A Midkine Promoter-based Conditionally Replicative Adenovirus for Treatment of Pediatric Solid Tumors and Bone Marrow Tumor Purgings

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ABSTRACT

The treatment of advanced neuroblastoma (NB) or Ewing’s sarcoma (ES) is one of the major challenges in pediatric oncology. Both malignancies are refractory to conventional therapies and have an extremely poor prognosis. High-dose myeloablative radiochemotherapy has auto-

tologous bone marrow or peripheral blood stem cell rescue is one of the most aggressive treatments attempted for these diseases but is often undermined by residual tumor cells contaminating the graft. Thus, in this approach, purging of tumor cells from the graft is key to the prevention of relapse after transplantation. We investigated a novel approach to eliminate tumor cells from the bone marrow or peripheral blood stem cell graft without causing stem cell damage through the use of a conditionally replicative adenovirus (Ad). ES and NB are sensitive to Ad infection, and advanced NBs express a high level of the growth/ differentiation factor midkine (MK). We confirmed in this study that ES cell lines (SK-ES-1 and RD-ES) are also sensitive to Ad infection and express high levels of MK. In contrast, CD34+ stem cells are refractory to Ad infection and express very little MK. A conditionally replicative Ad in which the expression of E1 is controlled by the MK promoter achieved good levels of viral replication in NB or ES and induced remarkable tumor cell killing. On the other hand, this virus caused no damage to CD34+ cells even after 3 h of infection at a dose of 1000 multiplicity of infection. We concluded that application of this replication-competent Ad to hematopoietic grafts could be a simple but effective procedure to achieve complete tumor cell purging.

INTRODUCTION

The development of CRAds designed to replicate exclusively in tumor cells has the potential to significantly advance the treatment of malignancy (1, 2). Two basic strategies have been used, each of which involves alterations in the expression of genes essential for Ad replication. The first approach takes advantage of the disordered cell cycle regulation in tumor cells. This dysregulation means that certain viral genes, which have cell cycle regulatory functions, become dispensable in tumor cells, for example the genes responsible for activating the cell cycle by blocking Rb protein or p53. Thus, these genes have been completely or partially deleted with the aim of rendering the viruses incapable of replicating in normal cells (3). AdΔ24 or dl1520 (ONY-XX-015; Refs. 4, 5) are examples of this approach. The second strategy is the replacement of Ad promoters with tumor-specific promoters to drive the expression of genes essential for Ad replication. CRAds controlled by α-fetoprotein (6) or prostate-specific antigen promoters (7) have already been applied to the treatment of hepatomas or prostate cancers, respectively.

MK is a heparin-binding growth factor identified as a product of a retinoic acid-responsive gene (8, 9). The effects of MK include a neurotrophic function, mitogenic activity in fibroblasts (10), antiangiogenic activity (11), migration-promoting activity (12), angiogenic transformation of fibroblasts (13). Of note, a close correlation between MK overexpression and tumorigenesis has been reported (16). The high expression level of MK observed in Wilms’ tumors has lead to the recognition that MK is one of the target genes for the Wilms’ tumor suppressor gene (WT1; Ref. 17). Furthermore, it has been reported that many kinds of malignant tumors express a high level of MK, for example, NBs (18), bladder carcinomas (19), lung cancers (20), breast cancers (21), esophageal cancers (23), and gastrointestinal cancers (24, 25). In the case of NB, Nakagawara et al. (18) reported that the MK is overexpressed in primary tumors as well as in cell lines, and strong expression of MK is correlated with poor prognosis. On the other hand, MK status in ES is still understudied. However, MK expression is not observed in mouse or human liver (10, 16), which is important in view of natural tropism of Ad for the normal liver. We established previously that the MK promoter could be used in an Ad vector where it retained the desirable features of high tumor activity and minimal activity in the liver, which enabled the mitigation of hepatic toxicity in a suicide gene approach to cancer therapy (26).

