Featured Article

Systemic Therapy of Malignant Human Melanoma Tumors by a Common Cold-Producing Enterovirus, Coxsackievirus A21

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Abstract

Purpose: **The incidence of malignant melanoma continues to increase worldwide; however, treatment of metastatic melanoma remains unsatisfactory, and there is an urgent need for development of effective targeted therapeutics. A potential biological target on the surface of malignant melanoma cells is the up-regulated expression of intercellular adhesion molecule (ICAM)-1 and decay-accelerating factor (DAF), relative to surrounding benign tissue. Coxsackievirus A21 (a common cold virus) targets and destroys susceptible cells via specific viral capsid interactions with surface-expressed virus receptors comprising ICAM-1 and DAF.**

Experimental Design: **The oncolytic capacity of a genetically unmodified wild-type common cold-producing human enterovirus (Coxsackievirus A21, CAV21) was assessed against *in vitro* cultures and *in vivo* xenografts of malignant human melanoma cells.**

Results: ***In vitro*** **studies established that human melanoma cells endogenously express elevated levels of ICAM-1/DAF and were highly susceptible to rapid viral oncolysis by CAV21 infection, whereas ICAM-1/DAF-expressing peripheral blood lymphocytes were refractile to infection.** ***In vivo*** **studies revealed that the tumor burden of nonobese diabetic severe combined immunodeficient mice bearing multiple s.c. melanoma xenografts was rapidly reduced by oncolysis mediated by a single administration of CAV21. The antitumor activity of CAV21 was characterized by highly efficient systemic spread of progeny CAV21, with oncolysis of tumors also occurring at sites distant to the primary site of viral administration.**

Conclusions: **Overall, the findings presented herein demonstrate an important proof of principle using administration of replication-competent CAV21 as a potential biological oncolytic agent in the control of human metastatic melanoma.**

Introduction

Malignant melanoma is often associated with early metastatic spread and a high level of resistance to current therapies, in particular chemotherapy. Nevertheless, a potentially specific and effective form of cancer control is offered by viral oncolysis, whereby viruses target and destroy tumor tissue (1–5). The simplest form of viral oncolysis is the direct lysis of malignant cells as a result of efficient multicycle viral replication, whereby the progeny virus produced is able to infect adjacent or distant cancer cells. The up-regulated expression of specific oncolytic virus receptors on the surface of tumor cells relative to surrounding normal tissue provides a discrete discriminatory mechanism for viral-mediated destruction of cancerous cells in environments of nonmalignant tissue (6, 7). One such potential target molecule is intercellular adhesion molecule (ICAM)-1, an immunoglobulin-like molecule that is widely accepted as a progression marker for malignant melanoma (8, 9). Elevated surface expression of ICAM-1 enhances the potential for melanocytes to interact with circulating lymphocytes via interactions with the integrin molecule lymphocyte function associated antigen-1, thus potentially providing a means by which melanomas undergo metastatic spread.

A further attractive target for oncolytic viruses is the glycosylphosphatidylinositol-linked glycoprotein decay-accelerating factor (DAF), a complement regulatory protein, whose presence counteracts the potentially destructive action of the complement system and whose expression is up-regulated on the surface of many tumors, including malignant melanocytes (10, 11). A subtle combination of surface-expressed ICAM-1 and DAF forms the cellular receptor complex for the human "common cold" enterovirus, Coxsackievirus A21 (CAV21), facilitating specific viral attachment, cell internalization, and subsequent rapid cell lysis (12). In this environment, DAF functions as a membrane sequestration receptor aiding in the capture and presentation of virions for interactions with ICAM-1 that facilitate changes in viral capsid conformation, permitting cell entry and subsequent lytic infection (13, 14). In general, enteroviral capsid-DAF interactions alone do not mediate lytic cell infection (15, 16). Interestingly, dual monoclonal antibody blockade of both ICAM-1 and DAF is required for total inhibition of CAV21 lytic infection of cancer cells (12). CAV21 has long been associated with common cold-like upper respiratory infection, and despite deliberate intranasal administration to human

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volunteers, development of dramatic illness has not been reported (17, 18). In this presentation, we highlight the potential application of a genetically unmodified wild-type strain of CAV21 as an effective oncolytic and therapeutic agent for the control of human malignant melanoma.

Materials and Methods

Cells, Viruses, and Antibodies. The melanoma cell lines SK-Mel-28 and ME4405 were obtained from Dr. S. Ralph (Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia) and Dr. G. Parmiani (Milan, Italy), respectively. The following cell lines were isolated in our laboratory: Mel-CV and Mel-FH (from metastases in lymph nodes); Miller (from an intestinal metastasis); and MM200 (from a primary melanoma).

RD (a heteroploid human embryonal cell line) rhabdomyosarcoma cells were obtained from the Entero-respiratory Laboratory, Fairfield Hospital (Melbourne, Victoria, Australia). The RD-ICAM-1 cell line was produced by stable transfection of RD cells with cDNA encoding the ICAM-1 molecule (12). MRC-5 cells (derived from human lung fibroblasts) were obtained from Bio-Whittaker.

All of the above-mentioned cell lines were maintained in DMEM containing 10% FCS.

Prototype strains of Coxsackievirus A21 (CAV21, Kuykendall strain), Coxsackievirus B1 (CVB1, Conn-5), Coxsackievirus B3 (CVB3, Nancy), and Echovirus 7 (E7, Wallace) were obtained from Margery Kennett (Entero-respiratory Laboratory, Fairfield Hospital). All viruses were propagated in RD-ICAM-1 cells, and infected cells were frozen and thawed to release the remaining intracellular virus particles. The virus-containing medium was clarified of cellular debris by centrifugation for 5 min at $1000 \times g$ and stored at −80°C.

