average of 71 times that of 12-hour values, whereas Ad5/3cox-2 was an average of 6 times that of 12-hour values. The replication of Ad5/3cox-2 conditionally replicative adenovirus was 2 to 10 times less than Ad5/3wt in liver at 36 hours, thus confirming reduced liver replication of conditionally replicative adenoviruses.

DISCUSSION

Novel models to evaluate toxicity and therapeutic efficacy of adenoviral gene therapies, particularly virotherapy with conditionally replicative adenoviruses, are urgently required. We report the use of human tissue slices for this purpose (38, 39). Using the Krumdieck thin-slice tissue slicer, we were able to show the viability of primary tumor slices over a 48-hour time period. We subsequently showed the fidelity of replication between known *in vitro* cell lines and their related xenografts. Finally, and most importantly, we established the ability of slices to show replication of adenovirus in human primary tumor as well as tumor/liver selectivity in human liver. This latter ability to study replication, specifically in the context of the major target organ of conditionally replicative adenovirus-based toxicities, potentially provides an important means to predict therapeutic index preclinically.

Cotton rats and pigs are the most permissive models of Ad5 replication (26); however, they are not sufficiently permissive (as measured by tissue viral titers relative to input dose) to serve as a model of replication-based toxicity. Murine and simian cells allow viral replication, but a late block prevents viral progeny production (27). With regards to direct evaluation of liver toxicity, before 1976, only small numbers of human primary hepatocytes could be cultured (28). Subsequently, isolated perfusion techniques were developed that used intact segments of liver perfused with culture media (29, 30). However, this is impractical with human liver for adenoviral replication because of the large volumes of viable liver required. Thus, these limits restricted preclinical analysis of conditionally replicative adenovirus-based hepatotoxicities to systems with limited relevance to actual human employment.

In addition to the difficulties studying liver toxicity, accurate modeling of primary tumor has been difficult as well. Barker *et al.* (31) developed a method to isolate primary tumor from ovarian cancer ascites for short-term culture. Lam *et al.* (32) developed a method of culturing primary human ovarian cells derived from ascites in a spheroid three-dimensional model that allowed analysis of conditionally replicative adenovirus replication. However, both are limited to tumor only (no assessment of liver replication) and also fail to represent the complex three-dimensional structure and heterogeneity of primary solid tumor. In an effort to address this issue as well as investigate the immune effects on virus function, Hallden *et al.* (33) developed primary murine carcinoma models. These consisted of nine murine carcinoma cell lines grown in xenograft fashion in immunocompetent mice. Subsequent adenoviral infection allowed for assessment of adenoviral function in the context of a competent immune system. However, this model is limited to murine carcinomas only, and there are a limited number of tumor types assessable in this manner. Also, there remains the inability to assess viruses intended for human use in a preclinical liver toxicity context.

The thin tissue slice (34) is thus an ideal stringent substrate system for achieving analysis of conditionally replicative adenoviruses. It is capable of representing the complexity of intact organs, and its methodology allows easier cross-conditionally replicative adenovirus comparisons. There is also the potential to use multiple organs from same donor (*i.e.*, normal tissue + tumor from same patient). Slices allow subsequent histologic evaluation in addition to biochemical evaluation methods–something not possible with current *in vitro* evaluation methods. There is also the concern of the efficient use of valuable human tissue samples, especially with hard to obtain tumors or organs

