Cancer Gene Therapy (2008) 15, 742–749
 0929-1903/08 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

Polyethylenimine-mediated *in vivo* gene transfer of a transmembrane superantigen fusion construct inhibits B16 murine melanoma growth

G Jeudy[1,2], F Salvadori[1], B Chauffert[1,3], E Solary[1], P Vabres[2] and J Chluba[1]

[1]*Inserm U866, IFR100, Faculté de Médecine, Dijon, France;* [2]*Dermatology Department, CHU Le Bocage, Dijon, France and* [3]*GF Leclerc Regional Anticancer Center, 1 rue du Professeur Marion, Dijon, France*

Immunotherapy has been proposed as a therapeutic strategy in advanced-stage melanomas in which other therapeutic options have little effect. The Staphylococcus enterotoxin A (SEA) has been used to stimulate an antitumoral immune response but its use is hampered by severe systemic side effects. Here, we show that SEA can be targeted to melanoma cells to limit these side effects. More specifically, we used a nonviral vector, the cationic polymer, polyethylenimine (PEI), to express a transmembrane SEA fusion construct (pSEA-TM) in B16F10-induced subcutaneous melanoma in mice. The efficacy of this *in vivo* transfection was enhanced by concomitant infusion of epinephrine to induce local vasoconstriction. In these conditions, repeated injections of pSEA-TM/PEI complexes elicited a significant response, as evidenced by tumor growth inhibition, without systemic adverse effects. T cell infiltration of the tumors, together with positive lymphocyte proliferation tests, suggested local and systemic immune responses. Altogether, PEI-mediated targeting of SEA to melanoma tumor cells *in vivo* efficiently stimulates the antitumor immune response without inducing the side effects observed with systemic administration of SEA.
Cancer Gene Therapy (2008) **15,** 742–749; doi:10.1038/cgt.2008.42; published online 11 July 2008

Keywords: melanoma; superantigen; Staphylococcus enterotoxin A (SEA), polyethylenimine (PEI), epinephrine

Introduction

The incidence of cutaneous melanoma as well as mortality from the disease are both increasing in western countries.[1–3] In the advanced stages, the efficacy of common therapeutic approaches such as chemotherapy and radiotherapy remains limited. As a result, the median survival of patients with metastatic melanoma is typically lesser than 12 months. In this setting, immunotherapy was proposed as an alternative approach,[4] and several strategies have been tested to increase tumor cell antigenicity and to stimulate antitumor immune cells.[5–8] For example, autologous T lymphocytes genetically engineered *ex vivo* can induce durable regression of established tumors when re-injected into patients.[9] To avoid *ex vivo* cell culture and engineering, alternative approaches, such as *in vivo* expression of immunostimulatory molecules in tumor cells have been proposed. Such techniques may be especially appropriate in cutaneous melanoma tumors, which are easily accessible for *in situ* treatment.

Superantigens (SAgs) are potent activators of $CD4^+$ and $CD8^+$ T cells. They bind to major histocompatibility complex (MHC) class II molecules at the surface of antigen-presenting cells and the complex interacts with a panel of T-cell β-chain regions. They cause rapid and massive proliferation of a large fraction of T cells (2–30%), together with the secretion of cytokines (for example, IFNγ, TNFα, IL-2 and IL-12).[10–12] This systemic T-cell activation and the induction of pro-inflammatory cytokines result in severe toxicity and is implicated in several human diseases, including toxic shock syndrome, scarlet fever and food poisoning.[13] Moreover, the applications of native SAg for cancer therapy are predicted to be hampered by missing expression of MHC class II in tumor cells and absence of therapeutic effects in mice injected with native Staphylococcus enterotoxin A (SEA) was reported.[14]

Different approaches using SAg have been tested to substitute the MHC II presentation and to enhance specific immunogenicity of tumor cells. SAgs were fused to targeting antibodies,[15–17] or expressed in tumor cells *ex vivo* to prepare tumor cell vaccines.[18–21] Another strategy consists in direct transfection of a SAg into the tumor,