The treatment of advanced NB or ES is one of the major challenges in pediatric oncology. Because both of these pediatric malignancies are refractory to conventional therapy and have an extremely poor prognosis, high dose myeloablative radiochemotherapy with autologous stem cell transplantation has been attempted (27–30). However, tumor cell contamination of the autologous hematopoietic graft is a significant clinical problem leading to recurrence after transplantation (31, 32). Although peripheral blood stem cell transplantation or immunomagnetic CD34+ selection (33, 34) has been used to deplete tumor cells, additional improvements in purging strategies are needed. Recently, investigators reported attempts to improve the efficacy of tumor cells purging in the case of breast cancers (35, 36) or multiple myelomas (37) through the use of replication-defective Ad vectors in a suicide gene approach or p53 antioncogene delivery. The rationale of using Ad vectors for the purging is that Ad shows high infection efficiency to tumor cells and low infectivity to hematopoietic cells. However, it is still questionable as to whether these anticancer strategies can transduce therapeutic genes to NB or ES cells with sufficient efficacy for an optimal therapeutic effect. This limitation is of particular importance in these tumor types, which have less cell-to-cell contact (and therefore less bystander effect; Ref. 38) and less abnormality of p53 (39, 40) compared with adult cancers. Thus, we hypothesized that a CRAd might overcome these limitations and deplete contaminating tumor cells more completely. Therefore, we investi-
gated the application of a MK promoter-based, replication-competent Ad for the treatment of these MK-positive tumors using a model system relevant to the consideration of stem cell purging for advanced NB or ES. First, we evaluated the MK expression level of the target tumors or CD34+ hematopoietic stem cells, then, based on these results, we assessed the efficacy of a CRAd in which expression of the essential E1 gene is driven by the MK promoter. Thus, this strategy effectively takes advantage of natural Ad tropism plus transcriptional control to achieve a highly synergistic improvement in the specificity of target cell killing.

**MATERIALS AND METHODS**

**Cells and Cell Culture.** The Wilms tumor G-401, NBY SK-N-SH, ES SK-ES-1 and the American Type Culture Collection (Manassas, VA) and cultured in the medium recommended by the manufacturer. The melanoma MeWo cell line was a kind gift of Prof. Ian. R. Hart (St. Thomas Hospital, London, United Kingdom) and maintained in DMEM with 10% FCS. The 911 cell line (41), an E1 transcomplementing helper cell line that allows for the replication of E1-deleted Ad vectors, was obtained from Dr. Alex J. van der Eb, University of Leiden, The Netherlands. These cells were maintained in DMEM with 10% FCS and used for initial virus generation and propagation. All of the medium and FCS used in this study was purchased from Mediatech/Celgro (Herndon, VA).

**RNA Preparation and RT-PCR.** Total cellular RNA of tumor cells or CD34+ cells was extracted from 103 or 2 × 106 cells, respectively, using an RNeasy kit (Qiagen). GeneAmp RNA PCR core kit (Applied Biosystems) was available for cDNA synthesis and PCR amplification of cDNA products. Oligonucleotides corresponding to the sense strand of human MK cDNA (5'-ATGCCGGACCGAGCTTCTCC-3': 1–20), the antisense strand of MK cDNA (5'-ATGCCGCTTGCCCCTGATG-3': 450–428; 22), in the sense strand of human glyceraldehyde-3-phosphate dehydrogenase cDNA (5'-TCATCACCACTCCTCA-3': 276–293), and in the antisense strand of glyceraldehyde-3-phosphate dehydrogenase cDNA (5'-CATCACGCCCAGATT-3': 638–655; 22, 42) were synthesized and used as primers for PCR. The PCR primers for MK transcript detection can discriminate MK transcripts from the genomic DNA. Total RNA (500 ng) of tumor cells or 50 ng of RNA of CD34+ cells were applied to a standard RT-PCR protocol. The PCR conditions were as follows: 20 cycles of denaturation (95°C, 1 min), annealing (58°C, 1 min), and extension (72°C, 1 min), for tumor samples or 27 cycles of the same PCR profile for CD34+ cell samples. PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide staining.

**Viruses and Viral Techniques.** The replication-competent Ad AdMKE1, including the adenoviral E1A region under the control of human MK promoter containing 27 bp of exon 1 and 2285 bp of the 5' flanking region of the human MK gene (43), was constructed using the "AdEasy" method (44) with incorporation of E1. The MK promoter we chose was too long to insert into an Ad genome without deletion of the adenoviral E3 region. Therefore, we constructed AdMKE1 with an E3 deletion by using the plasmid pShuttle as the backbone stimulating factor, and 10 ng/ml rh granulocyte macrophage colony-stimulating factor, and 10 ng/ml rh interleukin-6 (IL-6); StemCell Technologies Inc.). We counted hematopoietic colonies with >20 cells after 8 days of culture.

