

Ewing Sarcoma Family of Tumors Express Adenovirus Receptors and Are Susceptible to Adenovirus-Mediated Oncolysis

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Purpose: Attenuated viruses derived from adenoviruses (Ad) that kill tumor cells (oncolysis) are currently in clinical trials for selected cancers. Some cancers have proven resistant to Ad infection due to low expression of viral receptors. The authors sought to determine whether members of the Ewing sarcoma family of tumors (ESFTs) express Ad receptors and are sensitive to Ad-mediated oncolysis.

Methods: Using flow cytometry, the authors tested a panel of cell lines derived from ESFTs for expression of both the Ad receptor, coxsackie-adenovirus receptor (CAR), and the cellular mediator of Ad uptake, α_v -integrins, as well as for Ad-mediated gene transduction. Cell survival assays were used to assess the sensitivity to Ad-mediated oncolysis. Immunohistochemistry was used to assess CAR expression in primary tumors. mRNA levels of CAR in cell lines and tumor samples were also queried from a cDNA expression database.

Results: The ESFT cell lines expressed CAR and α_v -integrins, showed high levels of gene transduction, and were highly sensitive to viral oncolysis. Primary tumor samples were positive for CAR expression by immunohistochemistry. Microarray analysis confirmed CAR expression in ESFT cell lines and tumors.

Conclusions: Ewing sarcoma cells express the Ad receptors and are sensitive to Ad oncolysis. Treatment of Ewing sarcoma using conditionally replicative adenoviruses should be explored.

Key Words: Adenovirus—Ewing sarcoma—Gene therapy—Oncolysis—Osteosarcoma—Rhabdomyosarcoma.

Significant progress has been made in the treatment of the localized Ewing sarcoma family of tumors (ESFTs) during the past three decades through the use of multimodal therapy including chemotherapy, surgery, and radiation.

Unfortunately, some tumors are not amenable or are refractory to these therapies. In addition, current approaches have had little impact on the survival of patients with metastatic ESFTs. Our most recent hope, megadose chemotherapy with autologous stem cell support, has been disappointing (1). Exploration of novel therapies for ESFTs is therefore warranted.

Lytic viral infection of tumor cells (oncolysis) is being explored as a method of cancer therapy (2). Potential advantages of using viruses to kill tumor cells include their potency, their ability to spread from cell to cell, and most importantly the opportunity to molecularly engineer viruses to affect only selected cell types. Some viruses, such as Newcastle disease virus and reovirus, appear to have a natural predilection for tumor cells compared with normal cells (3,4). Most oncolytic viruses being explored as therapy for cancer, commonly derived from either herpes simplex virus type 1 (HSV-1) or adenovirus, have been genetically engineered to be selective for cancer cells (2). The primary methods of creating cancer-selective viruses have been either to mutate critical viral replication genes that are complemented by genes preferentially active in cancer cells (e.g., metabolic genes) or to place vital viral genes under the control of gene regulatory sequences preferentially active in cancer cells (e.g., prostate-specific antigen promoter) (5). Such targeted therapy would represent a new option in patients with disease that is refractory to chemotherapy or not amenable to traditional surgery and radiation, and could avert the short-term and long-term side effects that result from these traditional treatments.

In a study of pediatric sarcoma cells exposed to conditionally replicative HSV-1 viruses, we have shown that cells derived from rhabdomyosarcoma, osteosarcoma, and malignant fibrous histiocytoma were sensitive to HSV-mediated oncolysis (6). Curiously, however, cells derived from four different ESFTs were relatively resistant to oncolytic HSVs. The resistance was not due to poor viral entry, as demonstrated by robust gene transduction, but was likely due to the inability of the cells to metabolically support replication of attenuated HSV-1-derived viruses. We therefore sought to determine whether ESFT cells are sensitive to oncolytic adenovirus (Ad) infection as an alternative.

The oncolytic Ad perhaps best studied is the E1B-defective Ad strain dl1520 (ONYX-015), which is impaired

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in its ability to replicate in normal human cells (7,8). ONYX-015 replicates in and kills a number of different human tumor cells and has shown a safe clinical profile in human trials (9). A number of other conditionally replicative Ads (CRAds) are in various stages of development (10,11). Importantly, CRAds have been shown to be capable of causing inhibition of subcutaneous tumor growth at distant sites after intravenous administration (12,13). Addition of cytotoxic genes to the genomes of replicating viruses can further enhance the oncolytic potency of CRAds (14,15).