Correspondence: Professor J Chluba, 6 Bld Gabriel, Dijon 21000, France.
E-mail: Johanna.Chluba@u-bourgogne.fr
Received 11 January 2008; revised 16 April 2008; accepted 17 May 2008; published online 11 July 2008

which elicits local and systemic immune responses and leads to tumor regression without systemic toxicity.[22–24]

The present study was designed to test the ability of a synthetic cationic polymer, polyethylenimine (PEI), to deliver a SAg, namely the SEA, to tumor cells *in vivo* and to induce an antitumor immune response and tumor regression. For that purpose, a transmembrane SEA fusion construct (pSEA-TM) was complexed with PEI and injected, in the presence of epinephrine to improve *in vivo* transfection efficiency, into tumors induced by subcutaneous injection of a melanoma cell line into syngeneic mice.

Materials and methods

Cell culture

B16F10, a mouse melanoma cell line derived from C57BL/6 mice, was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA) and cultured at 37 °C in a humidified 5% CO_2 incubator in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, Cergy Pontoise, France) supplemented with penicillin at 100 U/ml, streptomycin at 100 µg/ml and amphotericin B at 250 ng/ml (Invitrogen, Cergy Pontoise, France).

SEA transmembrane expression vector

SEA gene from *Staphylococcus aureus* strain (Genbank accession number: M18970) was cloned in pTZ18R vector (a generous gift from Dr J Kappler, Denver, Colarado). A 707 bp fragment of the gene was amplified by PCR with primers including BamHI and XhoI restriction sites (forward primer: 5′-CCGCTCGAGACTTGTATATA AATAGAT-3′ and reverse primer 5′-CGCGGATCCA TGGAGAAAAGCGAA-3′) and subcloned into pDisplay (Invitrogen, Cergy Pontoise, France) which contains the human platelet derived growth factor receptor transmembrane sequence. The sequence of the resulting construct (pSEA-TM) was checked and plasmid DNA was amplified using the Endo Free Plasmid Maxi Kit (Qiagen, Courtaboeuf, France).

In vitro *transfection assays*

B16F10 cells were seeded on 24-well culture plates (900 µl of medium with 10% serum per well) 48 h before transfection to reach 60–70% confluence at the time of transfection. 2 µl of linear PEI (JetPEI, Ozyme, France) were added into 50 µl of 150 mM NaCl and complexed with 1 µg of pSEA-TM vector into 50 µl of 150 mM NaCl (final volume 100 µl) with a ratio of JetPEI to DNA phosphate of 5 (N/P = 5). The solution was homogenized and incubated for 15 min at room temperature, then directly added to the cells. The pDisplay empty vector was used as a control.

Indirect immunofluorescence

To check for SEA anchoring on the cell membrane, B16F10 cells were cultured on microscope slides (Sonic Seal, Dutscher, Brumath, France) and fixed in methanol at 4 °C for 10 min. Rabbit anti-SEA serum (Sigma-Aldrich, Lyon, France) was used at 1:20 dilution in phosphate buffered solution and flooded on cells at 4 °C for 1 h, rinsed and incubated with the second antibody (FITC-coupled rat anti-rabbit mAb, Dako, Trappes, France) at 4 °C for 1 h in the dark and visualized by fluorescence microscopy (Leica) or flow cytometry (Becton Dickinson, Le Pont de Claix, France).

