Cancer Immunol Immunother (1998) 46: 283–292

ORIGINAL ARTICLE

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Adenoviral transduction of melanoma cells with B7-1: antitumor immunity and immunosuppressive factors

Received: 21 December 1997 / Accepted: 16 March 1998

Abstract Previous studies in experimental models have demonstrated that the transduction of human or murine melanoma cells with the co-stimulatory B7-1 molecule induces effective antitumor immune responses. In order to develop B7-1 gene transfer as a therapeutic tool in the clinical management of melanoma, efficient means of in vivo gene transfer must be used. To this end we evaluated in vitro and in vivo immune responses associated with adenoviral transduction of murine and human melanoma cells with B7-1. Adenovirus-mediated transduction of human and murine melanoma cells with B7-1 leads to high-level transgene expression in vitro and in vivo and does not affect MHC class I and II expression. Adenovirus-delivered B7-1 induced antitumor immune responses, on the basis of observations that human melanoma cells transduced to express human B7-1 were able to co-stimulate allogeneic and autologous T cells to proliferate and that murine melanoma K1735 cells transduced to express murine B7-1 were rejected by syngeneic, immunocompetent mice. By contrast, intratumoral injection of an adenovirus encoding murine B7-1 failed to eliminate established murine melanoma (K1735) despite high-level transgene expression in tumor cells. Potent T cell inhibitory factor(s) secreted by both K1735 cells and select human melanoma cells may contribute to the failure to achieve protection in this setting. Thus, immune inhibitory melanoma-derived factors need to be taken into account when considering the clinical use of B7-1 immunotherapy.

Key words B7-1 · Adenovirus · Melanoma · T cell proliferation · Immunosuppression

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Introduction

Tumor cells display a variety of tumor-associated antigens that can be recognized by T cells [7, 25, 29]; however, most tumors evade cellular immune responses. T cell activation requires both an antigen-specific and an antigen-nonspecific signal, as described by the two-signal model of lymphocyte activation [18, 20, 28, 37]. The antigen-specific signal is provided by the interaction of the T cell receptor (TCR) with peptides presented by gene products of the major histocompatibility complex (MHC) of an antigen-presenting cell. A secondary signal is generated by a co-stimulatory molecule (e.g., B7-1 or B7-2) interacting with its cognate ligand on T cells. Transmission of an antigen-specific signal in the absence of co-stimulation often results in anergy or apoptosis of T cells [2, 16, 27, 37]. Tumor cells commonly express MHC molecules but do not usually express co-stimulatory molecules and may, therefore, fail to activate T cells. It has been shown earlier that the genetically engineered expression of co-stimulatory molecules such as B7-1 on tumor cells may allow them to function as antigen-presenting cells, effectively presenting tumor associated antigen and provoking immune recognition and elimination of tumor cells [38]. However, as noted by Chen et al. [9], the effectiveness of B7-1-mediated antitumor immunity is highly dependent on the tumor model chosen. The availability of tumor antigens [3, 42] or other tumor-derived agents may contribute to the variability seen in rodent tumor models.

In this study we have utilized adenoviruses expressing mB7-1 or hB7-1 (Ad.mB7-1 and Ad.hB7-l respectively) to transduce B7-1 expression in murine and human melanoma cells respectively. We show that in vitro use of Ad.hB7-1 and Ad.mB7-1 leads to high-level expression of functional B7-1 in murine and human melanoma cells respectively. This is associated with significantly decreased tumorigenicity upon injection into syngeneic mice. However, the growth of established tumors in immunocompetent mice is not affected by intratumoral injection of Ad.mB7-1 although significant numbers of tumor cells are transduced to express B7-1. Furthermore, we demonstrate that murine and human melanoma-derived factors strongly inhibit proliferation of T cells co-stimulated by B7-1-expressing cell lines. This is consistent with an important role for tumor-derived immunosuppressive factors in determining the efficacy of B7-mediated antitumor immunotherapy.

Materials and methods

Cells and mice

The murine melanoma cell line K1735 was originally obtained from Dr. J. Allison, Berkeley, Calif., and cultured in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Grand Island, N.Y.). Primary human melanoma cell lines WM3268, WM98-1, WM3182 and WM793 were derived from vertical-growth-phase lesions. The WM9, WM3122, WM1205, WM3172, WM3179 and WM3259 human melanoma cell lines were derived from metastases. All cell lines were gifts from Dr. M. Herlyn (Waster Institute, Philadelphia, Pa.). The melanoma cell lines were cultured in growth medium that consisted of MCDB-153 medium (Sigma, St. Louis, Mo.) containing 20% L-15, 2% FBS (Gibco) and 5 μg/ml insulin (Sigma). Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection and cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin (Gibco) and 0.2 mM proline (Sigma). CHO cells retrovirally transduced to express hB7-1 (CHOhB7-1) were kindly provided by Dr. P.S. Linsley (Bristol-Myers-Squibb Pharmaceutical Research Institute, Seattle, Wash.) and were grown in the same medium as CHO cells with the addition of 1 μM methotrexate (Sigma). Female Balb/c mice or C3H mice (Charles River Laboratories, Wilmington, Mass.) and severe combined immunodeficient (SCID) mice (Fox Chase Cancer Center, Philadelphia, Pa.) were used for experiments at 8 weeks of age or older.

Adenoviral transduction

The adenovirus containing the β-galactosidase reporter gene (Ad.lacZ) was constructed as described before [13]. Ad.mB7-1 and Ad.hB7-1 were constructed as described previously [39]. Briefly, the E1,E3-deleted adenoviral genomic DNA dl7001 or dl327 and plasmid DNA containing hB7-1 or mB7-1 were co-precipitated into 293 cells. The adenoviral genomic DNA was first digested with *Cla*1 and the plasmid DNA was linearized with *Nhe*l prior to co-transfection. Following homologous recombination, adenoviruses containing hB7-1 or mB7-1 were plaque-purified, expanded and purified through a cesium chloride gradient into viral stocks.

