HUMAN ADENOVIRUS REPLICATES IN IMMUNOCOMPETENT MODELS OF PANCREATIC CANCER IN SYRIAN HAMSTERS.

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Running title: Adenovirus replication in hamster cancer

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ABSTRACT

The pre-clinical evaluation of toxicity and antitumor effect of conditionally replicative (oncolytic) adenoviruses is hampered by the inability of human adenoviruses to replicate efficiently in murine cells. The Syrian golden hamster (*Mesocricetus auratus*) has been suggested as a permissive animal for adenoviral replication, and cancer cells lines derived from different hamster tumors are available. We provide evidence that wild type adenovirus type 5 is able to infect and replicate in the pancreatic cancer cells HaP-T1 and H2T both in vitro and in vivo. Determination of cytopathic effect, viral spread, progeny production and the expression of late viral proteins indicate that the complete viral cycle of adenovirus takes place, albeit less efficiently than in highly permissive human cancer cells A549 and HuH7. The intrahepatic inoculation of HaP-T1 and H2T cells gave rise to tumors in the liver of hamsters that resemble metastases of pancreatic cancer. The growth of HaP-T1-induced nodules was faster than those derived from H2T, but both of them caused progressive liver infiltration and peritoneal dissemination. When adenovirus was inoculated in these lesions, productive replication took place and newly formed infective virions could be recovered 4 days after administration. In conclusion, the Syrian hamster models described here offer the opportunity to evaluate the effect of oncolytic adenoviruses in an immunocompetent animal and may be a valuable tool in the pre-clinical evaluation of these agents.

Keywords: Adenovirus; replication; pancreatic cancer; liver; hamster.

INTRODUCTION

The use of replication-competent viruses to treat cancer is a classical strategy that has experienced a dramatic advance in the last decade, aided by the progress in the development of viral vectors for gene therapy (Parato *et al.*, 2005). This approach can contribute to the improvement of therapeutic regimes for malignancies that are refractory to current treatments, such as pancreatic cancer (Li *et al.*, 2004; Kasuya *et al.*, 2005) and others. The objective in the field is to obtain a virus that will complete its lytic cycle preferentially in cancer cells, whereas it is attenuated in normal cells. The release of a new viral progeny in the tumor will amplify the initial oncolytic effect and the process will continue until the tumor is destroyed or the virus is neutralized by the immune system and other host factors. Human adenovirus can be modified to achieve cancer specificity by different methods, including transcriptional control of viral genes and ablation of viral functions that can be complemented only in cancer cells (Ko *et al.*, 2005; Rein *et al.*, 2006). In addition, the antitumor effect can be improved by incorporation of therapeutic genes and the combination with other standard or experimental treatments. In fact, conditionally replicative adenoviruses (CRAd) are the most frequently used oncolytic viruses both in pre-clinical and clinical studies (Young *et al.*, 2006). The feasibility and safety of CRAds in human patients has been demonstrated in many clinical trials (Aghi and Martuza, 2005). However, the promising results obtained with early generation CRAds in animal models of liver and pancreatic cancer (Oonuma *et al.*, 2002; Takahashi *et al.*, 2002) have not been reproduced in humans, at least as single agents (Habib *et al.*, 2002; Hecht *et al.*, 2003; Makower *et al.*, 2003). Improved versions of CRAds are being generated, but the pre-clinical evaluation of these viruses is still hampered by the fact that human adenovirus does not replicate efficiently in mice and rats. The antitumor effect of CRAds is usually tested in tumor xenografts obtained by inoculation of human cancer cells in immunocompromised animals. Although this provides in vivo confirmation of the cytolytic properties of the virus and may validate the function of certain therapeutic genes, the xenograft models have important limitations. They cannot predict the influence of the host immune system, which is crucial to understand the biodistribution and duration of viral replication. In addition, many immunomodulatory cytokines commonly used as therapeutic genes are not fully functional in these animals. Finally, these models are not

suitable to assess the cancer specificity of oncolytic adenoviruses, since they will replicate preferentially in the tumor implant mainly because it is the only human tissue present in the animal. Some murine epithelial cancer cell lines that sustain adenoviral replication have been described (Ganly *et al.*, 2000; Hallden *et al.*, 2003), but few examples of animal models based on these cells are available.