In vivo *transfection assays*

The use of 6–8-weeks-old female C57BL/6 mice (Charles River Laboratories, L′Arbresle, France) housed under standard conditions (25 °C, 50% relative humidity, 12-h light/dark cycles) was approved by the local ethical committee. To establish subcutaneous tumors, 2×10^5 B16F10 cells in 200 µl of RPMI medium were injected subcutaneously into the left flank of each isofluorane-anesthetized mouse. 2 µl of *Invivo* JetPEI (Ozyme, St Quentin-en-Yvelines, France) were complexed with 10 µg of DNA in a 5% glucose solution (N/P = 10). The solution was quickly homogenized and left for 15 min at room temperature, then injected into the tumor (100 µl in 60 s). To determine the optimal conditions for *in vivo* gene transfer, we used a luciferase reporter vector, pEGFP/Luc (Clontech, Saint-Germain-en-Laye, France). When the tumors reached a diameter of 6–8 mm, a 29-G syringe (100 µl in 60 s) was used to inject into them either DNA/PEI complexes, DNA/PEI complexes added with epinephrine (1 mg/l) (Aguettant, Lyon, France) or PEI alone in 5% glucose solution. The mice were killed 24 h later and the tumors were collected, snap frozen, resuspended in reporter lysis buffer and homogenized with an Ultra-Turrax T25 (Janke & Kunkel, Germany) at 20 000 rpm for 30 s, lysed by three (−80 /37 °C) freeze/thaw cycles and the homogenates were centrifuged at 14 000 *g* for 4 min at 4 °C. 10 µl supernatant aliquots were mixed with 50 µl of luciferase assay substrate. Luciferase activity was measured on a luminometer (Microlite TLX1, Dynatech, France). Results are expressed as relative light units integrated over 10 s per mg of protein, using the bicinchoninic acid assay (Pierce, Paris, France). Luciferase activity observed in PEI alone control group represents luciferase background.

Tumor growth inhibition

When tumor diameters reached 6–8 mm, the mice were randomly assigned to one of four experimental groups (pSEA-TM/PEI complexes, empty pDisplay vector/PEI complexes, PEI alone and 5% glucose alone). Each group consisted of ten mice, which received intra-tumoral injections every fourth day, for a total of three injections, all of which included epinephrine. Tumor growth was monitored by calculating the tumor volume using the formula $V = lw^2/2$ where V is the volume, l is the length and w is the width.[25] When the tumor volume had reached 1500 mm^3 in glucose-treated mice, all mice were killed using isofluorane inhalation.

Histological and immuno-fluorescence analysis of tumors

Part of the collected tumors was formol-fixed and embedded in paraffin before hematoxylin-eosin staining and conventional microscopy examination. Part of the tumors was snap frozen in Tissue-Tek OCT compound (Euromedex, Souffelweyersheim, France), cryosectioned to a thickness of 7 μm, fixed in acetone, incubated with FITC-labeled anti- CD90/Thy-1 mAb (T cells) (Caltag Laboratories, Hamburg, Germany) at 1:25 dilution in phosphate buffered solution for 1 h in the dark and analyzed with a Leica fluorescence microscope.

Reverse transcriptase-polymerase chain reaction

Tumors snap frozen in liquid nitrogen were homogenized with an Ultra-Turrax T25 at 20 000 rpm for 30 s, and mRNA was isolated with Trizol reagent (Invitrogen, Cergy Pontoise, France). All RNA samples were treated with DNase 1 (Invitrogen, Cergy Pontoise, France) and reverse transcription was performed with the One-Step SuperScript II reverse transcriptase-polymerase chain reaction (RT-PCR) system (Invitrogen, Cergy Pontoise, France) using 1 μg of total RNA and amplification was done by standard PCR using the same primers as those used in SEA cloning. pSEA-TM containing plasmid was used as a positive control (100 pg plasmid/reaction) and β-actin cDNA PCR was used as a loading control (forward primer: 5′-AATTCATGGAGACAGACACAC TCC-3′, reverse primer: 5′-GATCCGTTTGGAGAAAG AGGTCAT-3′, PCR product: 486 bp). The PCR conditions using Taq DNA polymerase (Eppendorf, Le Pecq, France) were: initial denaturation at 94 °C for 2 min, amplification by 35 cycles (94 °C for 45 s, 59 °C for 45 s and 72 °C for 45 s) and final elongation at 72 °C for 10 min. The PCR products were separated on 1% agarose gel in Tris acetate EDTA buffer containing 0.5 μg/ml ethidium bromide.

Measurement of lymphocyte proliferation

The spleens were dissociated and splenocytes were harvested and cocultured with mitomycin-inactivated B16F10 cells (50 μg/ml for 1 h) and pSEA-TM transfected B16F10 cells at a ratio of 1:1 (10^5 cells per well) for 24 h in a 96-well plate. The lymphocyte proliferation rate was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Celltiter 96 Aqueous one solution cell proliferation assay, Promega, Cergy Pontoise, France). The absorbance was read at 490 nm on a Bio-Rad microplate reader.