The Ad cell host range is wide because the major Ad receptor, coxsackie-adenovirus receptor (CAR), is expressed in a variety of human tissues (16). The Ad fiber protein knob, which projects outward from the virion vertices, binds CAR as the first step in viral infection. Adenovirus internalization is mediated by binding of the fiber penton base to cellular $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins (17,18). Despite the prevalence of CAR, many cell types are poorly transduced by Ad. Low or absent CAR expression has been shown to limit the utility of Ad gene transfer and oncolysis for a number of cancers, including bladder cancer, prostate cancer, ovarian cancer, and rhabdomyosarcoma (19–22). Variations in clinical response of head and neck cancers after intratumoral injection of ONYX-015 are thought to be explained by variable CAR expression (23). Therefore, an important first step in the development of a CRAd treatment strategy for Ewing sarcoma is to determine the Ad receptor expression and oncolysis sensitivity of ESFT cells.

METHODS

Cells and viruses

Cell lines and culture conditions have been previously described (6,22). RD-LXSN was derived from the RD rhabdomyosarcoma cell line by stable transduction with a control retrovirus as described (22). RD-LXSN was previously shown to lack CAR expression and to be resistant to Ad oncolysis (22). Ramos (Burkitt lymphoma), HeLa (cervical carcinoma), and the ESFT cell line A673 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Although A673 is catalogued by ATCC as a rhabdomyosarcoma, it was derived from a human peripheral neuroepithelioma (24), and the presence of the EWS/FLI1 fusion protein (25) confirmed it to be a member of the ESFTs. The ESFT lines RD-ES and SK-ES, originally from ATCC, were kindly provided by Jeffrey Toretzky (University of Maryland, Baltimore, MD, U.S.A.). The previously characterized ESFT lines 5838 (26) and TC32 were also provided by Dr. Toretzky, and their identities were confirmed by sequence analysis of their EWS/ERG and EWS/FLI1 fusion proteins, respectively. A549 was derived from lung adenocarcinoma and was kindly provided by Richard Anderson (University of Iowa, Iowa City, IA, U.S.A.). An E1-deleted, replication-defective Ad express-

ing the firefly luciferase gene under control of the cytomegalovirus immediate-early promoter, AdLux (27), and a replication-competent adenovirus type 5, Adsub360 (Adwt), were purchased from the University of Iowa Gene Transfer Vector Core (Iowa City, IA, U.S.A.). Viral particle titers were determined by absorbance at 260 nm (28) and plaque-forming units by standard assays on HEK293 cells. The Adwt stock had 1×10^{12} virus particles/mL and 3×10^{10} plaque-forming units/mL.

Flow cytometry

For production of mouse monoclonal anti-CAR (RmcB) antibody, athymic nude mice were injected intraperitoneally with 0.3 mL 2,6,10,14-tetramethyl pentadecane (Sigma, St. Louis, MO, U.S.A.). Fourteen days later, mice were injected intraperitoneally with 5.0×10^6 RmcB hybridoma cells (ATCC). Three to 4 weeks after hybridoma injection, ascites fluid was harvested, pooled, and incubated at room temperature for 1 hour to allow clot formation. The clot was removed by centrifugation at 1100 g for 30 minutes at room temperature, and supernatant was stored at -70°C . To assess CAR expression in EWS cell lines, 1.0×10^6 cells in 500 μL phosphate-buffered saline were incubated with anti-CAR monoclonal antibody (RmcB) or mouse IgG1 isotype control (Pharmingen, San Diego, CA, U.S.A.) at 4°C for 30 minutes, after which the cells were washed with 2 mL phosphate-buffered saline. The cells were pelleted at $400 \times g$ for 7 minutes, resuspended in 500 μL phosphate-buffered saline, and incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG1 (Pharmingen). After incubation at 4°C for 30 minutes, the cells were washed and pelleted as described above, resuspended in 500 μL phosphate-buffered saline containing 1% paraformaldehyde, and analyzed with a FACSCalibur analyzer (BD BioSciences, San Jose, CA, U.S.A.). To assess α_v integrin expression in EWS cell lines, 1.0×10^6 cells in 500 μL phosphate-buffered saline were incubated with FITC-conjugated anti-CD51 monoclonal antibody (Immunotech, Marseille, France) or FITC-conjugated rat IgG1 isotype control (Pharmingen). After incubation at 4°C for 30 minutes, the cells were washed and pelleted as described above, resuspended in 500 μL phosphate-buffered saline containing 1% paraformaldehyde, and analyzed as above.