Among the species examined for human adenovirus replication, swine seems to be the most permissive (Torres *et al.*, 1996; Jogler *et al.*, 2006), but the lack of tumor models and economical considerations limits its general use in research laboratories. The cotton rat (*Sigmodon hispidusis*) is considered semi-permissive because intranasal inoculation of the virus achieves local replication and causes pneumonia (Pacini *et al.*, 1984). Tumor cells from cotton rat are available, and modest replication of human adenovirus has been described in vitro and in vivo (Toth *et al.*, 2005). An interesting alternative is the Syrian golden hamsters (*Mesocricetus auratus*), in which consistent adenovirus replication and release of new viral progeny in the lung has been observed (Hjorth *et al.*, 1988). Here we demonstrate potent replication of human adenovirus type 5 in pancreatic cancer cells from hamster in vitro and in vivo. The tumorigenic properties of HaP-T1 and H2T cells were compared after subcutaneous and intrahepatic inoculation and indicate different growth patterns which are suitable for in vivo testing. As a result, we describe here an immunocompetent model of pancreatic liver metastasis in which adenovirus can replicate efficiently. It has been recently reported that other hamster cell lines support different degrees of adenoviral replication and are tumorigenic after subcutaneous inoculation in hamsters (Thomas *et al.*, 2006). Interestingly, replication was not only observed in the inoculated site, but also in distant organs such as lung and liver. Our work expands these findings and supports the use of Syrian hamster as the basis for tumor models intended to evaluate the therapeutic potential of oncolytic adenoviruses.

MATERIALS AND METHODS

Cell lines and viruses

The cell lines HaP-T1 (German Collection of Micro organisms and Cell Culture, Cat. ACC 222) and H2T (courtesy of Dr. CM Townsend, University of Texas Medical Branch, TX, USA) are derived from pancreatic cancers induced by *N*-nitrosobis (2-hydroxypropyl) amine (BHP) in hamsters. The hamster kidney cell line Hak, the human lung cancer cell line A549 and the embryonic kidney human cell line HEK293 were obtained from American Type Culture Collection, Manassas, VA (cat. CCL-185, CRL-1573 and CCL-15 respectively). The human hepatocellular carcinoma cell line HuH-7 was obtained from Dr. Brechot (Inserm, Paris, France). MC38 cell line is a carcinogen-induced colon cancer developed in C57BL/6 mice (Corbett et al., 1975). All cells were maintained in DMEM supplemented with 10%FBS, 100 units/ml penicillin and 100 μg/ml streptomycin

The wild type human adenovirus type 5 (Ad5) was obtained from ATCC (Cat. VR-5). The AdCMVLuc is an E1-E3 deleted replication-deficient adenoviral vector expressing *Firefly Luciferase* under the control of the Cytomegalovirus promoter (Vector Biolabs, Philadelphia, PA, USA). The AdCMVLacZ is an equivalent vector expressing β-galactosidase (Mazzolini *et al.*, 2000). The amplification and purification of the viruses was performed using standard techniques, as previously described (Hernandez-Alcoceba *et al.*, 2000). Quantification of viruses was performed using the Adeno X Rapid titer kit (BD Bioscience), as specified by the manufacturer. Therefore, viral units are referred as infectious units (iu), which are equivalent to pfu. The titer of viral stocks used in this study was $6.2x10^{11}$ iu/ml ($5x10^{12}$ particles/ml) in the case of AdCMVLuc, and $2.3x10^{11}$ iu/ml ($7.8x10^{12}$ particles/ml) in the case of Ad5.