Statistical analysis

All experiments were performed in triplicate and results were presented as mean values ± s.e.m. of at least three independent experiments. Student's *t*-tests and non parametric Mann–Whitney tests were used to determine statistical significance (*P*-value at least <0.05).

Results

Expression of a functional SEA in pSEA-TM transfected B16F10 cells

A 707 bp fragment of the SEA gene from *Staphylococcus aureus* was fused with the transmembrane (TM) sequence of human Platelet-Derived Growth Factor Receptor. The functionality of the pSEA-TM encoding plasmid was checked by transfection in B16F10 murine melanoma cells, with the empty vector being used as a control. The expression of SEA at the surface of transfected cells was assessed by indirect immunofluorescence microscopy and flow cytometry using a rabbit polyclonal anti-SEA antibody (Figures 1a and b). When co-cultured with mouse splenocytes, SEA-expressing B16F10 induced a significantly higher level of T cell proliferation than did B16F10 cells transfected with the empty vector (Figure 1c; $P<0.001$).

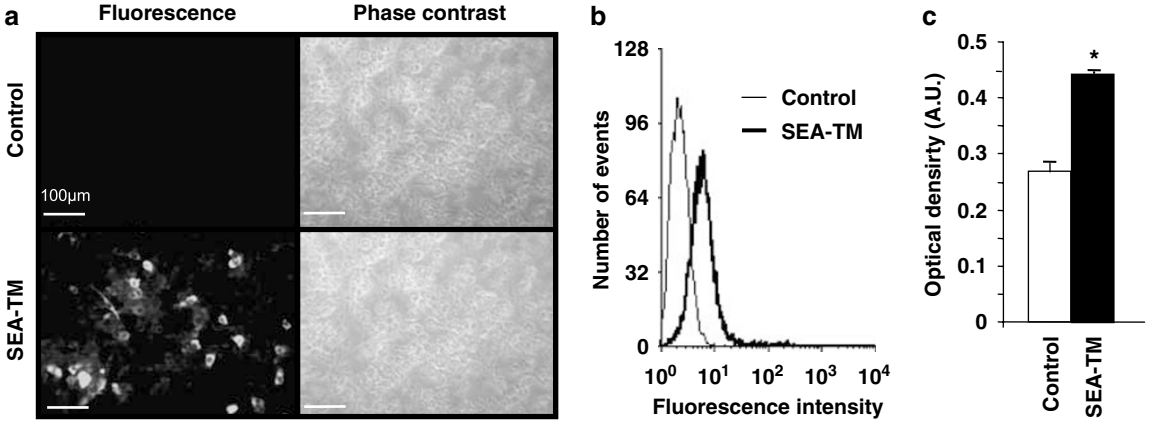

Figure 1 Expression of a functional SEA in pSEA-TM transfected B16F10 cells. B16F10 cells cultured *in vitro* were transfected with either the empty vector (control) or the pSEA-TM (SEA-TM) encoding plasmid, complexed to PEI. (**a**) Phase contrast and indirect immunofluorescence analyses of transfected cells, 24 h after transfection, using a polyclonal anti-SEA rabbit antibody. Scale bar = 100 μm. (**b**) Cells labeled with the rabbit anti-SEA antibody as in a were identified by flow cytometry. (**c**) Splenocytes were isolated from B16F10-tumor bearing mice and co-cultured at 1:1 ratio with mitomycin-inactivated control and SEA-TM expressing B16F10 cells before measuring cell proliferation using a 24 h MTS assay. Number of animals = 3. Each measurement was made in triplicate. Means ± s.e.m. are shown. *$P<0.001$, Student's *t*-test. SEA, Staphylococcus enterotoxin A; pSEA-TM, transmembrane SEA fusion construct; PEI, polyethylenimine.