Human tumor cells (3×10^6 cells) were transduced in vitro with Ad.hB7-1 or Ad.lacZ at a multiplicity of infection (m.o.i.) of 100 viral particles per cell and suspended in 3.5 ml growth medium containing 2% FBS. The murine melanoma cell line K1735 was transduced with Ad.mB7-1 or Ad.lacZ at a m.o.i. of 1000 to generate mB7-1/K1735 or lacZ/K1735 cells, respectively. After 24 h, fresh growth medium was added and, 3 days later, transgene expression was assessed by flow cytometry. Transduction and flow-cytometry studies were carried out three times without significant variation (representative data are shown). For in vivo transduction, WM9 tumors were established by injecting 1×10^6 WM9 cells in the right flank of SCID mice. When tumors reached 3 mm in their largest diameter (5–7 days after tumor cell inoculation), 5×10^9 plaque-forming units (p.f.u.) of Ad.hB7-1 or Ad.lacZ were injected intratumorally into one injection site (two animals per group).

Flow cytometry

Expression of human MHC class I and II molecules on human tumor cells was determined before and after adenoviral transduction by flow cytometry using monoclonal antibodies (mAb) W6-32 (Pel-Freeze, Rogers, Ariz.) and L243 (Becton Dickinson, Sunnyvale, Calif.) respectively. A fluorescein-isothiocyanate(FITC)-conjugated goat anti-(mouse Ig) (Sigma) antibody was used as a secondary antibody. Murine MHC class I and II expression was detected using anti-(mouse H-2k) and anti-(mouse I-Ak) mAb (Pharmingen, San Diego, Calif.) respectively, followed by FITC-labeled goat anti-(mouse IgG2a) or anti-(mouse IgG2b) antibodies respectively (Pharmingen). The expression of MHC class I and II molecules before and after transduction of tumor cells was assessed in two separate experiments. Human B7-1 expression on tumor cells or on single-cell suspensions of WM9 tumors after in vivo transduction with Ad.hB7-1 was detected using the BB-1/B7-1 mAb (Becton-Dickinson). B7-1 expression following in vivo transduction was analyzed on tumor cells from two different mice. For the analysis of murine B7-1, cells were incubated with an anti-(mouse CD80) antibody (Pharmingen) followed by a FITC-labeled goat anti-(rat IgG2a) antibody (Pharmingen). Ad.lacZ-transduced cells were incubated with fluorescein di-β-D-galactopyranoside (Molecular Probes, Eugene, Ore.) for 1 min at 37 °C followed by incubation on ice for 30 min. Stained cells (10 000 live cells) were analyzed in a FACScan flow cytometer (Becton Dickinson).

Tumor-conditioned medium (CM)

Tumor cells (3×10^6 cells) were cultured in 5 ml RPMI medium containing 10% heat-inactivated FBS at 37 °C for 24 h. CM was collected, centrifuged to remove cells, and stored in aliquots at –20 °C. CM from K1735 cells used in the anti-(transforming growth factor β) (anti-TGFβ) thymidine assay was collected after 3×10^6 K1735 cells had been incubated in 5 ml serum-free RPMI medium for 3 h.

Isolation of human peripheral blood mononuclear (MN) cells and purified T cells

Human MN cells were obtained from peripheral blood by Ficoll-Hypaque gradient centrifugation (Pharmacia, Uppsala, Sweden). Human T cells were isolated from the peripheral lymphocyte fraction by negative selection using a mixture of mouse mAb to human CD14 to eliminate macrophages, CD16 to eliminate natural killer (NK) cells, CD19 to eliminate B cells and MHC class II (L243 mAb) [44] followed by incubation with BioMag goat anti-(mouse IgG) magnetic beads (Perceptive Diagnostics, Cambridge, Mass.). The remaining cells were washed twice in RPMI medium containing 10% heat-inactivated FBS, counted and resuspended as described below. RPMI medium containing 10% heat-inactivated FBS was chosen for thymidine-incorporation assays because stimulated T cells were able to proliferate in this medium and mitomycin-C-treated tumor cells and CHO cells remained viable for 5 days. The purity of the T cell fraction (absence of antigen-presenting cells) was verified by their failure to proliferate in response to phytohemagglutinin (PHA, 5 μg/ml) alone. MN cells incorporated thymidine upon PHA stimulation (100 000 cpm), whereas purified T cells did not proliferate (1000 cpm). The absence of NK cells in the purified T cell fraction was assessed by flow cytometry using an anti-CD16 antibody. MN cells contained 3% CD16-positive NK cells and purified T cells contained less than 0.6% CD16-positive cells. Murine T cells were purified from C3H mouse splenocytes using a mouse T cell

recovery column kit according to the manufacturer's protocol (Cedarlane, Hornby, Ontario).

[^{3}H]Thymidine incorporation assay

Tumor cells and CHO cells (1×10^6) were treated with 100 μg mitomycin C (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 45 min at 37 °C in serum-free RPMI medium (Gibco). Cells were washed twice in RPMI medium (Gibco) and resuspended in RPMI medium containing 10% heat-inactivated FBS. Growth-arrested tumor cells and CHO cells remained viable in RPMI medium containing 10% heat-inactivated FBS. Cells were plated into 96-well round-bottom microtiter plates (Corning, Cambridge, Mass.) at 2.5×10^4 cells/well. Human T cells were added at 1×10^5 cells/well. Where indicated, PHA was used at a concentration of 5 μg/ml, or phorbol myristate acetate (PMA; 10 ng/ml; Calbiochem, La Jolla, Calif.) in combination with ionomycin (360 ng/ml, Sigma) was added. The fusion protein CTLA-4Ig was obtained from Dr. P.S. Linsley and used at a final concentration of 10 μg/ml. Negative controls included T cells in the presence or absence of PHA and tumor cells in medium alone. Positive controls included CHOhB7-1 cells and T cells in medium containing PMA/ionomycin or MN cells in medium containing PMA/ionomycin. Cells were incubated at 37 °C for 5 days, pulsed with 1 μCi [^{3}H]thymidine (ICN, Costa Mesa, Calif.) and harvested 18 h later. [^{3}H]Thymidine uptake was measured in a liquid scintillation counter. Data represent the means of quadruplicates. Each experiment was performed at least twice. Data are shown from a representative experiment. The stimulation index was calculated as follows: mean ^{3}H (cpm) in test wells/mean ^{3}H (cpm) of T cells. The same culture conditions were chosen for proliferation assays using MN cells. CM from melanoma cells and purified recombinant human TGFβ1 (rTGFβ1), rTGFβ2 or rTGFβ3 (R&D Systems, Minneapolis, Minn.) were added at the concentrations indicated when cultures were initiated. The inhibition of stimulated MN cells is presented as the effective dose that inhibits the proliferation by 50% (ED_{50}). The TGFβ concentration was plotted against the [^{3}H]thymidine counts of stimulated MN cells and the ED_{50} values were obtained by regression analysis. The thymidine assays using MN cells shown are representative of two experiments.