**RESULTS**

NB and ES Express High Level of MK. We wished to develop a strategy for the therapy of MK-positive pediatric tumors based on the use of a CRAd in which the MK promoter controls the expression of E1 (Fig. 1). In vitro, we first investigated a panel of tumor lines for MK expression. Candidate lines were investigated using an RT-PCR method as reported previously. We chose the G-401 and Daudi cells as positive and negative controls, respectively, as well as the MeWo melanoma line as a candidate Ad-infectable but MK-negative line. G-401, SK-N-SH, and two ES cell lines showed a strong PCR band of 450 bp corresponding to the MK protein. On the other hand, the band

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obtained from the MeWo line was barely detectable (Fig. 2A). On the basis of this result, we proceeded with additional evaluation using G-401, SK-N-SH, SK-ES-1, and RE-ES as MK-positive tumor lines and MeWo as a MK-negative tumor line.

**MK Promoter Retains Its Fidelity in the Ad Context.** In the next step we assessed the activity of the MK promoter driving the expression of the luciferase reporter gene in an Ad vector (AdMKLuc) in the various cell lines (Fig. 2B). The data of G-401 and SK-N-SH are consistent with our previous report (26). Daudi cells are omitted because of their refractoriness to Ad infection. In all of the lines, luciferase expression was achieved using the positive control AdCMVLuc vector. In the MK-positive lines, the luciferase activity induced by AdMKLuc showed comparatively high activity, especially in ES cell lines. Even in the SK-N-SH line, which has the lowest MK/CMV ratio of the MK-positive lines, the luciferase activity induced by AdMKLuc was 12% of that induced by AdCMVLuc. In contrast, the MK/CMV ratio of MeWo was 1.5%. Thus, these data confirm that the MK promoter retains its fidelity in the Ad vector.

**MK Promoter Driving CRAd Shows Replication Specificity in NB and ES.** To exploit the cell specificity of the MK promoter in a CRAd context, we then constructed a recombinant Ad (AdMKE1) in which the native E1 promoter is replaced with the MK promoter. The genomic structures of replication-competent Ads used in this study are depicted in Fig. 1. In addition to using the MK promoter to control E1 expression we also constructed a control vector (AdCMVE1) in which E1 expression is controlled by the CMV promoter. These viruses are deleted in the E3 region extending from 28130 to 30820 Ad5 nucleotides and the E1A promoter region extending from 342 to 488 Ad5 nucleotides. The deleted E1A promoter region, containing native E1A TATA box, was replaced with either the MK promoter or CMV enhancer/promoter to produce the viruses AdMKE1 or AdCMVE1, respectively.

To determine the specificity of replication of the AdMKE1, we infected the panel of MK-positive and negative lines and then used quantitative real-time PCR to determine the level of amplification of viral DNA. The nonreplicative AdMKLuc vector and wild-type Ad5 virus were used as negative and positive controls, respectively. We found that the rate of increase in DNA copy number for the AdMKE1 virus was higher than that of Adwt in every MK-positive cell line. In contrast, the copy number of AdMKE1 was 10 times less than that of Adwt in MeWo MK-negative cells (Fig. 3). In addition, the replication of AdMKE1 was relatively much less than the replication of a control-replicative Ad (AdCMVE1) in the MK-negative MeWo line than in the MK-positive lines. These results indicate that the MK promoter retains fidelity even in the replication-competent Ad.

**Specific Cell Killing Efficacy of MK Promoter Driving CRAd.** We next investigated the ability of AdMKE1 to achieve cell killing in the MK-positive lines using the MK-positive lines per se. However, despite this caveat, the profiles of the viral dose-response curves allowed assessment of the effect of replication-competent Ad infection on cell viability, and as an additional validation, studies were conducted in which cell number was determined by trypan blue exclusion and cell counting, which gave similar results (data not shown). For all of the MK positive lines, AdMKE1 had much better

