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 2-(4-sulfophenyl)-2H-tetrazolium, inner salt) ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-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 adenovirus systems 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-
efficient 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% CO₂ 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 Ads5/3lacI and Ads5/3cox-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 × 10⁶ 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% CO₂ 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 Ads5.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 × 10³, 1 × 10⁵, and 1 × 10⁶ 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 is 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. 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-gauge 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 copy numbers could be expected to be degraded by endogenous RNAs 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 × 10⁶–4.6 × 10⁷ 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. Ad5/3luc1 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 × 10³, 0.99 × 10⁴, and 0.13 × 10⁵ copies/ng RNA and rose to 3.6 × 10⁵, 11.4 × 10⁵, and 1.9 × 10⁶ 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 × 10⁵, 0.79 × 10⁶, and 0.42 × 10⁷ copies/ng RNA and rose to 0.40 × 10⁸, 8.79 × 10⁹, and 0.43 × 10⁷ copies/ng RNA after 36 hours in patients 1, 2, and 3, respectively.

Demonstration of Transcriptional Control Liver Off Phenotype of Conditionally Replicating 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
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 adenoviruses 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 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, Halden 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.
Because of the many slices available from a single sample, multiple experiments are possible from the 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 oncolytic 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 available.

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.

Fig. 3 Evaluation of adenoviral replication in human liver slices to determine toxicity. Human liver samples destined for transplant were 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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