Tumorigenicity

Three days after in vitro transduction, mB7-l-transduced and untransduced murine K1735 melanoma cells (1×10^6 cells) were injected subcutaneously into the right flank of syngeneic C3H mice (ten mice/group). Animals were monitored for tumor growth every other day and tumor sizes were determined by measurement with calipers. Tumor volume was calculated as follows: $V = l\times w^2/2$ (V = volume, l = length, w = width). When tumors reached 1 cm in the largest diameter, the mice were humanely killed. Animals surviving to day 55 were re-challenged with 1×10^6 K1735 cells subcutaneously in the left flank. Naive mice were injected with untransduced K1735 cells (1×10^6 cells/mouse, five mice) as controls in the re-challenge experiment. Animals were monitored for tumor growth until sacrifice or at least for 112 days. The tumorigenicity experiment was repeated using a mB7-l-expressing adenovirus derived from a different adenovirus clone. In the established murine melanoma model, K1735 melanoma cells (1×10^6 cells) were injected into the right flank of C3H mice (ten mice/group). When tumors reached a volume of approximately 27 mm^3, Ad.mB7-1 or Ad.lacZ (1×10^9 p.f.u. in a total volume of 50 μl) was injected at a single site into the tumor from a 27-gauge butterfly needle (Abbott Laboratories, North Chicago, Ill.).

Quantitative bioassay for TGFβ

The TGFβ concentrations in CM from K1735 cells before and after incubation (30 min at room temperature) with a pan-specific TGFβ-neutralizing antibody (R&D Systems) were detected by a quantitative bioassay [1] with minor modifications as described [4]. This assay was performed twice to verify reproducibility. It is based on induction of a truncated TGFβ-responsive promoter of plasminogen activator inhibitors, driving luciferase expression, and transfected into mink lung epithelial cells (a gift from Dr. D.B. Rifkin, New York University, N.Y.). Media containing serial dilutions of rTGFβ1, rTGFβ2 or rTGFβ3 (R&D Systems) were used as standards. Luciferase activity was detected in an ML 10 000 luminometer (Dynatech, Chantilly, Va.). The concentration of TGFβ in the samples was calculated by comparison to a standard curve.

Results

Transduction of murine and human melanoma cells with Ad.mB7-1 and Ad.hB7-1, respectively, leads to high-level transgene expression

As determined by flow cytometry, the murine (K1735) and human (WM9, WM98-1, WM3122, WM3179, WM3268, WM3259, WM793, WM1205, and WM3182) melanoma cell lines did not express B7-1 (Table 1). However, upon in vitro transduction with Ad.mB7-1 at a m.o.i of 1000, more than 98% of K1735 cells expressed mB7-1 after 3 days. The in vitro transduction of human WM9 cells with Ad.hB7-1 at a m.o.i. of 100 similarly yielded high levels of B7-1 expression (more than 98%) after 3 days. One week after in vitro transduction, B7-1 was expressed on 97% of the transduced WM9 cells, indicating the stability of the transgene expression in vitro. Additional human melanoma cell lines (WM98-1, WM3122, WM3179, WM3268, WM3259, WM793, WM1205, and WM3182) expressed B7-1 on at least 75% of the cells 3 days after in vitro transduction with Ad.hB7-1 (Table 1). In addition, selected cell lines were also examined on day 7. Adenoviral transduction at the m.o.i. chosen did not significantly affect cell viability as determined by trypan blue exclusion. To establish the efficiency of in vivo gene transfer, human melanoma WM9 xenografts were established in immunodeficient SCID mice and transduced in vivo by intratumoral injection of Ad.hB7-1. Flow cytometry of dispersed tumors showed that 19%–32% of the cells expressed human B7-1 on days 7, 14 and 21 (Fig. 1). The data represent the mean of two independent experiments. Taken together, these data show that adenoviral transfer induces high-level B7-1 expression in murine and human melanoma cells and that B7-1 expression persists at a constant level for the duration of in vitro assays and for several weeks in vivo.

MHC class I and II expression on human melanoma cells following Ad.hB7-1 transduction

Adenoviral proteins such as the adenoviral 19-kDa (E19) protein are known to down-regulate MHC expression [14]. Although the *E3* gene, which encodes the E19 protein, had been deleted from our adenoviral constructs, we sought to determine whether MHC expression on melanoma cells was modified by adenoviral gene transfer. Except for the WM3259 cell line, all untransduced human melanoma lines expressed MHC class I on their cell surface (Table

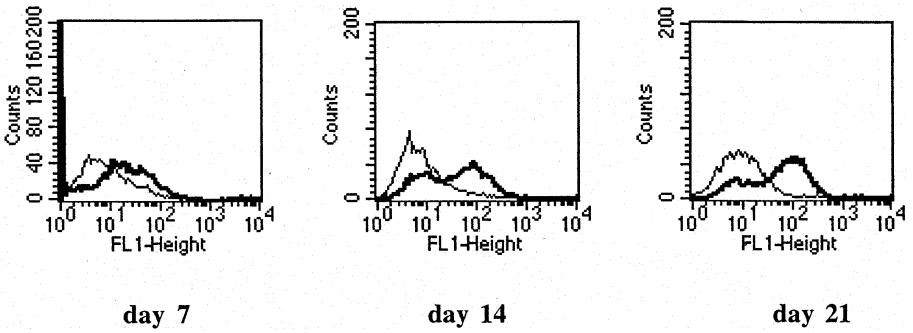

Fig. 1 Flow cytometry of WM9 cells on days 7, 14 and 21 after in vivo transduction with Ad.hB7-1. WM9 cells were injected into severe combined immunodeficient (SCID) mice to establish tumors. On day 0, the tumors were injected with Ad.hB7-1. On the assigned day, mice were killed and tumor cells were enzymatically dispersed. A representative histogram is shown for each assay time