![Fig. 1. Schema of the CRAd vectors used in this study. These vectors are constructed from E3 region-deleted Ad5 backbone and do not contain the Ad E1A promoter region expanding from nucleotide 324 to 488 of the Ad genome. Deletion of the E3 region was necessary, because the MK promoter we chose was too long to insert into the Ad genome without deletion of adenoviral E3 region. AdCMVE1 and AdMKE1 differ in the promoter driving E1A expression. Only AdMKLuc is the nonreplicating Ad control. We also use Adwt as a control.](image1)

![Fig. 2. A, evaluation of MK expression of the tumor cells. Lane 1, G-401 (Wilms tumor); Lane 2, SK-N-SH (NB); Lane 3, SK-ES-1 (ES); Lane 4, RD-ES (ES); Lane 5, MeWo (melanoma); Lane 6, Daudi cells (lymphoma). G-401 and Daudi cells are reported (26). Bars, SE. B, luciferase expression in cell lines. Infection of AdMKLuc or AdCMVLuc at a MOI of 50. The ratios of luciferase activity induced by AdMKLuc compared with AdCMVLuc (MK:CMV) are as follows: G-401, 0.35; SK-N-SH, 0.12; SK-ES-1, 0.75; RD-ES, 0.95; and MeWo, 0.015; bars, ± SE.](image2)

![Fig. 3. Assessment of viral DNA replication 24 h after infection. Because all of the vectors tested here contained complete E4 region, the viral DNA replication rate was evaluated by measuring the E4 copy number using the quantitative real-time PCR method. The nonreplicative AdMKLuc vector and Adwt virus were used as negative and positive controls, respectively; bars, ± SE.](image3)
cell-killing efficacy than the MK-negative MeWo line (Fig. 4). Furthermore, in ES cell lines, the effectiveness of AdMKE1 was comparable with AdCMVE1 or wild-type Ad5, as reflected by the similar profile of dose-response curves (Fig. 4, C and D). In aggregate these data demonstrated that AdMKE1 achieved potent, selective cell killing in the MK-positive lines.

To determine the capability of the purging strategy to accomplish eradication of clonogenic MK-positive tumors, treatment of these cells with AdMKLuc, AdMKE1, AdCMVE1, or wild-type Ad5, was followed by analysis for soft agar colony formation (Table 1). The SK-N-SH line was omitted, because it was not able to achieve colony formation in this assay per se. The data obtained by soft agar assay correlated well with the results of MTS assay. In MK-positive cells, the efficacy of eradicating tumor formation by AdMKE1 was comparable with that by AdCMVE1 or wild-type Ad5. It is noteworthy that almost complete prevention of colony formation was achieved in G-401 or RD-ES cells when the inoculum was infected with AdMKE1 at an MOI of 100 for 3 h. On the other hand, in the MeWo cells, efficacy of AdMKE1 seemed to be much less compared with AdCMVE1 or wild-type Ad5. Thus, these results were encouraging in regard to our aim of developing a purging strategy for MK-positive tumors.