The anti-ICAM-1 monoclonal antibody, WEHI, which is specific for the NH_2-terminal domain of ICAM-1, was supplied by Dr. Andrew Boyd (Queensland Institute for Medical Research, Brisbane, Queensland, Australia). Anti-DAF monoclonal antibody, IH4, which recognizes the third short consensus repeat of DAF, was a gift from Dr. Bruce Loveland (Austin Research Institute, Melbourne, Victoria, Australia).

Virus Infectivity Assays. Confluent monolayers of RD-ICAM-1 cells in 96-well tissue culture plates were inoculated with 10-fold serial viral dilutions (100 μl/well in quadruplicate) and incubated at 37°C in a 5% CO_2 environment for 48 h. Cell survival was quantitated by incubation with crystal violet/methanol solution [0.1% crystal violet, 20% methanol, and 4.0% formaldehyde in PBS (100 μl/well)] for 24 h, and after three washes with distilled water, the relative absorbance of individual wells was read on a multiscan ELISA plate reader (Flow Laboratories, McLean, VA) at 540 nm. Fifty percent viral end point titers were calculated using the method of Reed and Muench (19) by scoring wells as positive if the absorbance values were less than the mean minus 3 SDs of the control (no virus) wells.

***In Vitro* Viral Cell Lysis Assay.** The lytic activity of CAV21 in peripheral blood lymphocytes (PBLs) isolated (Ficoll gradient) from the blood of stage IV melanoma subjects and primary cultures of melanoma cells isolated from excised malignant melanoma tumors was tested in quadruplicate in ^{51}Cr release assays. Target cells were labeled with 100 μCi of $Na_2{}^{51}Cr0_4$ (New England Nuclear, Boston, MA) for 1.5 h at 37°C, washed three times, and resuspended in DMEM containing 10% FCS. CAV21 [50% tissue culture infectious dose ($TCID_{50}$) = $10^{7.4}$] was added to the target cells (approximately 10^5) in quadruplicate wells of a 24-well plate containing 1.0 ml of DMEM and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 18 h. For each target system, spontaneous release as well as maximal ^{51}Cr activity release was determined. After incubation, the supernatant was harvested and counted in an automated gamma counter. The percentage of lysis was calculated as follows: % specific cytotoxicity = [experimental release (cpm) − spontaneous release (cpm)]/[maximal release (cpm) − spontaneous release (cpm)].

Virus Purification. CAV21 was purified from stock preparations by velocity centrifugation in 5–30% sucrose gradients as described previously (20), and the peak infectious fractions were pooled, dialyzed against PBS, and stored at −80°C.

Flow Cytometry. DAF and ICAM-1 surface expression on surface of melanoma cells was analyzed by flow cytometry. Briefly, dispersed cells (1×10^6) were incubated on ice with the appropriate monoclonal antibodies (5 μg/ml in PBS) for 20 min. Cells were then washed with PBS, pelleted at $1000 \times g$ for 5 min, and resuspended in 100 μl of R-phycoerythin-conjugated $F(ab')_2$ fragment of goat antimouse immunoglobulin diluted 1:100 in PBS (DAKO A/S) and incubated on ice for 20 min. Cells were washed, pelleted as described above, resuspended in PBS, and analyzed for DAF/ICAM-1 expression using a FACStar analyzer (Becton Dickinson, Sydney, New South Wales, Australia).

Melanoma Xenotransplantation in Nonobese Diabetic (NOD)-Severe Combined Immunodeficient (SCID) Mice. All animal work was performed under guidelines approved by The University of Newcastle Animal Care and Ethics Committee. NOD-SCID mice were housed in pathogen-free quarters in the animal handling facility located within the university. SK-Mel-28 cells grown in DMEM containing 10% FCS were harvested, washed twice with DMEM, and resuspended in sterile PBS. Cell viability was assessed by trypan blue staining; only cell preparations with >95% viability were used for xenotransplantation. Before xenotransplantation, animals were anesthetized with i.p. injections of Rompun/Ketamine (50 mg/kg). For the monitoring of animals and measurement of tumor growth, animals were anesthetized with 3% isofluorane. Tumor cells were xenografted into the flanks of anesthetized 4–6-week-old female NOD-SCID mice by single or multiple s.c. injections of 2×10^5 cells. Melanoma xenograft growth was monitored daily and measured with calipers. Estimates of tumor volumes were calculated using the formula for a spheroid.

Results

***In Vitro* Lysis of Human Melanoma Cells.** Malignant melanoma cells characteristically express elevated levels of ICAM-1 and DAF (8–11). Because these up-regulated cell surface molecules constitute the cellular receptor complex for Coxsackievirus A21 (12), an enterovirus clinically associated with common colds (18), we investigated whether CAV21 could