Adenovirus gene transduction assay

Cells were plated at 3.0×10^5 cells/well in six-well dishes. The following day, medium was removed and 1 mL infection medium (plain medium supplemented with 2% fetal bovine serum) containing serial dilutions of AdLux was added. After a 90-minute incubation, 2 mL complete medium (plain medium supplemented with 15% fetal bovine serum) was added. Cells were harvested in cell lysis buffer (Tropix, Bedford, MA, U.S.A.) after 48 hours. Assays for luciferase activity were performed using the Luciferase Assay Kit (Tropix) according to the manufacturer's instructions on a Luminoskan Ascent microplate luminom-

eter (Labsystems, Helsinki, Finland). Data in all experiments represent the mean of three samples for each point. Protein concentrations were measured using the detergent-compatible Micro BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.).

Cell survival assay

Cells were plated in 96-well dishes at 4,000/well. After 24 hours, serial dilutions of Adwt were added to the medium. At the indicated days after infection, cell survival assays were performed using the CellTiter96 assay (Promega, Madison, WI, U.S.A.) per the manufacturer's instructions. Individual points were done in quadruplicate.

Immunohistochemistry

Clinical samples of ESFTs were identified from the archives of the Department of Pathology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, U.S.A. Samples were analyzed anonymously as approved by the Cincinnati Children's Hospital Medical Center Institutional Review Board. Sections stained with hematoxylin and eosin were reviewed as well as immunohistochemistry stains (e.g., CD99) to confirm the diagnosis of ESFT. In two patients, reverse transcription polymerase chain reaction was

used to confirm the presence of an ESFT-associated fusion protein transcript. Immunohistochemistry for CAR was performed as previously described (22), except that the antibody was diluted 1:4.

Microarray analysis

The relative expression levels of CAR, in 10 ESFT-derived cell lines and 13 tumor tissues, were extracted from a small round blue cell tumor gene expression database generated as previously described (29).

RESULTS

Expression of Adenovirus Receptors on Ewing Sarcoma Cells

Previous studies have shown that lack of CAR expression markedly hinders cell killing by CRADs for several cancers (19–22). Therefore, CAR expression appears to be an important predictor of Ad oncolytic efficacy. Here we used flow cytometry with an anti-CAR antibody (RmCB) to determine CAR expression on ESFT-derived cell lines. The A549 cell line, derived from a lung adenocarcinoma and previously shown to express high levels of CAR (22), was used as a positive control. The RD-LXSN cell line, previ-