Table 1 Colony Formation in Soft Agar

<table>
<thead>
<tr>
<th></th>
<th>G-401</th>
<th>RD-ES</th>
<th>SK-ES-1</th>
<th>MeWo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>93.7 (15.9)</td>
<td>148.0 (23.6)</td>
<td>329.7 (79.8)</td>
<td>242.0 (59.4)</td>
</tr>
<tr>
<td>MOI 20</td>
<td>82.0 (7.8)</td>
<td>85.3 (28.2)</td>
<td>182.3 (20.3)</td>
<td>228.7 (32.8)</td>
</tr>
<tr>
<td>AdMKLuc</td>
<td>1.3 (0.58)</td>
<td>63.7 (21.4)</td>
<td>58.3 (22.7)</td>
<td>173.7 (39.6)</td>
</tr>
<tr>
<td>AdMKE1</td>
<td>0</td>
<td>3.3 (2.5)</td>
<td>60.7 (13.4)</td>
<td>1.7 (1.5)</td>
</tr>
<tr>
<td>AdCMVE1</td>
<td>0</td>
<td>41.7 (6.0)</td>
<td>24.7 (16.7)</td>
<td>7.0 (2.0)</td>
</tr>
<tr>
<td>Adwt</td>
<td>0</td>
<td>0</td>
<td>5.3 (3.1)</td>
<td>0</td>
</tr>
<tr>
<td>MOI 100</td>
<td>16.3 (1.5)</td>
<td>34.0 (11.3)</td>
<td>116.7 (24.7)</td>
<td>67.3 (25.1)</td>
</tr>
<tr>
<td>AdMKLuc</td>
<td>0.7 (0.58)</td>
<td>0.3 (0.58)</td>
<td>20.0 (12.5)</td>
<td>30.0 (7.9)</td>
</tr>
<tr>
<td>AdMKE1</td>
<td>0</td>
<td>0</td>
<td>10.7 (5.5)</td>
<td>0</td>
</tr>
<tr>
<td>AdCMVE1</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Adwt</td>
<td>0</td>
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Data are means (±SD) of triplicate determinations.
MK Promoter Driving CRAd Confers No Damage to Hematopoietic Stem Cells. Having shown evidence of efficacy for the AdMKE1 approach for MK-positive tumors, the final step was to evaluate the potential toxicity of this approach for hematopoietic stem cells. The MK expression level in CD34+ cells was evaluated by RT-PCR as described before. CD34+ cells express very low levels of MK compared with G-401 (Fig. 5A). To investigate the potential toxicity of AdMKE1 infection on hematopoietic stem cells, we treated CD34+ cells with 1000 MOI of AdMKLuc, AdMKE1, AdCMVE1, or Adwt for 3 h and then cultured for 8 days (Fig. 5B). Compared with untreated (Mock) or nonreplication Ad-infected group (AdMKLuc), no reduction of hematopoietic colonies was observed in the group infected with AdMKE1. On the other hand, significant reduction of hematopoietic colonies was confirmed in the group infected with AdCMVE1 or wild-type Ad5. Compared with tumor cell lines tested in this study, CD34+ cells were actually less susceptible to the cell killing effect of AdCMVE1 or Adwt attributable to the poor Ad infectability of hematopoietic stem cells. Nevertheless, at high doses, toxicity was observed with the nonselective control viruses. Thus, the incorporation of the MK promoter into the CRAd strategy achieved a higher level of safety without compromising the basic efficacy against tumor cells. These data support our hypothesis that AdMKE1 would be a good candidate for bone marrow purging for NB or ES patients based on the combined attributes of Ad tropism as well as the transcriptional specificity of the MK promoter.

DISCUSSION

Conditionally replicative Ads, which show tumor specific replication and oncology, are promising new therapies for malignancies resistant to conventional treatments. In the current report, we demonstrate a strategy based on the use of a replication-competent Ad controlled by a tumor-specific promoter relevant to incurable pediatric solid tumors. Concurrently, we demonstrated that use of this replication-competent virus avoided causing damage to hematopoietic stem cells by virtue of both the low tropism of the virus for the cells as well as the low activity of the MK promoter in these cells.

The treatment of NB and ES is an area in which novel therapies are urgently needed. To our knowledge, this is the first report to address the question of using a CRAd approach for bone marrow purging. In addition, the method we presented herein, which involved only 3-h incubation of hematopoietic graft with CRAd, should be easily applicable to standard bone marrow transplantation protocols. Despite the fact that advanced NB and ES may show good initial responses to radiochemotherapy, aggressive recurrence frequently leads to the tragic loss of young lives. High-dose chemotherapy with autologous stem cell rescue has been introduced in an effort to overcome the high mortality caused by these devastating pediatric tumors (27–30). In this regard, complete removal of tumor cells contaminating the autologous hematopoietic graft has been a challenging problem but is essential for optimal outcome. Recently, several attempts at hematopoietic stem cell purging using nonreplicative Ad vectors have been reported, aimed at the treatment of breast cancer (35, 36), multiple myeloma (37), and prostate cancer (46). These approaches may be limited by the same issues that have compromised the direct application of Ad vectors to solid tumors, that is, poor penetration of the vector into the tumor mass. In this regard, the tumor cells in the bone marrow samples aspirated from tumor patients form clusters and cannot be expected to be effectively transduced by conventional replication-incompetent Ad. We speculate that the clusters of tumor cells justify the development of a CRAd approach to purging, allowing for improved intratumoral spread and penetration. Furthermore, it should be noted that NB and ES have fewer gap junctions (38) and less abnormalities of p53 (39, 40) compared with adult malignancies, thus making them less susceptible to the use of herpes simplex virus thymidine kinase (HSVTK) or p53 gene therapy approaches. We believe that the bone marrow purging of MK-positive pediatric solid tumors using AdMKE1 may be an ideal strategy, having the benefits of intratumor spread, and transductional and transcriptional specificity.