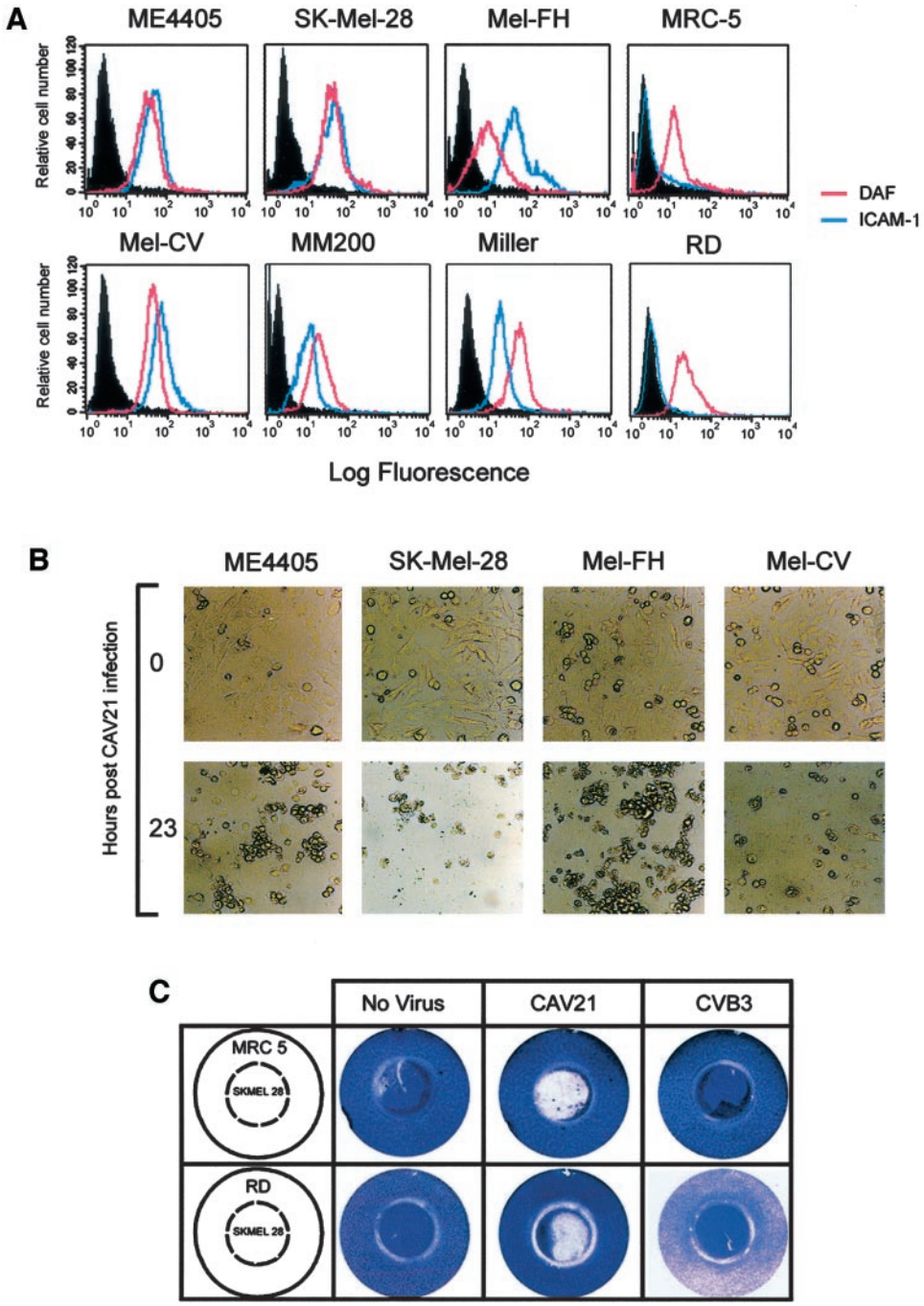

Fig. 1 Oncolytic destruction of human melanoma cells by Coxsackievirus A21. *A,* flow cytometric analysis of virus receptor expression. The *black solid histogram* represents the binding of the control antibody; intercellular adhesion molecule 1 expression is represented by the *blue histogram*; decay-accelerating factor expression is shown by the *pink histogram*. *B,* photomicrographs of CAV21-induced infection of *in vitro* cultures of human melanoma cells. Cell monolayers were infected with 1.0 $TCID_{50}$ (50% tissue culture infectious dose)/cell of CAV21 and monitored for cytopathic effect at 0 and 23 h after infection, and then photographed at a magnification of ×20. *C,* cocultures of human melanoma cells (SK-Mel-28) and nonmalignant lung fibroblasts (MRC-5) or malignant rhabdomyosarcoma (RD) cells in 6-well culture plates were infected with either of either 1.0 $TCID_{50}$/cell of CAV21 or CVB3. After a 48-h incubation, the cocultures were fixed and stained with a crystal violet/methanol solution, and cell viability was examined by microscopic examination.

infect and lyse a series of cell lines derived from malignant melanoma. Flow cytometric analysis indicated that the six investigated melanoma cell lines possessed abundant surface ICAM-1 and DAF with expression levels significantly higher than those observed on nonmalignant MRC-5 lung fibroblasts and malignant rhabdomyosarcoma RD cells (Fig. 1*A*). Monolayer cell cultures were exposed to the prototype strain of CAV21 (Kuykendall) at an input multiplicity of 1.0 $TCID_{50}$/cell and incubated at 37°C for 23 h. CAV21 infection produced extensive lytic cell destruction in all melanoma cultures, with the characteristic cytopathology associated with enterovirus infection (Fig. 1*B*). Monoclonal antibody blockage of the ICAM-1/DAF complex on the surface of SK-Mel-28 cells inhibited the binding of radiolabeled CAV21.[4] Yields of CAV21 in excess of

[4] D. R. Shafren, G. G. Au, and L. Beagley, unpublished data.

$10^{8.0}$ $TCID_{50}$/ml were obtained in these cultures, indicating not only that melanoma cells are susceptible to destruction after exposure to CAV21 but that the interaction produces abundant progeny virus.