Conditions for efficient gene transfection in mouse tumors

We used a luciferase reporter vector (pEGFP/Luc) to determine the best conditions for transfection of a nonviral construct using *invivo*JetPEI in melanoma tumors induced by subcutaneous injection of B16F10 cells into syngeneic mice. By measuring luciferase activity 24 h after transfection, the optimal efficacy was obtained by combining 10 μg of cDNA with 2 μl of PEI (N/P = 10) (Figure 2). To improve this efficacy, we used epinephrine, a vasoconstrictor commonly associated with local anesthetic agents to increase exposure length and efficacy. When combined with DNA/PEI, epinephrine (1 mg/l) significantly enhanced intratumoral transfection (Figure 2) without inducing any toxic side effect. These conditions, including addition of epinephrine, were those used in every subsequent experiment.

Intratumoral SEA inhibits tumor growth in mice

We then determined the *in vivo* antitumoral effects of SEA through the injection of pSEA-TM/PEI complexes into growing tumors, 10 days after tumor cell inoculation. Three control groups consisted of mice whose tumors were injected with empty vector/PEI complexes, with PEI alone and with 5% glucose solution, respectively. In order to ensure sufficient expression of SEA in tumors for the duration of the experiment, injections were repeated every fourth day. RT-PCR analysis permitted selective detection of pSEA-TM mRNA in tumors injected with the corresponding plasmid (Figure 3a). Four days after the first injection, a significant difference in tumor growth was observed between the SEA-TM treated and control groups, and this difference increased with time. At the end of the experiment, the mean volume of pSEA-TM treated tumors had decreased by 70–84% when compared with glucose treated tumors. Although to a lesser degree than that observed in tumors injected with pSEA-TM/PEI complexes, significant tumor growth reduction was observed in mice injected with the empty vector/PEI complex or with PEI alone as compared with those injected with glucose. (Figure 3b; $P<0.05$).

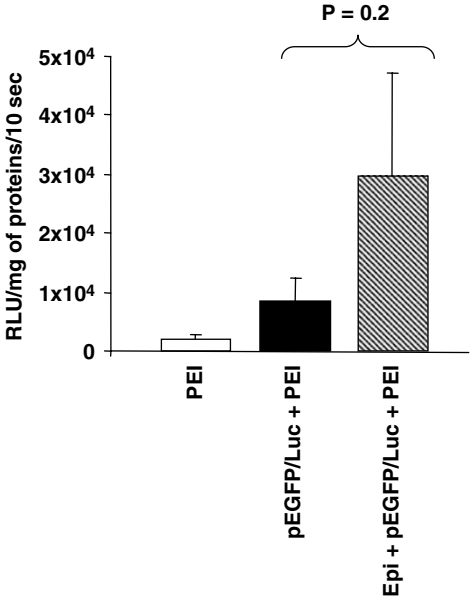

Figure 2 Optimal conditions for gene transfer into melanoma tumors in mice. Melanoma tumors obtained by subcutaneous injection of B16F10 cells in 8 week-old female, C57BL/6 mice were infused with 10 μg of pEGFP/Luc vector complexed with 2 μl of *in vivo* jetPEI, in the presence or absence of 1 mg/l epinephrine. Control tumors received only PEI and glucose 5%. Tumors were removed 24 h after intratumoral injection and assayed for luciferase activity. Results are expressed as RLU integrated over 10 sec per mg of protein and represent the mean ± s.e.m. values of three experiments in which each measurement was performed in triplicate ($P=0.2$; pEFGPLuc/PEI + Epinephrine compared with pEFGPLuc/PEI, Mann–Whitney test). PEI, polyethylenimine; RLU, relative light units.

Intratumoral SEA triggers a cellular inflammatory response

Histological analysis of hematoxylin/eosin stained tumor sections revealed diffuse infiltration of the tumor with