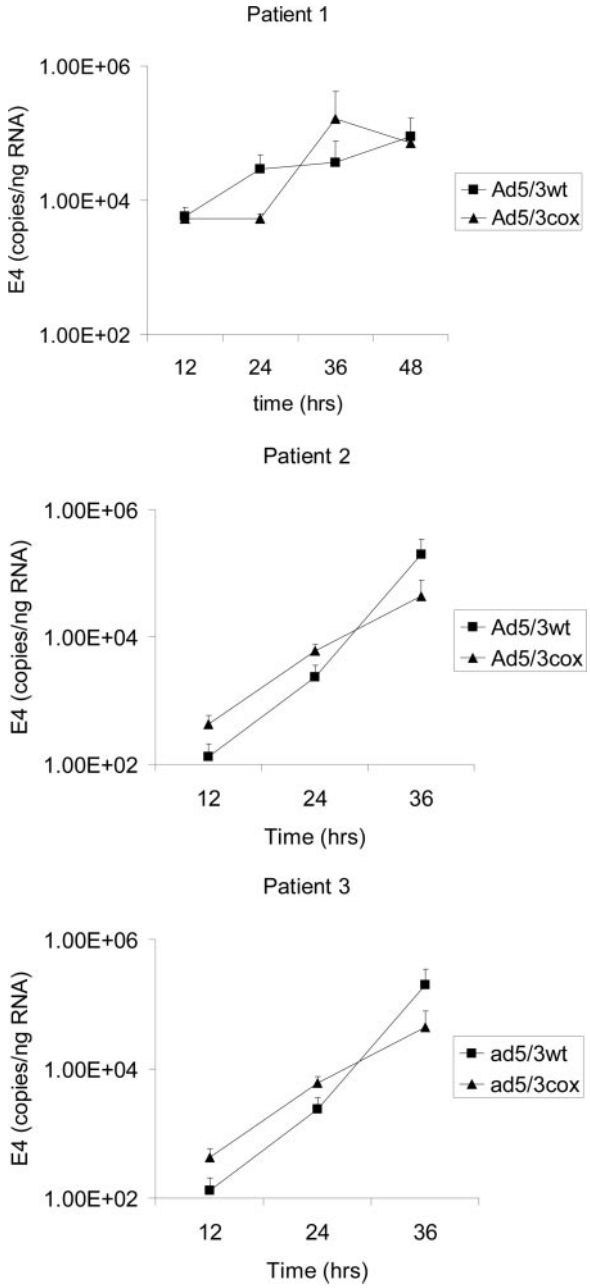

Fig. 2 Evaluation of adenoviral replication in primary human ovarian tumor slices derived from epithelial ovarian cancer patients. Human ovarian tumor slices were obtained from primary ovarian tumor with the Krumdieck thin-slice tissue slicer. Slices were infected with Ad5/3wt or Ad5/3cox-2 replicative adenoviruses to determine relative adenoviral replication at 12-, 24-, and 36-hour intervals. Quantitative reverse transcription-PCR of adenoviral E4 copy number determined replication of adenovirus. E4 copy number was calculated, and results are displayed in number of copies/ng RNA as determined by GAPDH expression. All data points are of triplicate slices; *bars*, ±SD.

(such as liver). Because of the many slices available from a single sample, multiple experiments are possible from same organ, and the experiments are controlled for variability in that all of the samples are from the same patient. We were able to obtain ~80 to 90 slices from a 2 × 2 cm cube of human liver. There is also the potential to study cell-cell interaction; a particularly interesting idea with regard to conditionally replicative adenoviruses and the "bystander effect" associated with other adenoviral gene therapies. Evaluation of combined chemosensitivity may also be possible because slices have been used to study the acute effects of cisplatin in human kidney slices (35).

There are currently two available thin-slice tissue slicers. The Krumdieck thin-slice tissue slicer incorporates a reciprocating blade design with circulating buffer flow. Attachments that allow oxygenation and cooling of the buffer solution are