The specificity of CAV21-induced lysis of cells expressing high levels of DAF and ICAM-1 was tested using cocultures of the human melanoma cell line SK-Mel-28 and nonmalignant lung fibroblasts (MRC-5, low DAF and little to no ICAM-1 expression; Fig. 1*A*) or malignant rhabdomyosarcoma cells (RD, low DAF expression and ICAM-1 negative). These cell cocultures were established in 6-well culture plates and infected with either CAV21 or Coxsackievirus B3 (CVB3) at multiplicities of 1.0 $TCID_{50}$/cell. CVB3 uses a cell receptor complex comprising DAF and the immunoglobulin-like molecule, the Coxsackie and adenovirus receptor (15, 21). After an incubation period of 48 h at 37°C, the plates were stained with a crystal violet solution to assess cell survival. The photomicrographs displayed in Fig. 1*C* indicate that CAV21 induces rapid oncolysis of the human melanoma cells without detectable evidence of lysis of surrounding DAF-expressing nonmalignant MRC-5 and malignant RD cells (Fig. 1*C*). The data confirm that CAV21-DAF interactions alone are not sufficient to mediate lytic cell infection, nor is direct cell contact with virally infected ICAM-1-expressing melanoma cells (Fig. 1*C*). In contrast, CVB3 induces lytic infection in the RD cells but does not lyse the melanoma or MRC-5 cell cultures, most probably because they lack detectable levels of Coxsackie and adenovirus receptor expression (data not shown).

In a further effort to the determine the specificity of the CAV21 infection, PBLs from four stage IV melanoma patients as well as first-passage *in vitro* cell cultures prepared from excised metastatic melanoma tumor masses were challenged with CAV21 ($10^{7.4}$ $TCID_{50}$/ml). Flow cytometric analysis revealed that all PBL cell preparations expressed strong levels of DAF and significant levels of ICAM-1, whereas in the primary melanoma cell cultures, high levels of both DAF and ICAM-1 were detected on the cell surface (Fig. 2*A*). In general, DAF expression was comparable between the PBLs and melanoma cells; however, ICAM-1 levels were significantly higher on melanoma cells than on the PBLs. CAV21-mediated cytolysis of PBLs and melanoma cells was assessed by using a standard ^{51}Cr release assay. After challenge with CAV21 for 18 h, microscopic examination revealed complete lytic destruction by CAV21 in all melanoma cultures (Fig. 2*B*), a result confirmed by the ^{51}Cr release assay, where levels of cytolysis approached 100% (Fig. 2*C*). In contrast, despite expressing significant amounts of DAF and ICAM-1, only background levels of cytolysis were observed in the PBLs after exposure to the same input dose of CAV21 (Fig. 2*C*).

Receptor Determined Specificity of CAV21-Mediated Melanoma Cell Oncolysis. Next we assessed whether common human enteroviruses other than CAV21, which also use a cell receptor complex consisting of DAF and an additional surface molecule for cell infection, could induce oncolysis of human malignant melanoma cells and/or RD cells. Briefly, 10^5 $TCID_{50}$ of CAV21 [cell receptor complex consisting of DAF/ICAM-1 (12)], CVB1/CVB3 [cell receptor complex consisting of DAF/Coxsackie and adenovirus receptor (15, 21)], and E7 (cell receptor complex consisting of DAF/unknown molecule[4]) were added to monolayers of MM200 (Fig. 1*A*) or RD cells (Fig. 1*A*) grown in 6-well culture plates. After incubation for 48 h at 37°C, the cell monolayers were stained with a crystal violet solution to assess cell survival. The RD cells used in this study express low levels of DAF and Coxsackie and adenovirus receptor but no ICAM-1 (Fig. 1*A*; Ref. 15), thereby potentially conferring susceptibility to infection by CVB1, CVB3, and E7 but not CAV21. The photomicrographs shown in Fig. 3*A* reveal that only CAV21 induces a detectable lytic infection in the melanoma cells (MM200). However, as anticipated, all of the enteroviruses except CAV21 lytically destroy RD cells.

To determine whether CAV21-induced cell lysis occurs in primary neoplastic cells, biopsy tissue from a malignant melanoma that had metastasized to a lymph node was challenged with CAV21 in an *in vitro* culture system. The malignant melanoma tissue was dissected into pieces (4 mm^3), which were individually placed into wells of a 24-well cell culture plate containing cell maintenance medium alone or culture medium containing either CVB3 (10^5 $TCID_{50}$) or CAV21 (10^5 $TCID_{50}$). All cultures were incubated at 37°C in a 5% CO_2 environment, and after a 72-h incubation period, the inoculated biopsy sections were examined microscopically for signs of viral-induced oncolysis. The photomicrographs presented in Fig. 3*B* highlight that neither the control (no virus) nor CBV3-inoculated biopsy material showed any microscopic signs of tissue destruction, in strong contrast to the widespread cell lysis and fragmentation of the tumor mass observed in the CAV21-inoculated culture.

***In Vivo* Oncolysis of Human Melanoma Xenografts by CAV21.** The *in vitro* data indicate that CAV21 selectively and specifically targets human melanoma cell lines via interaction with overexpressed ICAM-1 and DAF on the cell surface. To explore the possibility of lytically infecting preformed malignant melanoma tumors *in vivo* with CAV21, a series of animal challenge experiments was conducted. Initially, NOD-SCID mice bearing preformed melanoma xenografts received a single dose of PBS or CAV21 (10^3 $TCID_{50}$) by the intratumoral route, and tumor burdens were assessed over a 35-day period (Fig. 3*C*). Significant reductions in tumor burdens of mice treated with CAV21 were observed as early as 14 days after viral administration, with total tumor reduction achieved in an additional 7 days (Fig. 3, *C* and *D*). Mice treated with PBS were euthanized at 35 days after treatment because their tumor burden reached the upper limits imposed by the University Animal Ethics committee.