1). Upon adenoviral transduction, all melanoma cell lines remained MHC-class-I-positive, although the number of MHC-class-I-positive cells was reduced in two (WM9, WM3182) of the ten cell lines tested. Four melanoma cell lines expressed no or low levels of MHC class II molecules (WM3259, WM1205, WM3268, and WM3182). The number of MHC class II molecules on MHC-class-II-positive melanoma cells was not significantly altered upon adenoviral transduction. In summary, adenoviral transduction caused a slight reduction of MHC class I expression in select melanoma cells without altering MHC class II expression.

Proliferation of autologous and allogeneic human T cells stimulated with B7-1-expressing melanoma cells

To assess whether adenovirally transduced B7-1 was functional, we tested the ability of transduced human melanoma cells to co-stimulate purified autologous T cells in vitro. In the presence of PHA, three of four cell lines tested (Table 2), transduced with Ad.hB7-1, induced proliferation of autologous T cells, whereas parental cells (B7-1 negative) and Ad.lacZ-transduced cells did not (Table 2, Fig. 2). Note that the stimulation indices of T cells stimulated with B7-1 expressing WM9 cells in PHA-containing medium shown in Fig. 2 and Table 2 differ because they were obtained from separate experiments. The stimulation indices summarized in Table 2 are relative numbers and allow comparison only within one experiment. The dependence on B7-1 signaling was indicated by the ability of the soluble B7-1 antagonist CTLA-4Ig to block B7-1-induced proliferation (Fig. 2). However, in the absence of PHA there was no proliferative T cell response. Failure to proliferate in the absence of PHA is consistent with the absence of signal one. Next, we investigated the ability of human melanoma cells transduced with Ad.hB7-1 to co-stimulate allogeneic human T cells in vitro. As shown in Fig. 3 and Table 2, hB7-1-positive WM9, WM3179, WM98-1 and WM3248 cells provided a strong proliferative signal to allogeneic T cells in the absence of PHA. Variation of allogeneic proliferation was seen when different lymphocyte donors were used (Fig. 3 and Table 2). The stimulation indices are summarized in Table 2. The observed variation of stimulation indices when different tumor cell lines were used (Table 2) is likely a consequence of using lymphocytes from different blood donors. T cells co-cultured with untransduced and Ad.lacZ-transduced cells generally did not proliferate under the same conditions. Thus, adenovirally delivered B7-1 on human melanoma cells can provide functional co-stimulation to T cells.

K1735 cells transduced in vitro have diminished tumorigenicity

To address whether adenovirally encoded mB7-1 affects tumorigenicity in vivo, K1735 cells were transduced with Ad.mB7-1 or Ad.lacZ at a m.o.i. of 1000. After confirming mB7-1 expression by flow cytometry (more than 98%

Table 1 MHC class I and II expression of human melanoma cells before and after adenoviral transduction detected by flow cytometry. MHC class I and II expression was measured on day 3 and B7-1 expression was measured on day 3 or 7

Cell line	Before adenoviral transduction, expression (%) of:				After adenoviral transduction, expression (%) of:			
	B7-1 day 3	B7-1 day 7	MHC class I	MHC class II	B7-1 day 3	B7-1 day 7	MHC class I	MHC class II
WM9	1	0	99	99	99	98	44	75
WM98-1	5	3	99	99	97	92	98	99
WM793	3	2	100	94	98	97	88	91
WM1205	0	ND	100	2	98	ND	94	2
WM3122	1	3	97	97	98	97	92	92
WM3179	0	ND	86	20	75	ND	92	36
WM3182	5	ND	89	16	95	ND	69	10
WM3259	2	ND	1	1	80	ND	1	1
WM3268	3	ND	99	44	77	94	99	54

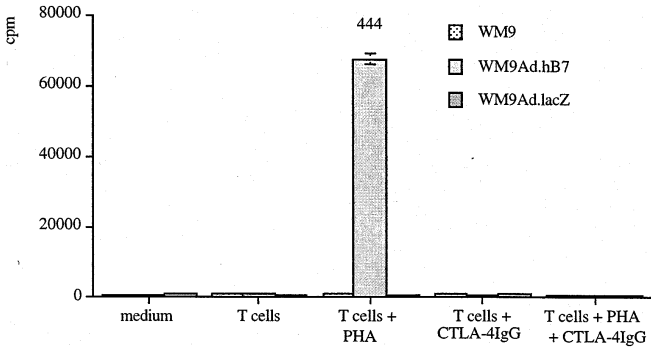

Fig. 2 Thymidine incorporation by autologous T cells co-cultured with Ad.hB7-1-transduced WM9 cells. Stimulation indices (greater than 1) of autologous T cells co-cultured with untransduced or adenovirally transduced WM9 cells are indicated at the top of each bar. Transduced WM9 cells co-stimulate autologous T cells only in the presence of phytohemagglutinin (*PHA*)