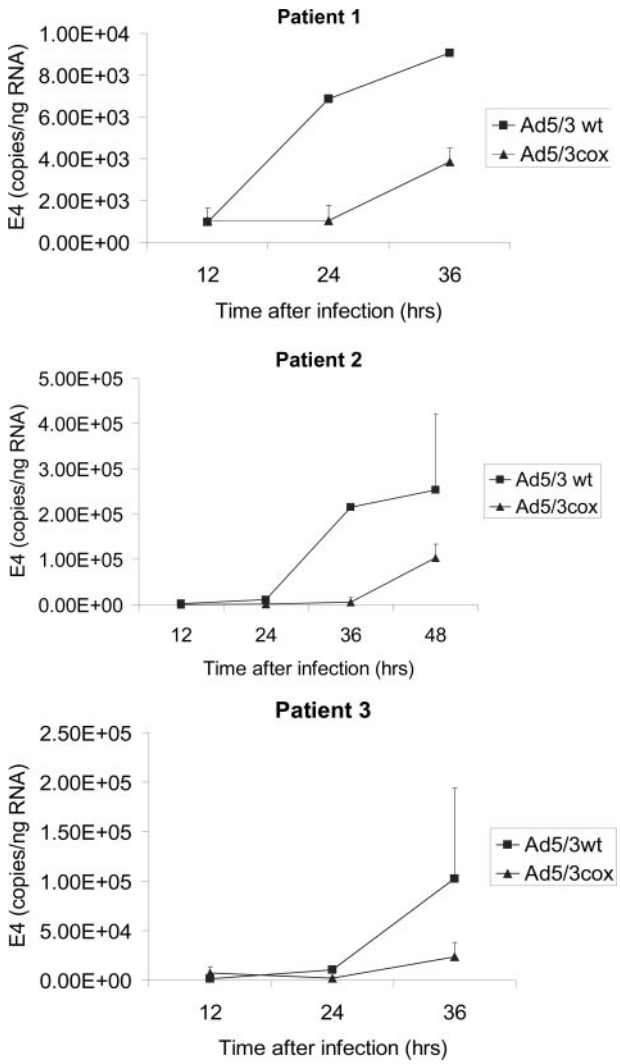

Fig. 3 Evaluation of adenoviral replication in human liver slices to determine toxicity. Human liver samples destined for transplant was obtained and thin-slices created with Krumdieck thin-slice tissue slicer. Slices were infected with either Ad5/3wt or Ad5/3cox-2 replicative adenoviruses to determine relative adenoviral replication at 12-, 24-, and 36-hour time points. Quantitative reverse transcription-PCR for adenoviral E4 copy number was calculated to determine adenoviral replication, and results are displayed in number of copies/ng RNA as determined by GAPDH expression. All data points of triplicate slices; *bars*, ±SD.

available as accessories. There is also the Brendel-Vitron system, which incorporates a rotating blade, and oxygenation and cooling of the buffer solution are standard. Either system is adequate for the purpose of developing precision tissue slices for subsequent culture.

Optimal liver slice thickness and culture conditions have been reviewed previously (20, 21). We choose 250 μmol/L for an optimal balance of handling and viability of our slices. Oxygenation of hepatocytes has been a concern; however, one must realize that too high an oxygen concentration may have deleterious effects on the tissue and alter tissue phenotype. In this regard, we chose normal oxygen concentrations and a submerged media dynamic rocking plate culture system. It has been shown (21) that liver slices consume only 0.3 to 1% of dissolved oxygen per minute; therefore, it is unlikely that oxygen availability is the limiting factor in slice viability. Of additional note, tissue to be used must be handled like transplant tissue, with careful respects paid to keeping it sterile, cold (4°C), submerged in University of Wisconsin solution, and rapid transport to slicing/culture. Cyropreservation of sliced tissue has been reported, but most have only reported viability for ≤24 hours, and only 60 to 70% of initial viability (21, 36, 37). Cold storage of human liver tissue in University of Wisconsin solution is possible for up to 24 hours; however, slice viability from this tissue is relatively unknown. It is important for future implementation of thin slices, especially as longer culture times are demanded, that attention is paid to the viability of the slices. Several methods of evaluating viability have been described, including ATP content, K^+ retention, lactate dehydrogenase leakage (liver), protein synthesis, and MTS reduction. Of these, only MTS and lactate dehydrogenase leakage do not require destruction of the slice tissue, which would make subsequent analysis of adenoviral replication problematic.

In conclusion, novel models for evaluation of *in vivo* toxicity with new generation infectivity enhanced gene therapy vectors are needed. Thin-slice tissue culture is an ideal method for *ex vivo* preclinical toxicity and infectivity analysis because it allows evaluation of conditionally replicative adenoviruses in normal human liver samples as well as in primary tumors derived from cancer patients, in which the *in vivo* structure and composition of liver and heterogeneity of human solid tumors is maintained. This allows us to extrapolate the therapeutic efficacy and toxicity for clinical translational purposes to be used as a therapeutic index for human clinical trials. Future studies will evaluate improved, long-term culture environments as well as "tumor-on/liver off" quantitative indices.