In the next series of experiments, the fundamental strategy was to determine whether intratumoral (Fig. 4*A*), i.p. (Fig. 4*B*), or i.v. (Fig. 4*C*) administration of a single dose of replication-competent, wild-type CAV21 (10^3 $TCID_{50}$) would facilitate an efficient level of viral oncolysis and reduce tumor burden in NOD-SCID mice bearing multiple preformed flank melanoma xenografts. In these experiments SK-Mel-28 cells (2×10^5) were inoculated into NOD-SCID mice s.c. at two sites, in the upper and lower flanks. The introduction of two preformed tumors in the animal model allowed systemic virus spread and distant tumor targeting to be determined in a simple manner. In the group of mice receiving a single intratumoral injection of virus into one of the two tumors, volume reduction in the noninjected tumor would indicate qualitatively the ability for systemic spread. When the xenograft volumes reached approx-

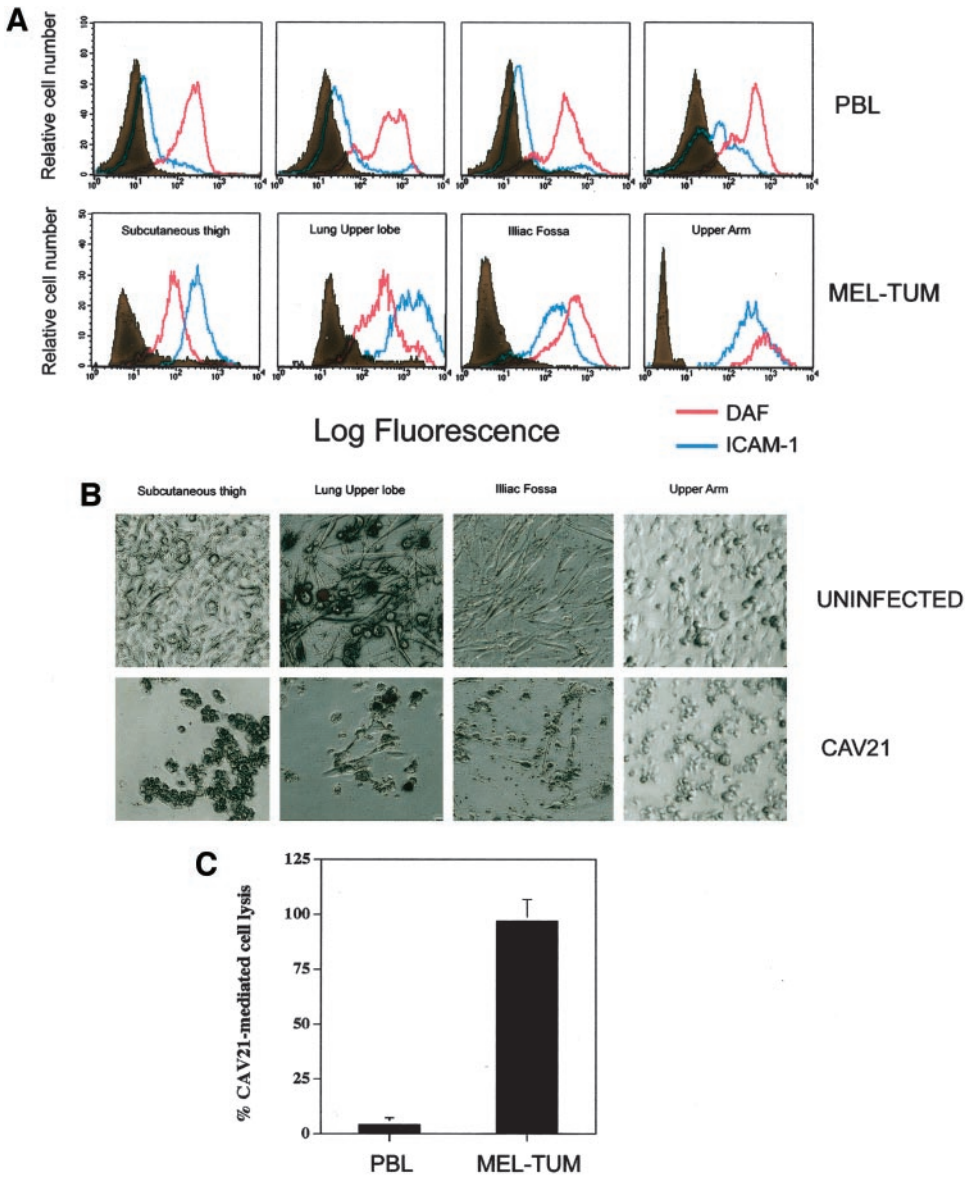

Fig. 2 Lytic infection of primary passage melanoma (MEL-TUM) cells and peripheral blood lymphocytes (PBLs) from stage IV melanoma subjects. *A*, flow cytometric analysis of virus receptor expression. The *black solid histogram* represents the binding of the control antibody; intercellular adhesion molecule 1 expression is represented by the *blue histogram*; decay-accelerating factor expression is shown by the *pink histogram*. *B*, photomicrographs of CAV21-induced lytic infection of primary *in vitro* cultures of human melanoma cells from biopsy samples. Cell monolayers were infected with 1.0 $TCID_{50}$ (50% tissue culture infectious dose)/cell of CAV21 for 18 h, and the cell monolayers were then inspected for signs of cell lysis and photographed at a magnification of ×20. *C*, *in vitro* cell lysis of primary cultures of malignant melanoma cells and PBLs. CAV21 ($10^{7.4}$ $TCID_{50}$) was added to the ^{51}Cr-labeled melanoma cells or Ficoll gradient-purified PBLs from stage IV melanoma subjects. After incubation for 18 h at 37°C, the supernatant was harvested and counted in an automated gamma counter. The percentage of lysis was calculated as follows: % specific cytotoxicity = [experimental release (cpm) − spontaneous release (cpm)]/[maximal release (cpm) − spontaneous release (cpm)].