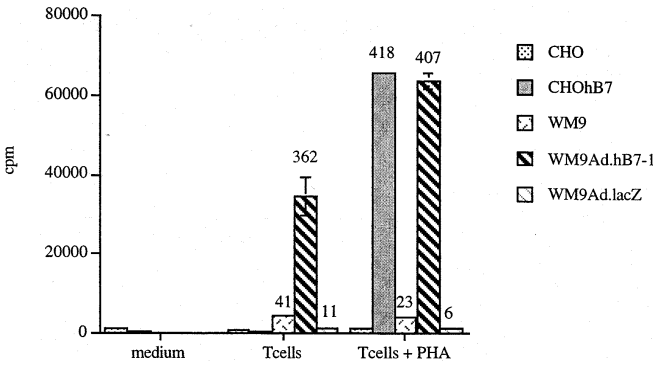

Fig. 3 Thymidine incorporation by allogeneic T cells stimulated with Ad.hB7-1-transduced WM9 cells. Stimulation indices (greater than 1) of allogeneic T cells co-cultured with untransduced or adenoviral transduced WM9 cells are indicated at the top of each bar. Thymidine incorporation demonstrated that WM9 cells transduced with Ad.hB7-1 are able to co-stimulate allogeneic T cells to proliferate. *CHO* Chinese hamster ovary cells

positive, data not shown) 3 days after gene transfer, mB7-1/K1735 cells were injected subcutaneously into the right flank of C3H mice. Mice injected with untransduced K1735 cells or lacZ/K1735 cells served as controls. Mice injected with untransduced tumor cells died or needed to be killed because of their tumor burden within 34 days. All mice injected with mB7-1/K1735 cells and only 30% of the mice receiving lacZ/K1735 cells survived until day 55 (Fig. 4). The animals surviving immunization with mB7-1/K1735 cells (ten mice) or lacZ/K1735 cells (one mouse) were re-challenged with parental K1735 cells injected subcutaneously on the left flank on day 55 after the first tumor cell inoculation. Naive mice injected with the same cells served as a concurrent positive control in the re-challenge experiments (not shown). The experiment was terminated on day 120. The mouse immunized with lacZ/K1735 cells did not survive the re-challenge, whereas three out of ten mice immunized with mB7-1/K1735 cells survived the re-challenge with parental K1735 to day 120. The tumorigenicity experiment was repeated using a different adenovirus clone expressing mB7-1. By day 120, 30% of the animals injected with mB7-1/K1735 cells had developed tumors,

Table 2 Stimulation of allogeneic and autologous T cells responding to B7-1-expressing human melanoma cells in the presence (+) and absence (–) of phytohemagglutinin (*PHA*) T cell proliferation is reported as the stimulation index (mean ^{3}H in test wells/mean ^{3}H of T cells)

Cell line	Stimulation of responding T cells			
	Autologous		Allogeneic	
	– PHA	+ PHA	– PHA	+ PHA
WM9 (–)	0.2	0.3	8.3	1.9
WM9Ad.lacZ	1.3	0.8	0	1.4
WM9Ad.hB7-1	1.2	77.5	163.0	11.2
WM98-1 (–)	ND	ND	0.5	0.5
WM98-1Ad.lacZ	ND	ND	1.8	1.4
WM98-1Ad.hB7-1	ND	ND	14.2	21.7
WM3122 (–)	0.1	1.7	0	4.4
WM3122Ad.lacZ	0	2.1	0	4.7
WM3122Ad.hB7-1	0	2.4	0	14.3
WM3172 (–)	0.4	9.7	0.2	0.2
WM3172Ad.lacZ	0.1	9.1	0.2	0
WM3172Ad.hB7-1	0.5	247.7	2.3	41.4
WM3179 (–)	ND	ND	ND	ND
WM3179Ad.lacZ	0	0	0	ND
WM3179Ad.hB7-1	0	37.4	45.3	ND
WM3248 (–)	ND	ND	ND	ND
WM3248Ad.lacZ	ND	ND	0	0
WM3248Ad.hB7-1	ND	ND	21.2	18.6
WM3268 (–)	ND	ND	ND	ND
WM3268Ad.lacZ	ND	ND	0.3	0.8
WM3268Ad.hB7-1	ND	ND	0.6	20.0

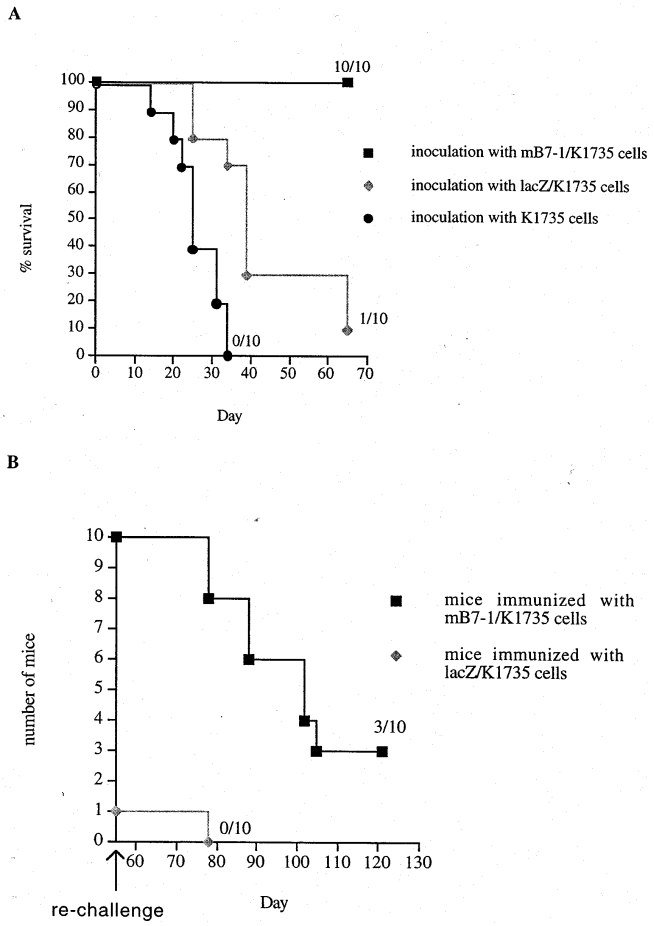

Fig. 4A, B Tumorigenicity and protective immunity of K1735 cells transduced with Ad.mB7-1. **A** Mice were inoculated with live K1735 cells (●), mB7-1/K1735 cells (■) or lacZ/K1735 cells (◆) in the right flank. The numbers at each line represent the fraction of animals surviving initial tumor cell inoculation. By day 34, all mice injected with untransduced K1735 cells had been killed due to their tumor burden. One mouse receiving lacZ/K1735 cells and all mice that had been inoculated with mB7-1/K1735 cells survived. **B** On day 55, mice surviving the initial tumor cell inoculation were re-challenged with live, untransduced K1735 cells in the left flank. Numbers at each line represent the fraction of animals surviving the re-challenge as assessed on day 112. Upon re-challenge, the mouse injected with lacZ/K1735 cells did not survive (◆) and three of the ten animals immunized with mB7-1/K1735 cells demonstrated protective immunity (■)

whereas 100% of the mice receiving lacZ/K1735 cells had developed tumors (data not shown). Taken together, these results demonstrate that expression of mB7-1 on tumor cells leads to their rejection upon inoculation into most or all animals and induces long-lasting systemic immunity in only a minority of mice.