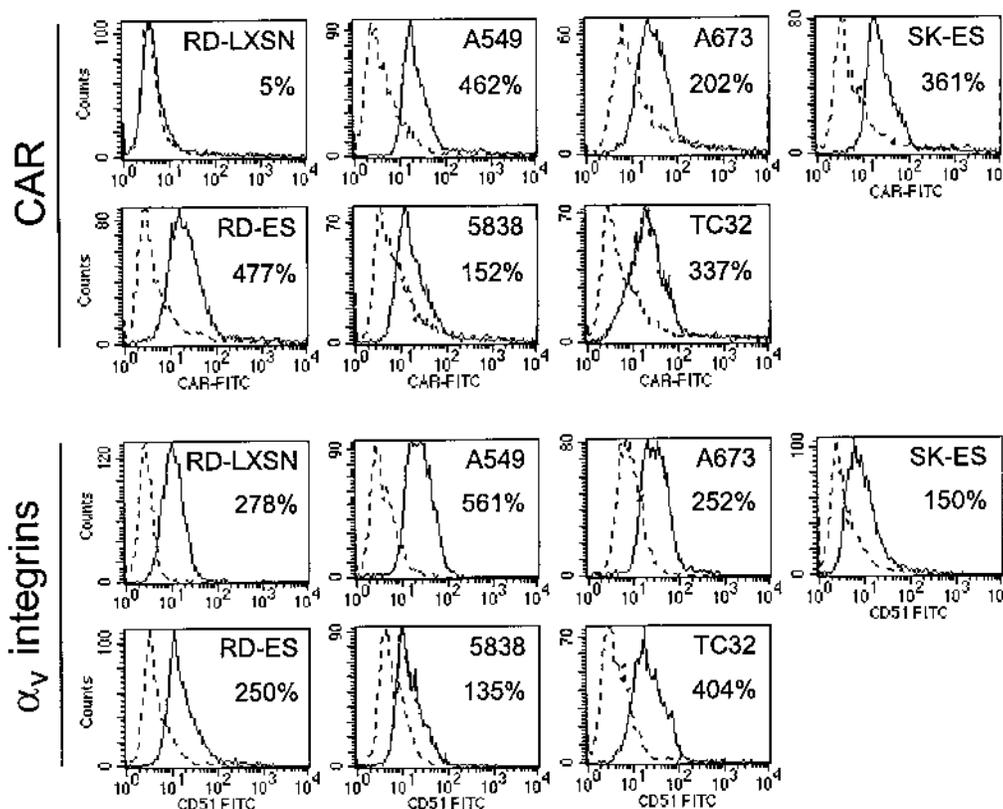


FIG. 1. Analysis of expression of the adenovirus cellular receptors on Ewing sarcoma family of tumors cells. Cells were stained with anti-coxsackie-adenovirus receptor antibody (RmCB antibody, *bold lines*) or anti- α_v -integrin antibody (anti-CD51, *bold lines*) and analyzed by flow cytometry. A549 was a positive control cell line for both, and the rhabdomyosarcoma-derived cell line RD-LXSN was a negative control for coxsackie-adenovirus receptor. The *dotted curve* (left) in each panel is the isotype control. The percentage is the change in median fluorescence relative to the isotype control.

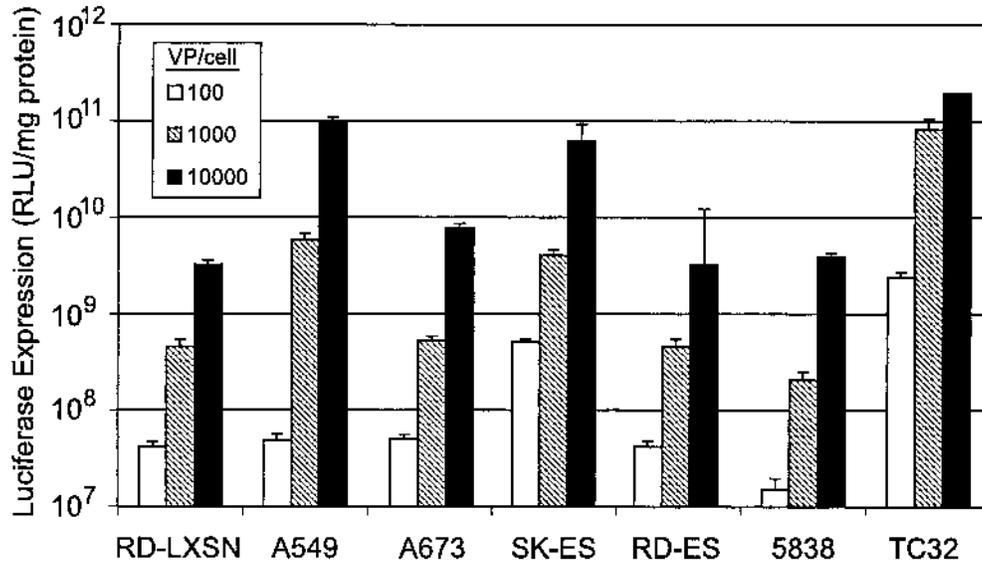


FIG. 2. Susceptibility of Ewing sarcoma family of tumors cells to adenoviral gene transduction. Cells were transduced with AdLux expressing the luciferase gene at three different concentrations as indicated (VP, virus particles). Luciferase expression was determined at 48 hours after infection and normalized to protein concentration. Bars indicate the average of triplicate points; standard deviations are indicated.