imately 200–400 mm^3, the animals were divided into six groups (5 animals/group) to investigate the three different routes of administration. For ethical reasons, animals in the PBS-treated groups were terminated from the experiment at day 17 due to the considerable increase in tumor burdens. However, at this time all mice receiving CAV21, irrespective of the route of administration, exhibited significant reductions in the tumor burden of both the primary and secondary flank xenografts (Fig. 4). By 30 days after CAV21 administration, tumors could not be detected in any of the mice receiving CAV21. Serum viral loads of approximately 10^5 to 10^6 $TCID_{50}$/ml (viral titers significantly higher than input virus levels) were observed in CAV21-injected mice as determined by real-time PCR and standard cell culture infectivity assays.[4] Similar levels of tumor reduction by multiple-route CAV21 administration to mice bearing melanoma xenografts of the malignant line ME4405 (Fig. 1*A*) were also observed.[4] Overall, these data highlight that CAV21 exhibits efficient systemic spread, as evidenced by the oncolysis of tumors at sites distant to the primary site of viral administration.

***In Vivo* Oncolysis of Human Melanoma Xenografts by Various Input Multiplicities of CAV21.** Having established that semipurified preparations of CAV21 administered via intratumoral, i.v., and i.p. routes were successful in reducing melanoma tumor burden, we then investigated the antitumor efficacy of gradient-purified CAV21 over a range of input multiplicities. Preformed single melanoma xenografts on the upper flanks of NOD-SCID mice (approximately 400–600 mm^3) were injected via the intratumoral route with four varying input multiplicities of CAV21 ranging from 10^1 to 10^5 $TCID_{50}$. All CAV21 dosage levels were extremely active in significantly reducing melanoma xenograft mass (Fig. 5). The predilection of CAV21 for the lytic infection of melanoma cells was confirmed