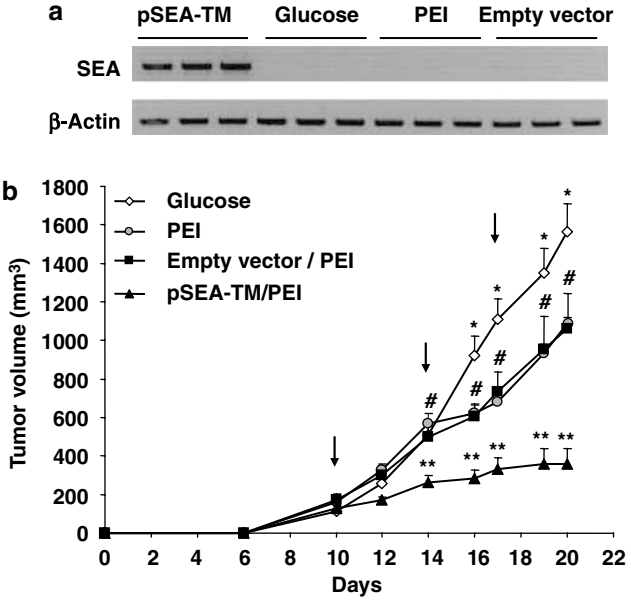

Figure 3 Intratumoral pSEA-TM gene delivery inhibits tumor growth in mice. Melanoma tumors were obtained by subcutaneous injection of B16F10 cells in C57BL/6 mice and were injected at day 10 with either pSEA-TM/PEI complexes or the empty vector/PEI complexes or PEI alone or 5% glucose solution. (**a**) Tumors were removed 20 days after the first injection and SEA-TM mRNA expression was analyzed by RT-PCR. β-actin mRNA was used as a control. Results from three mice per group are shown. (**b**) Injections were repeated every fourth day (arrows) and tumor volume was measured at regular time points until sacrifice. (▲) pSEA-TM/PEI complexes; (■) empty vector/PEI complexes, (○) PEI alone, (◇) 5% glucose solution. $n=10$ per group. ($^{**}P<0.001$; $^{\#}P<0.05$: significant tumor growth inhibition of pSEA-TM group compared with the 5%-glucose-solution group and to empty vector/PEI and PEI groups, respectively; $^{*}P<0.05$: significant tumor growth inhibition of empty vector/PEI and PEI groups compared with 5% glucose solution group, Mann–Whitney test). pSEA-TM, transmembrane SEA fusion construct; PEI, polyethylenimine; RT-PCR, reverse transcriptase-polymerase chain reaction.