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REFERENCES

1. Nettelbeck DM, Curiel DT. Tumor-busting viruses. Sci Am 2003; 289:68–75.

2. Nettelbeck DM. Virotherapeutics: conditionally replicative adenoviruses for viral oncolysis. Anti-Cancer Drugs 2003;14:577–84.

3. Alemany R, Balague C, Curiel DT. Replicative adenoviruses for cancer therapy. Nat Biotechnol 2000;18:723–7.

4. Gomez-Navarro J, Curiel DT. Conditionally replicative adenoviral vectors for cancer gene therapy. Lancet Oncol 2000;1:148–58.

5. Curiel DT. The development of conditionally replicative adenoviruses for cancer therapy. Clin Cancer Res 2000;6:3395–9.

6. Barnes MN, Coolidge CJ, Hemminki A, Alvarez RD, Curiel DT. Conditionally replicative adenoviruses for ovarian cancer therapy. Mol Cancer Ther 2002;1:435–9.

7. Vecil GG, Lang FF. Clinical trials of adenoviruses in brain tumors: a review of Ad-p53 and oncolytic adenoviruses. J Neuro-Oncol 2003; 65:237–46.

8. Nemunaitis J, Ganly I, Khuri F, et al. Selective replication and oncolysis in p53 mutant tumors with ONYX-015, an E1B–55kD gene-deleted adenovirus, in patients with advanced head and neck cancer: a phase II trial. Cancer Res 2000;60:6359–66.

9. Lamont JP, Nemunaitis J, Kuhn JA, Landers SA, McCarty TM. A prospective phase II trial of ONYX-015 adenovirus and chemotherapy in recurrent squamous cell carcinoma of the head and neck (the Baylor experience). Ann Surg Oncol 2000;7:588–92.

10. Nemunaitis J, Cunningham C, Buchanan A, et al. Intravenous infusion of a replication-selective adenovirus (ONYX-015) in cancer patients: safety, feasibility and biological activity. Gene Ther 2001;8: 746–59.

11. Douglas JT, Kim M, Sumerel LA, Carey DE, Curiel DT. Efficient oncolysis by a replicating adenovirus (ad) in vivo is critically dependent on tumor expression of primary ad receptors. Cancer Res 2001;61: 813–7.

12. Ganly I, Kirn D, Eckhardt G, et al. A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. Clin Cancer Res 2000;6:798–806.

13. Witlox AM, Van Beusechem VW, Molenaar B, et al. Conditionally replicative adenovirus with tropism expanded towards integrins inhibits osteosarcoma tumor growth in vitro and in vivo. Clin Cancer Res 2004;10:61–7.

14. Kawakami Y, Li H, Lam JT, et al. Substitution of the adenovirus serotype 5 knob with a serotype 3 knob enhances multiple steps in virus replication. Cancer Res 2003;63:1262–9.

15. Bauerschmitz GJ, Lam JT, Kanerva A, et al. Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. Cancer Res 2002; 62:1266–70.

16. Shinoura N, Yoshida Y, Tsunoda R, et al. Highly augmented cytopathic effect of a fiber-mutant E1B-defective adenovirus for gene therapy of gliomas. Cancer Res 1999;59:3411–6.

17. Lamfers ML, Grill J, Dirven CM, et al. Potential of the conditionally replicative adenovirus Ad5-Delta24RGD in the treatment of malignant gliomas and its enhanced effect with radiotherapy. Cancer Res 2002;62:5736–42.

18. Wildner O, Blaese RM, Morris JC. Therapy of colon cancer with oncolytic adenovirus is enhanced by the addition of herpes simplex virus-thymidine kinase. Cancer Res 1999;59:410–3.

19. Krumdieck CL. A new instrument for the rapid preparation of tissue slices. Anal Biochem 1980;104:118–23.

20. Parrish AR, Gandolfi AJ, Brendel K. Precision-cut tissue slices: applications in pharmacology and toxicology. Life Sci 1995;57: 1887–901.