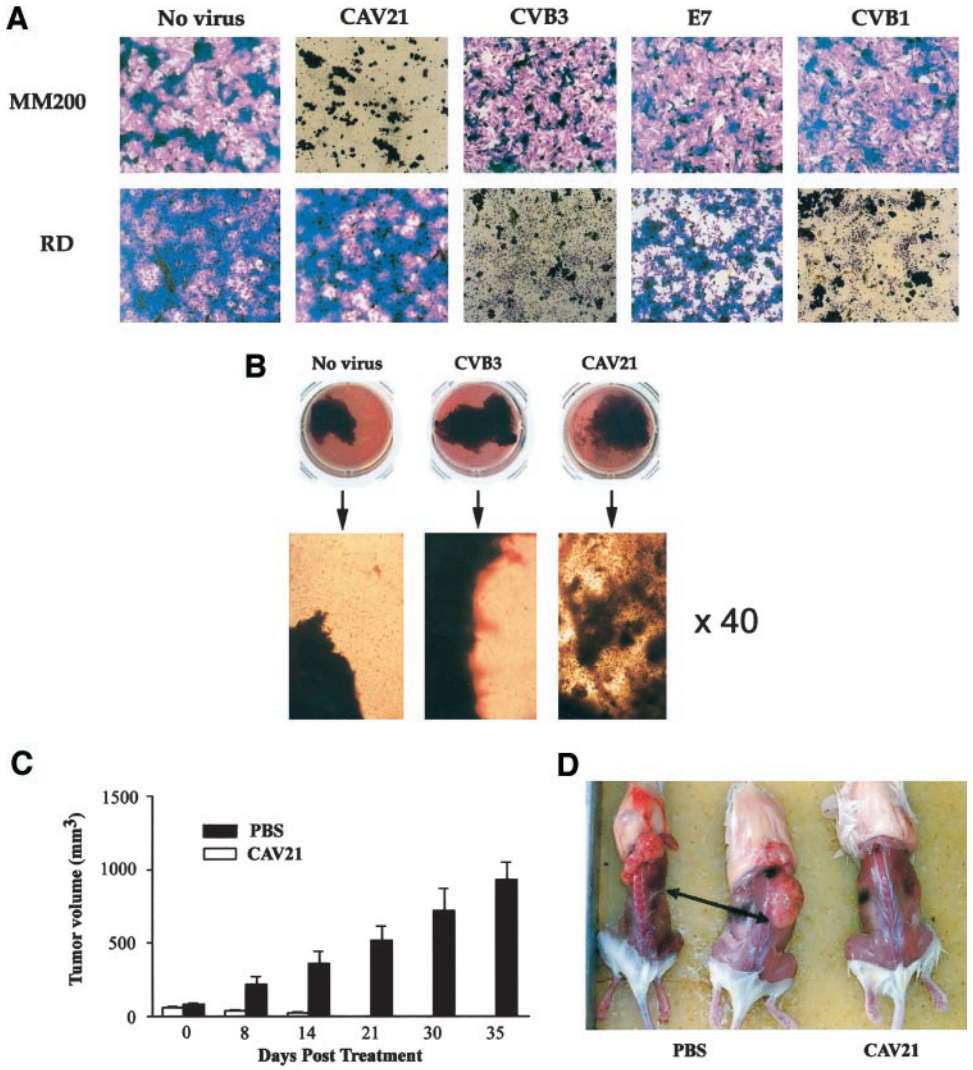

Fig. 3 Intercellular adhesion molecule 1/decay-accelerating factor expression mediates CAV21 oncolysis of melanoma cells. *A*, monolayers of MM200 or RD cells in 6-well culture were inoculated with 10^5 $TCID_{50}$ (50% tissue culture infectious dose) of the following representative human enteroviruses: CAV21; Coxsackievirus B3 (CVB3); echovirus 7 (E7); or Coxsackievirus B1 (CVB1). After incubation for 48 h at 37°C, the cell monolayers were fixed and stained with a crystal violet solution to assess cell survival. *B*, primary malignant melanoma tissue (~4-mm^3 pieces) were individually placed into wells of a 24-well cell culture plate containing cell maintenance medium alone, culture medium containing 10^5 $TCID_{50}$ CVB3, or medium containing 10^5 $TCID_{50}$ CAV21. All cultures were incubated at 37°C in a 5% CO_2 environment, and after a 72-h incubation period, the inoculated biopsy sections were examined microscopically for signs of viral-induced oncolysis. *C*, nonobese diabetic, severe combined immunodeficient mice bearing s.c. tumors (approximately 100 mm^3) growing on the flanks after injection with 2×10^5 SK-Mel-28 cells received intratumoral injection with a single dose of CAV21 (10^3 $TCID_{50}$) or PBS. The average relative tumor sizes were measured externally with calipers and are expressed as the means of five treated mice ± SD. *D*, photograph illustrating the tumor burden of s.c. melanoma xenografts on the flanks of nonobese diabetic, severe combined immunodeficient mice at 35 days after intratumoral injection with CAV21 (10^3 $TCID_{50}$) or PBS.

by the comparable level of tumor burden reduction seen in mice given 10 $TCID_{50}$ and mice given CAV21 at 10- to 10^4-fold higher input multiplicities (Fig. 5).

Discussion

Presently, patients terminally ill with malignant metastatic melanoma frequently do not respond to conventional anticancer regimes such as chemotherapy, radiotherapy, or immunotherapy. The demonstrable selective susceptibility of their tumor cells to lysis by CAV21 offers a potential opportunity to retard and possibly control the spread of the malignancy in such subjects. Rapid and efficient oncolysis of *in vitro* melanoma cell cultures and of *in vivo* melanoma xenografts in SCID mice after a single dose of wild-type CAV21 further highlights the potential of this genetically nonmodified virus to be a therapeutic agent for such malignancies.

A major finding of this study was that intratumoral administration of CAV21 to one of several preformed xenografts produced oncolysis of xenografts distant to the infection site. Thus, the appeal of CAV21 as an antimelanoma treatment is

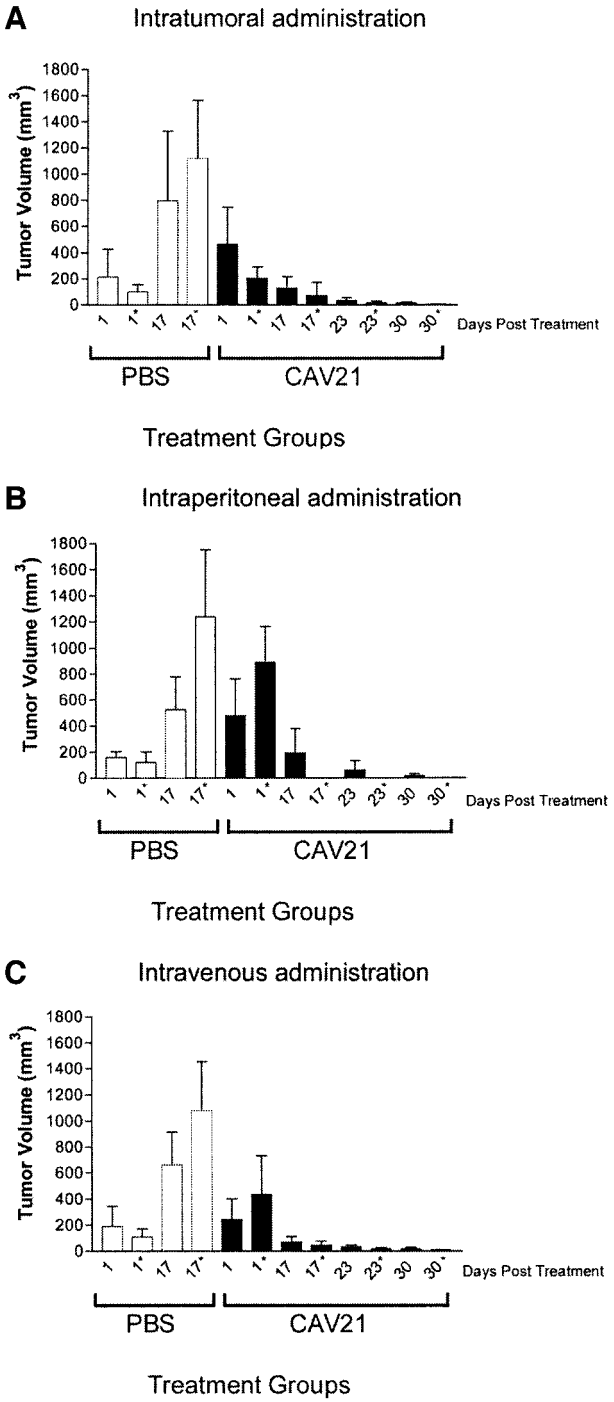

Fig. 4 Oncolytic activity of Coxsackievirus A21 on multiple flank human melanoma xenografts via different routes of administration. Nonobese diabetic, severe combined immunodeficient mice bearing two s.c. tumors (200–400 mm^3) growing in distinct sites on the flanks after injection with 2×10^5 SK-Mel-28 cells received injection with a single dose of CAV21 [50% tissue culture infectious dose ($TCID_{50}$) = 10^3] via the (*A*) intratumoral (injection of one tumor only), (*B*) i.p., and (*C*) i.v. routes. The relative tumor sizes measured externally with calipers are expressed as the means of five treated mice ± SD. ∗ represents the secondary melanoma xenograft.