Animals

Female athymic (*nu/nu*) mice (5-6 weeks of age) were purchased from Charles River Laboratories (Barcelona, Spain). Female Syrian (Golden) hamsters (*Mesocricetus Auratus*; *HSD HAN: AURA,* 5 weeks of age) were obtained from Harlan, Barcelona Spain. Athymic mice were kept in individually-ventilated cages and received autoclaved drinking water and food ad libitum. Hamsters were maintained in the same kind of cages after administration of adenoviruses. Manipulation of the animals was performed

in a biosafety type II laminar flow cabin. All procedures were carried out following protocols approved by the local ethical committee in accordance with recommendations for proper care and use of laboratory animals.

***In vitro* viral replication assay**

For virus burst size determination, cells ($2x10^4$ /well) were cultured in 24-well plates overnight and infected with Ad5 in DMEM supplemented with 2% FBS. Throughout this work, the MOI (multiplicity of infection) is defined as the amount of infectious units (iu) per cell. This parameter was adjusted for each cell line to obtain more than 50% of cells infected with the initial viral dose. Adenovirus was removed after 2 h, cells were washed 2 times with phosphate-buffered saline solution (PBS) and incubated in DMEM supplemented with 2% FBS for additional 48h or 96h. Then cells were collected and lysed by three cycles of freezing and thawing. For determination of virus release in the culture medium, infections were performed at lower MOI in order to preserve viability of the monolayer for the entire duration of the experiment. Virus was removed 12h after infection, and supernatant was collected at different time points until day 9 post-infection. The amount of viable viruses present in cell lysates or supernatants was determined by the end-limiting dilution method in HEK293 cells. The result is expressed as infectious units (iu)/cell, considering the number of cells plated at the beginning of the experiment. The accuracy of the end-limiting dilution method was confirmed with the Adeno X Rapid titer kit (BD Bioscience).

***In vitro* cytotoxicity assays**

Cells were plated at $5x10^3$cells/well in 96-well plates before infection. Twenty-four h later, media were removed and replaced with DMEM supplemented with 2% FBS. Cells were infected by exposure to adenovirus preparations diluted to the indicated MOIs in 100 µl volume. After 2 h incubation 100 µl of fresh medium were added. Cell viability was determined 5 or 7 days after infection by the MTT assay (Sigma). At the time of analysis, 40 µl of Thiazolyl blue (MTT 5 mg/ml) solution were added to the cell cultures in 60 µl of medium. Four h later, media were removed and precipitated MTT salts were solubilized into 50 µl of 0.04 N HCl diluted in isopropanol. Quantification was performed by reading absorbance at 590 nm in spectrophotometer. The data were analyzed using the Prism GraphPad program.

Western Blotting

Cells were seeded in a 6-well plate at a density of $1x10^5$ cell/plate, cultured for 24 h and infected with each indicated virus in DMEM supplemented with 2%FBS. After 2 h the cells were washed with PBS and new medium was added. One and 3 days after infection, cells were harvested and suspended in TNE buffer (50 mM Tris pH 7.5, 5 mM EDTA, 100 mM NaCl) supplemented with 1% Igepal and a cocktail of protease inhibitors (Complete Tablets, Roche). After sonication, the lysate was cleared by centrifugation and protein concentration was determined using the Bio-Rad protein assay. Equal amounts (10 ug) of total protein were separated on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane (Amersham Bioscience). Immunoblots were processed according to standard procedure, using 1: 10000 primary monoclonal antibodies against fiber (Lab Vision Corporation, USA) or 1:5000 of anti-GA3DPH antibody (Biogenesis), washed and incubated with 1:5000 peroxidase-conjugated goat anti-mouse IgG (Pierce). Detection was performed by Western Lighting chemiluminescence reagent (Perkin Elmer Life Sciences). All antibodies were incubated in PBS-Tween 0,05% with milk 1% during 2 hours (primary antibodies) or 1 hour (secondary antibody).

Detection of viral proteins by immunohistochemistry.