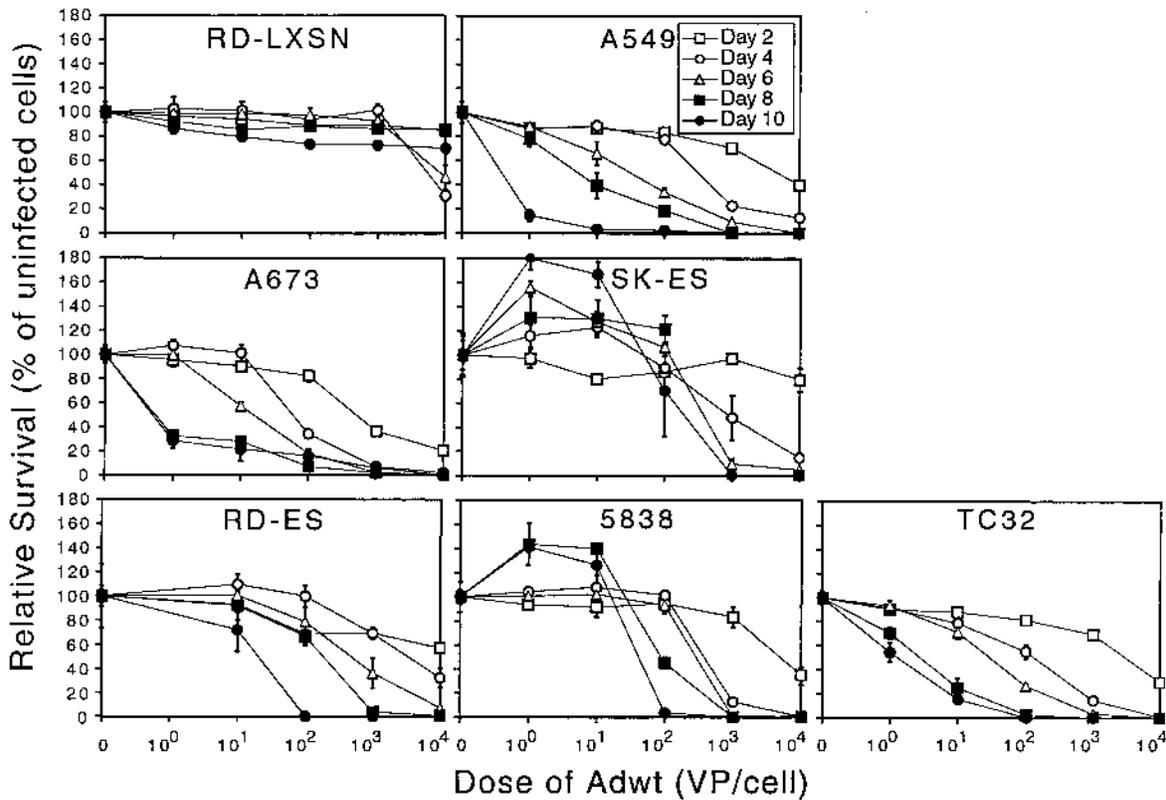


FIG. 3. Oncolysis of Ewing sarcoma cells by wild-type adenovirus. Cells were transduced with adenovirus type 5, Adsub360 (Adwt), and followed for survival. Results are shown as a percentage of viable uninfected cells. Dose-response curves are shown for the different days indicated, except with RD-ES, which were done on days 3, 4, 5, 6, and 10, respectively. RD-LXSN is a rhabdomyosarcoma line resistant to adenovirus oncolysis for comparison. Values represent the mean of four samples for each point. Standard deviations are shown, but many are obscured by the data symbols. A repeat experiment gave similar results.

ously shown to express only low levels of CAR (22), was used as a negative control. All of the ESFT cell lines expressed CAR (Fig. 1). The percentage shift in the median fluorescence of the cell population with RmcB compared with the isotype control was used to quantitate relative CAR expression. Expression of CAR was similar on RD-ES and A549 cells (477% vs. 462% shift in median fluorescence). It was also highly expressed on SK-ES (361%) and TC32 (337%) cells, and less highly expressed on A673 (202%) and 5838 (152%) cells.