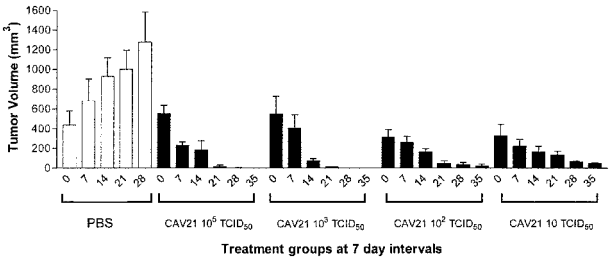

Fig. 5 Dose response of the oncolytic activity of Coxsackievirus A21 on flank human melanoma xenografts. *A*, nonobese diabetic, severe combined immunodeficient mice bearing s.c. tumors (400–600 mm^3) growing on the flanks after injection with 2×10^5 SK-Mel-28 cells received intratumoral injection with a single dose of CAV21 at the following input multiplicities: 10^5; 10^3; 10^2; and 10 $TCID_{50}$ (50% tissue culture infectious dose). The relative tumor sizes measured externally with calipers are expressed as the means of five treated mice ± SD.

enhanced by its apparent sensitivity, whereby direct and rapid oncolysis is produced by input virus loads as low as 10 $TCID_{50}$/tumor (approximately 200–500 mm^3). This sensitivity can be attributed both to the presence on tumors cells of the specific virus receptor uptake complex ICAM-1/DAF and to the rapid production of abundant progeny virus, which is then available for systemic viremic spread to distant metastases. Additional data supporting the use of CAV21 as an oncolytic agent of malignant melanoma were supplied by the findings that primary cell suspensions isolated directly from a number of surgically excised metastatic tumors of end-stage melanoma subjects exhibited high-level DAF/ICAM-1 surface expression and were susceptible to rapid oncolysis during CAV21 infection (Fig. 2). In contrast, PBL preparations from stage IV melanoma subjects that expressed significant levels of DAF and ICAM-1 were refractile to lytic infection by CAV21. The dose of CAV21 used to challenge the PBL cells was approximately 200-fold higher than levels of CAV21 detected in the blood of viremic mice-bearing melanoma xenografts after intratumoral injections of CAV21 (data not shown). The inability of CAV21 to infect the PBLs, despite their expression of significant levels of ICAM-1, may be due to inadequate viral internalization or the lack of cellular factors required for efficient viral capsid uncoating and viral replication. In addition, radiolabeled virus binding and growth kinetic assays revealed attachment of CAV21 to the surface of PBLs but no subsequent production of progeny virus.[4] The observation that CAV21 bound to DAF on the surface of PBLs is unable to mediate cell lytic infection confirms previous observations that DAF acts as a membrane sequestration receptor, accumulating and enhancing presentation of CAV21 virions for subsequent interactions with ICAM-1 (15, 22, 23).

The majority of viruses currently used in oncolytic strategies are genetically modified to reduce virulence and increase specificity for neoplastic cells (3, 5, 6, 24, 25). One such recombinant virus is PV1(RIPO), an intergeneric recombinant poliovirus bearing internal ribosome entry site elements from a common cold human rhinovirus constructed to reduce the neuropathogenicity of the poliovirus for application in the oncolysis of malignant gliomas (6). However, the CAV21 used in this

study is wild type, is not genetically modified, and is significantly attenuated, lacking neuropathogenicity.

Thus, natural infection with CAV21, a common cold enterovirus, induces mainly upper respiratory infection. Deliberate intranasal administration of CAV21 to many volunteers has not led to development of dramatic disease or clinical signs of infection other than those experienced during natural CAV21 exposure (17, 18). The animal experiments presented herein suggest that for immunocompetent late-stage melanoma patients, the benefits of CAV21 destruction of metastatic melanoma tissue may far outweigh the potential risks. In addition to the CAV21-mediated tumor destruction, it is possible that specific tumor antigens released from the melanoma cells during viral oncolysis may stimulate favorable antitumor responses. Furthermore, it is anticipated that immune recognition of melanoma cells may well be enhanced when bearing CAV21 antigens produced during the viral infection process. However, immune system recognition of CAV21 poses a potential barrier to efficient systemic viral spread. In general, the presence of neutralizing antibodies from prior natural or deliberate exposure to wild-type strains from which the recombinant oncolytic viruses have been constructed is a hindrance to systemic spread of all oncolytic viruses (25). In the case of CAV21, pilot serological analysis of patients possessing malignant melanoma and healthy laboratory staff indicated a protective level of serum antibody in $<$10% of samples.[4]

Taken together, the present study suggests that CAV21-mediated oncolysis of malignant melanomas may be a promising new approach to treatment of metastatic melanoma. The major advantages for the selection of CAV21 as a potential oncolytic agent lie in its mild pathogenicity in humans, immense predilection for lytic infection of malignant melanoma cells, *in vivo* amplification of low viral input doses for targeting of noninjected tumors, nonrequirement for genetic modification, and the low prevalence of neutralizing antibody in potential tumor-bearing patients. One further potential advantage that CAV21 offers is the ability to prepare highly purified virions directly from genetically characterized full-length infectious cDNA clones. Studies involving the intratumoral administration of wild-type CAV21 for control of metastatic late-stage melanoma are planned in our institution.

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