For the analysis of viral replication and spread *in vitro*, cells were seeded in a 24-well plate at the concentration of $2x10^5$ cells/well. Twenty for hours later, they were infected with Ad5 at different MOIs. At different times after infection, the cell monolayers were fixed with cold methanol for 10 min at -20°C, washed three times with PBS + 1%BSA and incubated for 1 h at 37°C with a 1:1000 dilution of a mouse anti-adenovirus monoclonal antibody blend (Chemicon International, USA). Then, cells were washed 3 times with PBS + 1% BSA, and incubated for 1 h at 37°C with a 1:1000 dilution of a goat anti-mouse antibody conjugated with horseradish peroxidase (Pierce, USA). After 3 washes with PBS + 1%BSA, cells were incubated for 10 min with a DAB substrate (DakoCytomation, Denmark) to reveal the positive cells.

Establishment of pancreatic tumors in nude mice and hamsters

Xenografts were induced in the flank of athymic (*nu/nu*) mice by subuctaneous inoculation of 10^7 A549, 10^6 Hap-T1 or 10^7 H2T cells resuspended in 50 µl of 0.9% saline solution. The appearance of tumors were monitored twice a week and tumors

measured by a calliper. The volume was calculated using the following formula: $(Dxd^2)/2$, where D and d are the major and minor diameters, respectively. The viruses were injected intratumorally when tumors reached a volume of 600-700 mm^3.

In the case of subcutaneous tumors in hamsters, 10^6 Hap-T1 or 10^7 H2T cells were resuspended in 50 µl saline and injected into the flank. The monitorization of tumor growth was performed as described above.

For the establishment of liver metastases of pancreatic cancer, hamsters were laparotomized and $2x10^6$ Hap-T1 cells or $6x10^6$ H2T cells were injected in a single liver lobe in a volume of 50 µl saline using a Hamilton syringe. Animals were anesthetized by inhalation of isofluorane and received analgesia after surgery. Viruses were injected intratumorally following the same procedure.

Quantification of viral replication in vivo

Following a single intratumoral injection of Ad5, animals were sacrificed at different times post-infection and tumors were immediately frozen in liquid nitrogen. For the extraction of viable adenoviruses, samples were thawed on ice, and 100-400 mg of tumor tissue was homogenized in 1 ml of complete HEK293 growth medium. After 3 cycles of freezing and thawing, samples were centrifuged at 10,000 g for 5 min and the supernatant containing adenovirus was recovered. Quantification of viable particles was performed by incubation of the tumor extracts with HEK293 cells for 48 h and identification of infected cells by immunohistochemistry using a mouse anti-adenovirus monoclonal antibody blend (Chemicon International, USA).

Quantitative PCR

Infected cells were harvested, subjected to 3 cycles of freezing and thawing and DNA was isolated using a High Pure Viral Nucleic Acid Kit (Roche) according to the manufacturer's instructions. Two µl of each sample were used for quantification of viral copies. PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Ltd) with SYBR green fluorophore. The primer sequences used for amplification of Adenovirus E4 gene were 5'-CTAACCAGCGTAGCCCA-3' (forward) and 5'-TGAGCAGCACCTTGCATTTT-3' (reverse). Reactions were performed in a total volume of 20 µl including 10 µl 2X iQ SYBR Green Supermix (BioRad), 0.6 µl of each primer at the concentration of 10 µM and 2 µl of template. Parameters of PCR included an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 15

sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and one cycle of 72°C for 4 min. Melt analysis was performed to determinate the specificity of the PCR amplification. To calculate the amount of copies/μg DNA, a standard curve of purified Ad5 genomes was prepared.