Ad.mB7-1 does not induce rejection of established K1735 tumors

To address the efficacy of B7-1 transfer in an in vivo gene-transfer setting, we determined the ability of Ad.mB7-1 to eradicate established K1735 tumors following intratumoral

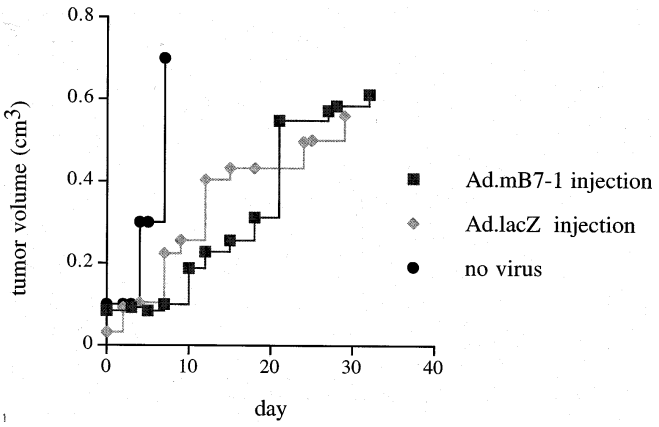

Fig. 5 Tumorigenicity of K1735 tumors after adenoviral injection into established tumors. The tumor growth rate of adenovirus (Ad.mB7-1 or Ad.lacZ)-injected tumors is diminished, but there was no regression of established K1735 tumors

injection of Ad.mB7-1 or Ad.lacZ. All animals treated this way had to be killed by day 33 after inoculation because of progressive tumor growth. There was no difference in the rates of tumor growth between animals injected with Ad.mB7-1 and Ad.lacZ-injected animals (Fig. 5). Tumors injected with either adenoviral vector did grow more slowly than uninjected tumors (the mean time to death being 20 days in adenovirus-injected mice compared to 6 days in uninjected mice, Fig. 5). These results indicate that expression of mB7-1 on established tumors is insufficient to induce tumor regression.

Melanoma cells produce soluble factors that inhibit the proliferation of murine T cells and human mononuclear cells

One mechanism by which tumor cells can down-modulate immune responses is the production of immunosuppressive factors [41]. Earlier work has shown that K1735 cell extracts contain T-cell-inhibitory activities [31]. To account for soluble factors secreted by K1735 cells that could counteract antitumor immunity elicited by B7-1 expression, we determined whether CM from K1735 cells affected proliferation of murine and human T cells or MN cells. As shown in Fig. 6A, several dilutions of CM from K1735 cells strongly inhibited thymidine incorporation of murine T cells stimulated with PMA/ionomycin. Titration of this effect showed that proliferation of stimulated T cells was blocked by CM from K1735 cells up to a dilution of 1:4000. Furthermore, an antiproliferative effect was observed when CM from K1735 was added to human MN cells activated by PHA (data not shown). Similarly, CM from human WM9 cells inhibited the proliferation of human MN cells stimulated with PHA (data not shown). By contrast, CM from the human metastatic melanoma cell lines WM3179 and WM3122 did not inhibit the proliferation of mitogen-stimulated MN cells significantly (data not shown). Thus,

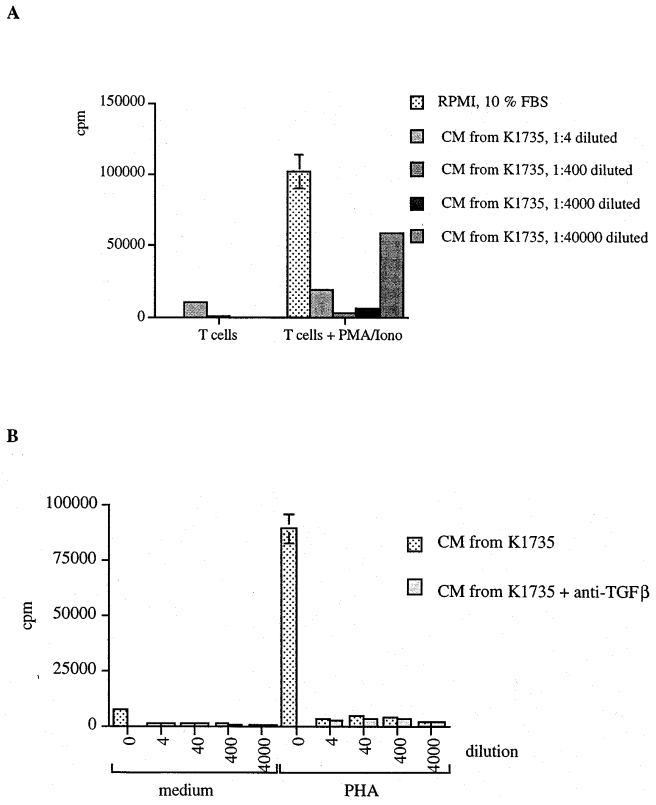

Fig. 6A T cell proliferation in the presence of conditioned medium (*CM*) from K1735 cells. K1735 cells produce soluble factor(s) that inhibit the proliferation of murine T cells. **B** Thymidine incorporation by mononuclear (MN) cells incubated with transforming-growth-factor-β(TGFβ)-depleted CM from K1735 cells. K1735 CM used in **A** was collected after the cells had been cultured for 24 h in medium containing 10% fetal bovine serum (*FBS*), whereas K1735 CM used in **B** was collected after the cells had been cultured for 3 h in serum-free medium. Pre-treatment of CM from K1735 cells with a TGFβ-neutralizing antibody does not alleviate the inhibitory effect on the proliferation of PHA-stimulated MN cells. *PMA/Iono* phorbol myristate acetate/ionomycin

the murine melanoma K1735 cell line and human WM9 cell line secrete soluble factors capable of inhibiting T cell proliferation.