We also analyzed the expression of the Ad coreceptors, α_v -integrins, on the ESFT-derived cell lines by flow cytometry using the anti-CD51 antibody. Again, A549 was used as a positive control, and the cell line Ramos, previously shown to lack α_v -integrin expression (22), was used as a negative control (not shown). All ESFT-derived cells expressed cell surface α_v -integrins (see Fig. 1). A549 cells had the highest expression (561% shift in median fluorescence), with TC32 also showing robust expression (404%). Expression on A673 (252%) and RD-ES (250%) was greater than expression on SK-ES (150%) and 5838 (135%).

Adenovirus transduction of Ewing sarcoma cells

To assess the relative susceptibility of ESFT-derived cells to Ad transduction and gene transfer, we used a virus expressing the firefly luciferase gene under the control of the strong promoter from the immediate-early gene of cytomegalovirus (AdLux). Luciferase expression was quantitated by enzymatic assay (Fig. 2). RD-LXSN cells were included as a CAR-negative cell line, and A549 cells were again used as a positive control. Different virus loads spanning three logs were tested. Luciferase expression was highest in TC32 cells (greater than A549). Expression in SK-ES cells was similar to A549 and ~1 log less in the other four

cell lines (RD-LXSN, A673, RD-ES, 5838). These data suggest that ESFT-derived cell lines are readily but variably transducible by human Ad.

Sensitivity of Ewing sarcoma cells to adenovirus oncolysis

We have previously shown that rhabdomyosarcoma cell lines with low CAR expression were relatively resistant to Ad-mediated oncolysis (22). Because the ESFT cells expressed robust levels of both CAR and the α_v -integrins, we predicted they would be sensitive to oncolysis by Ad.

Cell lines derived from ESFTs were exposed to various levels of wild-type Ad and followed for survival (Fig. 3). The CAR-negative rhabdomyosarcoma cell line, RD-LXSN, was again included for comparison, and A549 cells served as a positive control. All of the ESFT lines were sensitive to Ad oncolysis. A complete cytopathic effect was achieved in all cultures by day 10 with 1,000 viral particles/cell (equivalent to a multiplicity of infection of 30) and in several cells with 100 viral particles/cell (multiplicity of infection 3; RD-ES, TC32, and nearly in A673 and 5838). The dose required to kill 50% of the cells (IC_{50}) extrapolated from these curves at day 6 indicated that A673 and TC32 were the most sensitive (~10 and ~20 viral particles/cell, or multiplicity of infection 0.3 and 0.6, respectively). The other cell lines were moderately sensitive, with IC_{50} values for RD-ES of ~700 and for both SK-ES and 5838 of ~300 viral particles/cell (multiplicity of infection 21 and 9, respectively). The E1-deleted, nonreplicative Ad (AdlacZ) had no effect on survival of these cell lines (data not shown).

Coxsackie-Adenovirus Receptor Immunohistochemistry in Primary Human Ewing Sarcoma Family of Tumors

The expression of CAR has been shown to correlate with Ad gene transfer and oncolysis for a variety of cancers (22,23,30). To determine whether primary ESFTs express CAR and might therefore be susceptible to Ad-mediated oncolysis, we performed immunohistochemistry using the anti-CAR antibody (RmcB) on archived specimens from seven ESFTs in six patients (in one patient, both the primary biopsy sample and the bone marrow metastasis were evaluated). Four of the ESFT samples were from primary diagnostic biopsy samples, two were from biopsy samples of metastatic disease, and one was from a biopsy sample of a recurrent lesion. Adenoidal epithelium was used as a positive control. All samples stained positively for CAR (Fig. 4). Five samples stained strongly (4+) and diffusely, and two had more focal, intermediate staining (2+). All showed membrane staining and six of the seven also showed cytoplasmic staining; in two, the membrane staining was accentuated relative to cytoplasmic staining.