RESULTS

Replication of adenovirus in pancreatic cancer cells from hamster. The HaP-T1 and H2T cell lines are derived from pancreatic cancers induced in hamsters by injection of the carcinogen *N*-nitrosobis (2-hydroxypropyl) amine (BHP) (Townsend *et al.*, 1982; Saito *et al.*, 1988). The replication of wild type adenovirus type 5 (Ad5) was analyzed in these cell lines, together with other reference cells that include the human cancer cells A549 (lung cancer), and HuH7 (hepatocellular carcinoma), the hamster kidney cell line HaK and the murine cancer cells MC38 (colon cancer). We calculated the viral progeny obtained 48h after infection of the cells with Ad5. The MOI (multiplicity of infection, defined as the number infectious particle per cell) was adjusted for each cell line to obtain at least 50% of cells infected with the initial viral dose in our assay conditions. This means MOI 50 for HaP-T1, H2T, HaK and HuH7, MOI 10 for A549, and MOI 200 for MC38. This estimation was obtained by exposing the cells to a replication-deficient adenoviral vector expressing the reporter gene β-galactosidase, and quantifying the infected cells by X-Gal staining (not shown). In figure 1A we represent the amount of infective viral particles accumulated in the cells 48h after infection. A wide range was observed among all cells types, from virtually no virus detected in the murine cells MC38 to approximately 2,000 iu/cell in the human cancer cell A549. In the case of pancreatic cancer cells from hamster, HaP-T1 produced 280 iu/cell, whereas H2T had the lowest yield at this time point (0.4 iu/cell). In agreement with recent reports (Thomas *et al.*, 2006), we observed viral progeny production also in the hamster kidney cells HaK (15 iu/cell), although less efficiently than in HaP-T1. When a replication-deficient adenoviral vector was used a control (AdCMVLuc), virtually no viral progeny was detected under these assay conditions (not shown). These data support the notion that Ad5 can replicate in hamster cancer cell lines, but important differences in efficacy were observed between them. Therefore, additional experiments

were carried out in order to determine if the moderate (HaP-T1) or low (H2T) viral progeny observed in hamster cell lines was indeed due to a productive viral cycle. In figure 1B we represent the viral progeny production of HuH7, HaP-T1 and H2T cells over time. The progressive increase indicates that several rounds of viral production and re-infection take place in the cells cultures. To confirm that the entire viral cycle is completed in hamster cells, we analyzed the presence of new viral particles liberated in the supernatant of the cultures. In figure 1C we show that the amount of virus released from HaP-T1 and H2T cells increases over time up to 9 days after the initial infection with Ad5. When compared to the human cells HuH7, viral progeny production was significantly lower during the first 6 days. At day 9 the amount of virus present in the supernatant continued to increase in hamster cells. In contrast, viral release reached a plateau in HuH7 cells, probably due to a complete destruction of the cell monolayer at this late time point. In the case of the MC38 cells, liberation of virus was undetectable. Finally, we performed quantitative PCR using primers specific for the E4 region to monitor the production of new viral genomes in the cells. In figure 1D we can see the accumulation of viral copies from 12 to 48h post-infection both in human and hamster cells, with lower copy number in the case of H2T. Stabilization of the copies from 48 to 96h may be due to the equilibrium between cell lysis and re-infection of neighbouring cells in the monolayer.

Expression of adenoviral proteins in hamster cells. In order to better characterize the viral cycle of adenovirus in hamster cells, we analyzed the expression of late viral proteins, which are only produced after the replication of the adenoviral genome has been completed. We performed immunohistochemical detection of capsid proteins in cultures of HaP-T1, H2T and A549 cells that had been infected with Ad5 for different periods of time. The result shown in figure 2A demonstrates not only that viral proteins are expressed in hamster cells, but also that the number of positive cells increases from day 2 to day 4. This indicates the release of new generations of viruses from the cells infected at the beginning of the experiment, and spread in the monolayer. In contrast, virtually no expression could be detected in the murine cell line MC38 at any time point, indicating a blockade in the viral cycle. The expression of the fiber protein was analyzed by Western blot in lysates of cells infected by Ad5 at different times (figure 2B). In agreement with the previous results, we could detect increasing amounts of the protein in hamster and human cell lines, but not in the murine cells MC38, nor in human cells infected with an E1-deleted, replication-deficient adenoviral vector (AdCMVLuc).