TGFβ does not account for the inhibitory effect of the CM from K1735 cells

TGFβ is an immunomodulatory cytokine known to be expressed by humans [35, 45] and mice [6]. Melanoma cells secrete primarily biologically inactive, latent TGFβ in vitro, which consists of TGFβ complexed with part of its precursor polypeptide, which can be converted to the active form by proteolytic cleavage [35]. In support of a potential immunosuppressive role of melanoma-derived TGFβ in our system, we observed that bioactive human rTGFβ1, rTGFβ2 or rTGFβ3 inhibited proliferation of PHA-stimulated MN cells in a dose-dependent manner. The inhibition of the proliferation of PHA-stimulated MN cells is reflected by the ED_{50} values of rTGFβ1, rTGFβ2 or rTGFβ3 ranging between 0.1 ng/ml and 1.5 ng/ml (data not shown). Similar results were obtained when PMA/ionomycin was used for the activation of MN cells (data not shown). We therefore determined whether tumor-derived, secreted TGFβ could contribute to the antiproliferative effects of CM from K1735 cells on MN cell and T cell proliferation. The concentrations of TGFβ produced by K1735 cells were determined by luciferase bioasssay. CM from K1735 cells was collected from cells exposed to serum-free medium for 3 h. Serum-free medium was used for the detection of TGFβ because this function is altered by the presence of growth factors and retinoids that are present in serum. Under serum-free conditions, K1735 cells did not change their morphology, growth rate and their ability to produce inhibitory factors. As measured by bioassay, 3×10^6 K1735 cells (in 5 ml medium) secreted total TGFβ protein at a level of 4.5 ng/ml, of which less than 3% (0.1 ng/ml) was in the bioactive form. When CM from K1735 cells was diluted 1:4000, the bioactivity of TGFβ was below the threshold of detection of the bioassay (20 pg/ml), indicating that TGFβ produced by K1735 cells does not account for the immunoinhibitory effect since the inhibitory effect could be seen at this dilution. In addition, the elimination of TGFβ from CM from K1735 cells with a TGFβ pan-specific neutralizing antibody failed to reverse the inhibition of the proliferation of mitogen-stimulated MN cells (Fig. 6B).

Discussion

Gene-transfer-based vaccination has recently emerged as a promising avenue to elicit immune responses to malignant tumors. The application of these strategies to human tumors requires efficient gene delivery systems. Adenoviral gene delivery provides an attractive option to direct gene expression to tumor cells because a wide variety of tumor cell types can be transduced at high efficiency, adenoviruses can be produced at high titers and they have low toxicity [5]. Using adenovirus vectors to express the co-stimulatory molecule B7-1 on murine and human melanoma cells, we were able to transduce a high fraction of human and murine melanoma cells in vitro. In addition, we were able to transduce 19%–32% of tumor cells within an established tumor (27 mm^3) by intratumoral injection. This compares favorably with gene delivery by liposomes [40], by retroviruses [46] or in the form of naked DNA [40]. We further demonstrate that adenoviruses encoding the co-stimulatory B7-1 molecule can be successfully used to elicit immune responses to human and murine melanoma in vitro and in vivo. We have addressed a number of potential problems associated with the use of adenovirus vectors as expression vectors and the generation of B7-expressing melanoma vaccines. The modulation of MHC class I expression required for antigen presentation was examined. We provide evidence of the function of B7-1 expressed after adenoviral transduction on K1735 cells. Furthermore,

tumor-derived inhibitory factor(s) that interfere with T cell responses were detected.

The adenoviral 19-kDa protein (E19), which is encoded by the *E3* gene is known to down-regulate MHC class I expression [14]. Down-regulation of MHC class I on virally transduced cells may serve to impair antigen presentation and inhibit the immune response to transduced cells. To avoid this potential problem, the vectors used in the present study have *E3* deletions. Only slight down-regulation of MHC I expression was observed in only two of ten transduced human melanomas. MHC expression in those two cell lines was reduced but not abolished by adenoviral transfer. These results suggest an E19-independent mechanism by which adenovirus can affect MHC expression; however, this effect is small.

The second major consideration for B7-l-mediated immunotherapy is the ability of transduced cells to express functional B7-1 and to co-stimulate T cells. We demonstrated that adenovirally encoded B7-1 is functional, as determined by its ability to provide co-stimulation to autologous and allogeneic T cells. Four B7-l-transduced cell lines stimulated autologous T cell proliferation in the presence of PHA although not in the absence of PHA. Yang and colleagues were able to demonstrate cytotoxic T lymphocyte activity against melanoma cells using autologous peripheral blood lymphocytes cultured in vitro with freshly isolated and transduced B7-1-expressing tumor cells [49]. The different in vitro systems and different T cell responder populations may account for the difference in the responses seen in these two studies. In addition, the patient donors used in our studies had their T cells collected and tested at least 3 years after surgical removal of the melanoma metastases. Thus, it is likely that the frequency of T cells that are capable of recognizing melanoma antigens was diminished. All of the human melanoma cells transduced with Ad.hB7-1 stimulated allogeneic T cell proliferation in medium containing PHA (Fig. 2, Table 2). Five of seven Ad.hB7-1-transduced cell lines exhibited an allogeneic proliferative response in the absence of PHA. Thus, functional co-stimulation as assessed in vitro was achieved using our vector system.