21. Olinga P, Meijer DKF, Slooff MJH, Groothuis GMM. Liver slices in in vitro pharmacotoxicology with special reference to the use of human liver tissue. Toxicol In Vitro 1997;12:77–100.

22. Olinga P, Groen K, Hof IH, et al. Comparison of five incubation systems for rat liver slices using functional and viability parameters. J Pharmacol Toxicol Methods 1997;38:59–69.

23. Kanerva A, Mikheeva GV, Krasnykh V, et al. Targeting adenovirus to the serotype 3 receptor increases gene transfer efficiency to ovarian cancer cells. Clin Cancer Res 2002;8:275–80.

24. Yamamoto M, Davydova J, Wang M, et al. Infectivity enhanced, cyclooxygenase-2 promoter-based conditionally replicative adenovirus for pancreatic cancer. Gastroenterology 2003;125:1203–18.

25. Rivera AA, Wang M, Suzuki K, et al. Mode of transgene expression after fusion to early or late viral genes of a conditionally replicating adenovirus via an optimized internal ribosome entry site in vitro and in vivo. Virology 2004;320:121–34.

26. Prince GA, Porter DD, Jenson AB, et al. Pathogenesis of adenovirus type 5 pneumonia in cotton rats (Sigmodon hispidus). J Virol 1993;67: 101–11.

27. Lucher LA. Abortive adenovirus infection and host range determinants. Curr Top Microbiol Immunol 1995;199(Pt 1):119–52.

28. Guillouzo A, Oudea P, Le Guilly Y, et al. An ultrastructural study of primary cultures of adult human liver tissue. Exp Mol Pathol 1972; 16:1–15.

29. Meijer DK, Keulemans K, Mulder GJ. Isolated perfused rat liver technique. Methods Enzymol 1981;77:81–94.

30. Melgert BN, Olinga P, Weert B, et al. Cellular distribution and handling of liver-targeting preparations in human livers studied by a liver lobe perfusion. Drug Metab Dispos 2001;29:361–7.

31. Barker SD, Casado E, Gomez-Navarro J, et al. An immunomagnetic-based method for the purification of ovarian cancer cells from patient-derived ascites. Gynecol Oncol 2001;82:57–63.

32. Lam JT, Bauerschmitz GJ, Kanerva A, et al. Replication of an integrin targeted conditionally replicating adenovirus on primary ovarian cancer spheroids. Cancer Gene Ther 2003;10:377–87.

33. Hallden G, Hill R, Wang Y, et al. Novel immunocompetent murine tumor models for the assessment of replication-competent oncolytic adenovirus efficacy. Mol Ther 2003;8:412–24.

34. Bach PH, Vickers AE, Fisher R, et al. The use of tissue slices for pharmacotoxicological studies: the report and recommendations of ECVAM workshop. Angera, Italy: European Centre for the Validation of Alternative Methods; 1996.

35. Fisher RL, Sanuik JT, Gandolfi AJ, Brendel K. Toxicity of cisplatin and mercuric chloride in human kidney cortical slices. Hum Exp Toxicol 1994;13:517–23.

36. Olinga P, Merema MT, Hof IH, et al. Effect of cold and warm ischaemia on drug metabolism in isolated hepatocytes and slices from human and monkey liver. Xenobiotica 1998;28:349–60.

37. Glockner R, Rost M, Pissowotzki K, Muller D. Monooxygenation, conjugation and other functions in cryopreserved rat liver slices until 24 h after thawing. Toxicology 2001;161:103–9.

38. Rots MG, Elferink MGL, Oosterhuis D, et al. A clinically relevant *ex vivo* toxicity assay using human liver slices to determine specificity of replication of oncolytic viruses. Mol Ther 2004;8:512.

39. Rots MG, Elferink MGL, Gommans WM, et al. Precision cut tissue slices: A novel ex vivo toxicity and specificity assay for the preclinical screening of gene transfer vectors. Mol Ther 2003;7:56.

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