Cytotoxic effect. We next analyzed the ability of Ad5 to cause cytopathic effect and death of cancer cells, which is the main property of oncolytic adenoviruses. The viability of the cells was analyzed several days after infection with different MOIs, to allow several rounds of viral replication. To ensure that the cytotoxicity was due to viral replication and spread in the cultures, we included as a control the replication-deficient adenovirus AdCMVLuc under the same conditions. This virus only reduced the viability of the cells when used at high MOIs (exceeding 200 virus/cell, not shown), which may reflect direct toxicity instead of virus replication. The same effect was observed in the murine cell line MC38 after infection with Ad5 (figure 3). In contrast, hamster cells were efficiently killed by Ad5 at relatively low MOIs, and this effect was increased over time (not shown). In agreement with data obtained on the study of viral replication (figure 1), the most permissive cell line was the human A549, followed by HaP-T1 and H2T.

Replication of adenovirus in pancreatic cancer xenografts. Once replication of adenovirus was demonstrated in vitro in HaP-T1 and H2T cells, we initiated experiments to determine if these cells were suitable to establish relevant cancer models for oncolytic adenovirus evaluation. These viruses are currently evaluated in human tumor xenografts growing in nude mice, where it has been demonstrated that adenovirus can replicate and produce viral progeny. In order to compare the performance of human and hamster cells, we established tumor xenografts by subcutaneous injection of A549, HaP-T1 and H2T cells in nude mice. Tumors were inoculated with $5x10^8$ iu when they reached a volume of approximately 350-700 mm^3, and mice were sacrificed at different time points. Under these experimental conditions, no consistent antitumor effect was observed in any case. The presence of viable viral particles was determined in tumor lysates by titration on 293 cells. As expected, viral progeny was detected at days 4 and 6 post-infection in A549-induced tumors, ranging from 10^5 to 10^6 iu/mg tumor (Figure 4A). The viral yield obtained after infection of the A549 tumors with AdCMVLuc was below 10^3 iu/mg, and this was considered as the background for this assay in our experimental conditions for these cells. When the tumors were lysed 6 hours after infection a similar background level of virus was observed (data not shown), indicating that the initial viral load is rapidly cleared and the viruses detected the subsequent days correspond to new generations of virions produced inside the tumor. In the case of

hamster cells, the viral progeny exceeded 10^4 iu/mg in most of the animals at day 4, with values close to the human cells at day 6 (Figure 4B), indicating active viral replication in vivo.

Growth of pancreatic cancer cells in immunocompetent animals. The tumorigenicity of HaP-T1 and H2T cells in Syrian hamsters was initially compared by subcutaneous inoculation of the cells and direct measurement of tumor growth (Figure 5A). When 10^6 cells were injected, HaP-T1 gave rise to tumors in most of the animals with a latency of 2 weeks approximately. The tumors grew progressively for more than a month, until the hamsters were sacrificed for ethical reasons. In contrast, 10^6 H2T cells produced detectable tumors only two months after inoculation (not shown). When 10^7 H2T cells were inoculated, tumors appeared 3 weeks later and grew slowly for almost 2 months. With the aim to establish a model of experimental liver metastases, we analyzed the ability of the cells to form tumors after intrahepatic injection in the hamsters. Based on the previous results, we inoculated $2x10^6$ HaP-T1 or $6x10^6$ H2T cells into a single hepatic lobe. One month later, the appearance and behaviour of the animals remained normal. However, laparotomy revealed extensive tumor growth in the site of injection and peritoneal infiltration in the case of HaP-T1 (Figure 5C, lower panel). At this moment, H2T-induced tumors were smaller (average 200 mm^3, Figure 5B) and limited to the inoculation site. However, animals sacrificed 2 months after cell implantation revealed extensive tumor infiltration in the liver and peritoneum (Figure 5D, left lower panel). In addition, lung metastases were observed in more than 80% of the animals at this late time point (figure 5D, right lower panel). In both cases, histopatological analysis confirmed the existence of malignant cells suggestive of pancreatic cancer metastatic to the liver (Figures 5C and D, upper panels). In a separate experiment, hamsters inoculated intrahepatically with $2x10^6$ HaP-T1 cells were laparotomized 2 weeks later and tumors measured. The average size at this point was 800 mm^3 (Figure 5B).