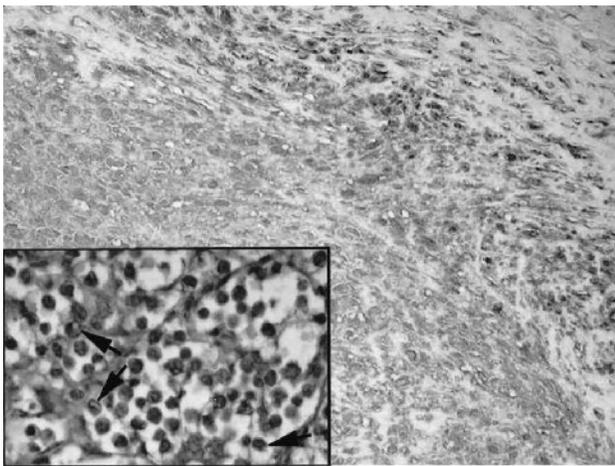


FIG. 4. Immunohistochemical staining for coxsackie-adenovirus receptor in primary Ewing sarcoma family of tumors. In this example, most sarcoma cells in the main mass (lower left) and at the interface with adjacent collagen (upper right) stained with anti-coxsackie-adenovirus receptor antibody. The inset (lower left) demonstrates membrane accentuation (arrows) found in some samples.

Microarray Analysis of Coxsackie-Adenovirus Receptor Expression in Ewing Sarcoma Family of Tumor Cell Lines and Tumors

We wished to confirm the expression of CAR in ESFTs using an additional method besides immunohistochemistry. Microarray technology has enabled the simultaneous analysis of the expression of many genes in a given tissue and has been performed on a panel of childhood sarcomas as part of the small round blue cell tumor database (29). On query of the small round blue cell tumor microarray expression database, CAR expression was found to be positive (arbitrarily defined as >0.1 relative red index, with the average of all expressed genes normalized to an index of 1) in 10 of 11 (91%) ESFT cell lines and in 25 of 28 (89%) ESFT tumors in the microarray.

DISCUSSION

Our data show that cell lines derived from ESFTs express the attachment and internalization receptors for Ad, are readily transduced by Ad, and are susceptible to oncolysis by replication-competent Ad. We also found that primary ESFTs express the major Ad attachment receptor. Because clinical response to oncolytic Ad is thought to correlate with CAR expression, these results suggest that conditionally replicative Ads may be efficacious for the treatment of ESFTs.

For some of the cell lines, there was a relationship between Ad receptor expression, gene transduction, and oncolysis, but the correlation was not absolute. The three cell lines most susceptible to Ad gene transduction (TC32, A549, SK-ES) also had the highest CAR expression. Two of these (TC32, A549) were the most sensitive to oncolysis, but SK-ES was among the least sensitive (along with 5838). A673 showed moderate expression of CAR and was among the least susceptible to transduction but was highly sensitive to oncolysis (equivalent to TC32). 5838 showed the lowest CAR and the lowest transduction and tied for the lowest oncolysis sensitivity. As previously shown, the CAR-negative rhabdomyosarcoma cells (RD-LXSN) were essentially resistant to oncolysis. None of the differences could be accounted for by differences in integrin expression. Some of the lack of correlation of transduction with receptor expression and sensitivity might be accounted for by differences in promoter activity in different cell lines. Even so, although our findings suggest that ESFTs are susceptible to Ad infection and oncolysis, none of the parameters measured was absolutely predictive of the degree of sensitivity.

We have recently discovered that most sarcomas are killed by oncolytic viruses derived from HSV-1 (6). The ESFT cells, however, were relatively resistant to HSV-1-mediated oncolysis (presumably because the HSV-1 neurovirulence gene, ICP34.5, is deleted in these viruses but is required for virus growth in some neuro-derived cells). Our

data suggest that Ad-based oncolytic strategies will be more useful for ESFT than HSV-based treatments.

Clinical trials with ONYX-015 suggest that CRAd vectors can be safely administered intratumorally, intra-arterially, and intravenously (31). Although ONYX-015 has not yet been shown to have clear efficacy as a single agent, it appeared to enhance the antitumor effect of chemotherapy (32). The low CAR expression by tumor cells in these trials may have contributed to low clinical response rates (23). Genetic modifications of virion proteins have shown to enhance oncolysis by facilitating virus-cell binding (21,22, 33-35) and may be one strategy to increase clinical effectiveness. Increased efficacy with replicative Ads could also be realized by choosing appropriate, sensitive tumor types for therapy. Our data suggest that ESFTs may be suitable clinical targets for Ad-mediated oncolytic therapy.

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