To our knowledge, this is the first study that investigates the tumorigenesis of adenovirus-mediated B7-1 expression on K1735 cells. We have shown that Ad.mB7-l-transduced tumor cells were less tumorigenic than untransduced tumor cells or tumor cells transduced with Ad.lacZ. There was no tumor growth in the mice receiving mB7-1/K1735 cells under conditions where untransduced cells were fatal. The group of animals receiving lacZ/K1735 cells demonstrated delayed tumor growth compared to mice injected with untransduced tumor cells, and one mouse survived the initial tumor inoculation. The prolonged survival generated in mice exposed to lacZ/K1735 cells suggests a role for viral antigens or β-galactosidase in the induction of an immune response. Others have demonstrated an immune response to tumors expressing β-galactosidase [11, 19] and this may be further augmented by the co-expression of viral antigens [50]. Tumor cells transduced with Ad.mB7-1 provided protection against re-challenge with parental tumor cells that did not express viral proteins. This protection is consistent with an immune response to tumor-associated antigens. The response to viral antigens may have contributed to the protection. However, we have previously shown that mB7-1 expression in the absence of viral antigens can induce a protective immune response to parental K1735 cells [12]. Several studies address the inability of B7-l-expressing melanoma cells to induce systemic immunity. Mice that rejected B7-1-expressing B16 melanoma cells were not protected against re-challenge by wild-type cells [48]. It has been postulated that B7-1 expression on tumor cells confers direct killing by NK and $CD8^+$ T cells resulting in tumor rejection but not in the induction of systemic immunity. Li et al. demonstrated protection and short-lasting immunity against K1735 melanoma cells only when mice were immunized with tumor cells co-expressing B7-1 and CD48 [24]. Townsend et al. studied the induction of protective immunity in detail [43]. By day 60 after immunization with B7-l-expressing K1735 cells, the protective immunity against re-challenge with parental tumor cells was reduced by 50%. Others found that re-challenge with CL62 melanoma cells on day 60 was effective in inducing systemic immunity [30]. Taken together, these results suggest that induction of immune responses to viral antigens does not impair development of specific anti-melanoma immune responses and immunity can be developed after re-challenge on day 55.

In reference to the potential of adenoviral vectors as vaccine vehicles, we demonstrate that adenoviral transduction of K1735 melanoma cells with mB7-1 results in tumor rejection in vivo and the generation of an effective memory response. The ability of B7-1 expression on tumor cells to induce tumor rejection has been reported to depend on the immunogenicity of the tumor cell line used [9]. Specifically, different investigators have reported varying results in different murine tumor model systems. Chen and colleagues retrovirally transfected both immunogenic (E6B2) and non-immunogenic (B16) melanoma cells to express mB7-1 and showed complete rejection of E6B2 cells and complete tumorigenicity of B16 cells. Chen and colleagues also observed no effect of forced B7-1 expression on the tumorigenicity of K1735 cells [8]. In contrast, Townsend and Allison demonstrated complete rejection of K1735 cells that were transfected with a B7-1 expression vector [42]. We have shown previously that 70% of mice injected with retrovirally transduced mB7/K1735 cells survived [12]. Finally, Chong and colleagues reported a delayed appearance of tumors when mB7/K1735 cells transfected by calcium phosphate precipitation were used with only 15% survival [10]. The differences in protection from tumorigenicity may be due to underlying variations in the tumorigenicity of the different K1735 clones used and/or in different levels of transgene expression [10]. Nevertheless, compared with other vector systems, adenoviral transduction of K1735 cells with mB7-1 provides a viable option to elicit effective co-stimulation and suppress tumorigenicity in this tumor model.

Although Ad.mB7-1 transduction caused complete rejection of the live B7-l-transduced cells, only partial pro-

tection (30%) was achieved after re-challenge of these mice with parental tumor cells. Other investigators have described varying degrees of systemic immunity from live vaccines expressing B7-1 [10, 17, 23]. Chong and colleagues even reported a decrease in systemic immunity to B7-1/K1735 cells as compared to parental K1735 cells [10]. Furthermore, established K1735 tumors (27 mm^3) intratumorally injected with either adenovirus construct progressed more slowly than the untransduced tumors (Fig. 5). The in vivo transduction of K1735 tumors with Ad.mB7-1 did not result in tumor regression and there was no difference in growth rate from that of Ad.lacZ-injected controls. Previously we have shown that interleukin-12 (IL-12) could augment the efficacy of prophylactic vaccination with B7-l-expressing tumor cells [12]. However, when intratumoral injection of K1735 tumors was combined with the administration of recombinant IL-12 (125 ng/dose, daily doses on days 2–6 and on days 9–13 after virus injection), we did not see a growth rate or survival difference between mice treated with Ad.mB7-1 and IL-12 and the control mice (data not shown). Therefore, vectors that confer B7-1 expression combined with IL-12 treatment may not be as useful in promoting rejection of established tumors as previously hoped [32, 33].

One possible explanation for this is that the tumours established at the time of injection were growing too quickly to be eliminated by an immune response engendered by the Ad.mB7-1 injection. Alternatively, established tumors may secrete higher concentrations of tumor-derived factors (compared to tumor cell vaccines) and thereby limit the effectiveness of the immune response. Production of immunosuppressive mediators by tumor cells is a widespread phenomenon [41] and could limit the efficacy of intratumoral immunization. Therefore, we investigated whether murine and human melanoma cells produce and secrete immunosuppressive factors that could interfere with an effective T cell response. We demonstrate that CM from murine K1735 cells and human WM9 cells inhibit the proliferation of human MN cells stimulated by PHA. This inhibition was shown to be potent, as this activity was observed at CM dilutions exceeding 1:4000. Melanoma cells and other tumor cells secrete a variety of immunosuppressive factors including TGFβ [21], IL-10 [47], prostaglandins [22], leukotrienes [36], colony-stimulating factor [51] and others [15, 26, 31, 34].

In this study we have excluded any role for melanoma-derived, secreted TGFβ in inhibiting T cell proliferation. Prostaglandin E_2 (PGE_2), another known tumor-derived immunosuppressive factor [22], is not produced by K1735 cells at detectable levels, as analyzed by PGE_2/enzyme immunoassay. The exact nature and mechanism of the factor(s) secreted by K1735 cells remain under investigation. Specific strategies to circumvent the immunosuppressive effects of individual tumors may need to be devised for effective B7-1-mediated immunotherapy.

Acknowledgement This work was supported by Public Health Service grants PO1-CA25874 and R01-CA67776 from the National Institutes of Health.

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