Lack of toxicity for hematopoietic cells is a key property for any Ad-based bone marrow purging strategy. However, prevention of hepatic toxicity is also another important aspect (47, 48) even in ex vivo strategies. Tumor cells infected with replication-competent Ad may release new viruses in vivo. Should this occur, there would be a potential for in vivo toxicity, especially in the liver, because this is the predominant site of Ad vector localization after systemic injection. In this regard, we reported previously the potential benefits of using MK promoter for cancer gene therapy based on the ability to avoid hepatic injury attributable to the very low level of promoter activity in this organ (26). Because AdMKE1 showed specificity in both replication rate and cytotoxicity as shown in MK-positive and negative cells, it can be expected to render less toxicity to the liver compared with AdCMVE1 or wild-type Ad5. However, despite the selectivity of AdMKE1 for replication in MK-negative cells, the degree of control is not absolute, because some toxicity could be seen even in the MK-negative MeWo cells. We believe this arises as a result of a low level of expression of E1 attributable to the promoter activity of the LITR and/or encapsidation signal. Thus, additional improvements in specificity could be achieved before clinical application. These approaches could entail the use of insulator sequences between the LITR and MK promoter (49) or by placing other essential viral genes in addition to E1 under MK promoter control (e.g., E4). Nevertheless, the degree of toxicity we observed in the MK-negative line was significantly less than that for the positive lines. At this time, the

Fig. 5. A, evaluation of MK expression level in CD34+ cells. Total RNA (50 ng) was analyzed using RT-PCR. Lanes 1 and 4, G-401; Lanes 2 and 5, CD34+ hematopoietic cells; Lanes 3 and 6, Duadi cells. Very scant bands corresponding to MK transcripts were observed in CD34+ and Duadi cell samples. B, hematopoietic colony formation derived from CD34+ cells infected with Ad vectors. Hematopoietic colonies were counted after 8 days of culture in Methocult GF H4434 (0.9% methylcellulose in Iscove’s modified Dulbecco’s medium, 30% fetal bovine serum, 1% BSA, 3 units/ml of rh erythropoietin, 100 μmol/l 2-mercaptopethanol, 2 mmol/l l-glutamine, 50 ng/ml rh stem cell factor, 10 ng/ml rh granulocyte macrophage colony-stimulating factor, and 10 ng/ml rh IL-3). Data are means of triplicate determinations; bars, ± SE.
correlation between toxicity in putative negative cells in \textit{in vitro} assays and the degree of control needed to prevent damage to normal tissues \textit{in vivo} is uncertain. Replicative viruses already in clinical trial have shown at least some low level of viral production and/or toxicity in negative cell lines \textit{in vitro} (7, 50–54). Unfortunately, at this time, no suitable animal models exist for the assessment of CRAd toxicity \textit{in vivo}.

The application of a MK-promoter based CRAd for NB or ES therapy may have utility beyond stem cell purging. Clinical trials of immunogene therapy for NB based on Ad gene transduction have been reported (55). In this study the authors used irradiated autologous NB cells transduced with the gene for IL-2 as an immunostimulatory agent. However, a new trial using unirradiated autologous cells has been proposed to maximize immunogenicity. The protocol seeks to use unirradiated cells as the vaccine, thus raising safety concerns (56). It might be plausible to coinfect cells with both the MK CRAd and a vector carrying an immunostimulatory IL-2 gene, thus ensuring the eventual clearance of the injected cells but also potentially improving overall immunostimulatory effect. The potential application of a CRAd to tumor vaccine strategies is an area that warrants additional investigation. In this regard, most of the malignancies refractory to conventional treatments are MK-positive tumors (18–25), thus, a MK promoter-based approach would be rational.

We believe that the data presented here provides a basis for the additional development of replication-competent Ad strategies based on the MK promoter for the therapy of pediatric and adult cancers.

ACKNOWLEDGMENTS

We thank Prof. Ian R. Hart for providing us with the MeWo melanoma cell line. We also thank Lioudmila Kaliberova, Ramon Alemany, Dirk M. Nettelbeck, and Kiyoshi Kawakami (Kagoshima City Hospital, Kagoshima, Japan) for their excellent technical support and expert advice.

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