Replication of adenovirus in liver metastases of pancreatic cancer in hamsters. Based on the previous results, we established liver metastases by injection of HaP-T1 or H2T in the liver of hamsters. The Ad5 virus ($2x10^9$ iu) was injected intratumorally when HaP-T1 and H2T lesions had evolved for 14 and 30 days, respectively. Four days later, animals were sacrificed and the amount of viable adenoviruses was quantified in tumor

extracts. As shown in figure 6, both types of tumors yielded detectable viral progenies, exceeding 10^3 iu per mg of tissue on average. In contrast, no viruses could be detected when the AdCMVLuc virus was injected in this kind of tumors growing in immunocompetent hamsters. Since the background due to this replication-deficient vector is lower in this model than in the xenografts growing in athymic mice, the viral yield observed with Ad5 clearly indicates active viral replication in vivo. Interestingly, the amplification of Ad5 in tumors derived from H2T cells is as efficient as in HaP-T1 tumors, despite the lower replication capacity observed in vitro.

DISCUSSION

The field of oncolytic adenoviruses needs new animal models to test the efficacy, specificity and toxicity of these agents. The human tumor xenografts in nude mice certainly provide proof of concept of viral oncolysis, but these models tend to over-estimate the therapeutic potential of replication-competent adenoviruses. The information obtained in early clinical trials indicate that the viruses used so far are safe, but at the same time it suggests that combination treatments and viruses with higher oncolytic potency will be needed to achieve clinically relevant effects. In addition, new strategies include the use of "armed" viruses expressing immunostimulatory and other molecules whose effects depend on the interaction with the host. All these considerations have stimulated the search for immunocompetent animal models in which adenovirus can replicate. In mice, the blockade of human adenovirus cycle is mainly due to post-entry steps, since many murine cells can be efficiently infected (Ganly *et al.*, 2000). The permissivity for adenovirus replication varies depending on the cell type in different species, including humans (Jogler *et al.*, 2006). For instance, moderate replication has been observed in certain murine cancer cell lines that can be used to establish tumors in syngeneic C57BL6 animals (Hallden *et al.*, 2003). Although this is an advance versus xenograft models, replication of the virus does not take place in the host tissues. Only limited replication in the liver of CBA mice was reported after high doses of human adenovirus were injected systemically (Duncan *et al.*, 1978). The cotton rat model is closer to achieve the objective, but there are few tumor cell lines available, the ones tested have limited permissivity (Toth *et al.*, 2005), and the subcutaneous injection of oncolytic adenoviruses in these animals showed no replication in target organs such as lung and liver (Wildner and Morris, 2002). In contrast, during the course of our experiments it has been described that the intratumoral administration of adenovirus in Syrian hamsters led to systemic dissemination and replication in these organs (Thomas *et al.*, 2006). Productive replication of a recombinant oncolytic adenovirus lacking most of the E3 region was demonstrated in malignized kidney cells (HaK), cells from a ductus deferens leyomiosarcoma (DDT1 MF-2) and from pancreatic cancer (PC1). Our results expand the list to other pancreatic cancer cell lines (HaP-T1 and H2T), suggesting that many other hamster cells may have

the same characteristic. A relatively lower virus burst size was observed in HaK cells in our study compared with the results reported by Thomas *et al*. They report 10 times less virus production relative to A549 cells, whereas we found a 100 fold reduction. This discrepancy may be due to the different viruses evaluated. We chose to study the replication of wild type human adenovirus 5 instead of a particular CRAd in order to acquire general knowledge that will not depend on specific genetic modifications. In fact, changes in the E3 region may have a profound impact on the replication and persistence of CRAds in vivo (Torres *et al.*, 1996; Wang *et al.*, 2003). We have studied different aspects of the viral cycle which may be relevant for the oncolytic effect of adenoviruses. Under our assay conditions we observed higher progeny production of Ad5 in HaP-T1 cells than in H2T or the previously described HaK cells. The virus burst size was lower than in the human cancer cells A549 and HuH7, but was definitely higher than in the murine cell line MC38, in which virtually no viral production was detected. Viral genomes and newly formed virions accumulated over time inside HaP-T1 and H2T cells, and viable particles could be detected in the supernatant of the cells, indicating the successful completion of the viral cycle. In addition, the spread of the virus was demonstrated in cellular monolayers by immunohistochemical detection of late gene expression, in contrast to the murine cells MC38. In agreement with its lower permissivity for replication, H2T was more resistant to the cytopathic effect of Ad5, despite having equivalent infection rate. Adenovirus cycle takes place in H2T cells, but it seems to be retarded in time and reduced in intensity. Surprisingly, when tumors were generated in athymic mice by subcutaneous inoculation of these cells, the amount of virus recovered several days after the intratumoral injection of Ad5 was similar for HaP-T1 and H2T tumors. The same was observed when the viral progeny was analyzed 4 days after the injection of Ad5 in liver metastases induced by intrahepatic injection of the hamster cells in immunocompetent recipients. Thus, the discrepancy between in vitro and in vivo data seems to be a general characteristic of the cells, and does not depend on the host. H2T-induced tumors are generally softer and easier to inject than HaP-T1 tumors, and necropsies performed in recipient animals confirmed that HaP-T1 cells induce more fibrosis. This characteristic may hamper the intratumoral distribution of the virus and explain a relatively low viral progeny, compared with the less permissive H2T cells. Another possible explanation is that the relatively attenuated cytopathic effect of adenovirus in H2T cells triggers lower innate antiviral responses in vivo and extends the chances of viral replication. In any case, both types of tumors

allowed the in vivo replication of Ad5 with an efficacy less than one order of magnitude lower than the highly permissive A549 tumors in nude mice. This property was maintained in the immunocompetent hamsters, whereas a replication-deficient vector showed virtually no viral progeny under the same conditions. Interestingly, the ability of Ad5 to replicate in hamster cells is not limited to cancer cell lines, since this was recently observed in primary cells from normal liver and to a lesser extend lung (Jogler *et al.*, 2006). The same study pointed out that swine is the most permissive animal among all species studied (mouse, cotton rat, rabbit, guinea pig, woodchuck and hamster), and this should be taken into account for the development of more advanced animal models in the future. Nevertheless, the hamster combines an acceptable permissivity for adenovirus with other advantages like easy handling and the availability of cell lines from different cancers. Some carcinogen-induced pancreatic cell lines like the ones used in this study are especially relevant because they reproduce many genetic abnormalities found in the human disease, like mutations in the K-ras oncogene (Erill *et al.*, 1996) and inactivation of tumor suppressor genes (Muscarella *et al.*, 2001). In addition, they are able to recapitulate the natural history of the human disease in the animal, with infiltrative growth and ability to produce distant metastases (Morioka *et al.*, 2000a; Morioka *et al.*, 2000b; Morioka *et al.*, 2000c). We have characterized the growth patterns of HaP-T1 and H2T cells after subcutaneous or intrahepatic injection in hamsters. In both conditions, HaP-T1 grew rapidly, and in the case of liver metastases the animals had to be sacrificed one month after implantation for ethical reasons. H2T tumors grew more slowly but they had the same invasive and metastatic ability. In fact, the slower progression of the intrahepatic tumor allows extended survival of the animals and facilitates the appearance of distant metastases in the lungs. Therefore, this model may be more suitable for the evaluation of experimental treatments that are active through long periods of time. In summary, the Syrian hamster is an interesting platform for the development of cancer models in which oncolytic adenoviruses can be tested.

AKNOWLEDGEMENTS

We thank Dr. CM Townsled (University of Texas Medical Branch, USA) for the H2T cell line, and Dr. Brechot (Inserm, Paris, France) for the HuH-7 cells. This work was supported by UTE-Proyecto CIMA and grant SAF-2003-08385 from the Spanish Department of Science and Technology. RHA is a recipient of a Ramon y Cajal program from the Spanish Department of Science and Technology.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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