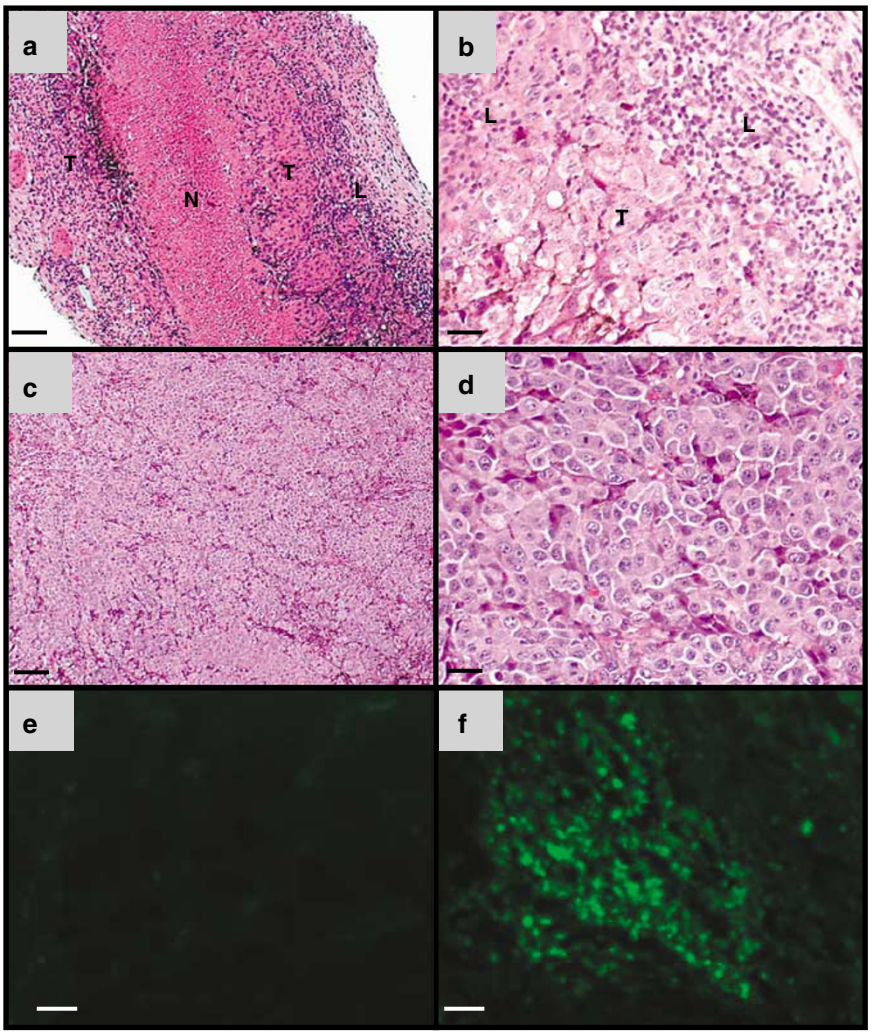

Figure 4 Histological and fluorescence analysis of tumors. (**a**–**d**) Tumors were removed, formol-fixed and embedded in paraffin before hematoxylin-eosin staining. (**a** and **b**) Tumors injected with pSEA-TM/PEI complexes were collected 20 days after injection. T: tumor tissue; L: lymphocytes; N: necrosis. (**c** and **d**) Glucose-treated tumors exhibited dense tumoral tissue with few inflammatory cells. (**a** and **c)** Magnification $\times 50$, scale bar $= 200\,\mu$m. (**b** and **d)** Magnification $\times 200$, scale bar $= 50\,\mu$m). (**e** and **f)** Tumors were harvested, snap frozen in Tissue-Tek OCT compound and cryosectioned for immunofluorescence analysis using a FITC-labeled anti-mouse CD90/Thy-1 antibody (**e**) tumors injected with 5% glucose solution; (**f**) pSEA-TM/PEI treated tumors. pSEA-TM, transmembrane SEA fusion construct; PEI, polyethylenimine.

mononuclear white cells, including lymphocytes and macrophages, together with necrosis in pSEA-TM treated tumors (Figures 4a and b), which was not observed in control tumors injected with 5% glucose solution (Figures 4c and d). In addition, tumors injected with pSEA-TM were markedly infiltrated by $CD90^+$ T cells (Figure 4f), this was not observed in tumors injected with 5% glucose solution (Figure 4e).

Intratumoral SEA stimulates the proliferation of splenic lymphocytes

At the end of the experiment, the animals were killed and their splenocytes were co-cultured with mitomycin-inactivated control and pSEA-TM transfected B16F10 cells. The proliferative response of lymphocytes from pSEA-TM treated mice was higher than that of other groups when stimulated with pSEA-TM transfected compared with nontransfected cells (Figure 5, $P<0.05$).

Discussion

This study demonstrates that *in situ* gene transfer of pSEA-TM complexed with PEI and epinephrine is a simple and effective immunogene-therapy approach for melanoma with the added benefits of low toxicity and limited side effects. It allows expression of SEA at the membrane of tumor cells, and membrane-bound SEA on tumor cells elicits a significant decrease in tumor growth associated with a local (intratumoral infiltration of T cells) as well as systemic (increase in splenic lymphocyte proliferation) immune response.

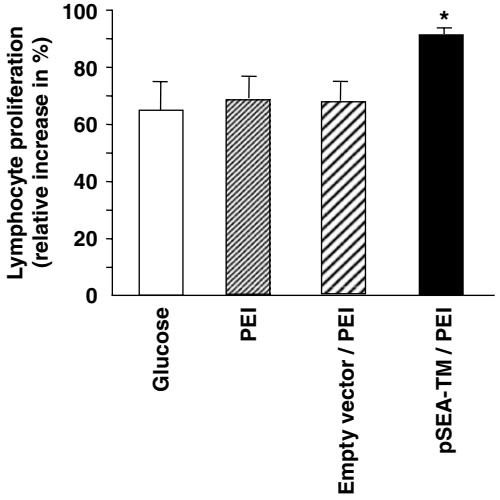

Figure 5 Intratumoral SEA transfection stimulates systemic lymphocyte proliferation. Tumors were injected as described in Figure 3. Splenocytes were collected at the time of animal sacrifice and cultured with control and SEA-expressing B16F10 cells. Results represent the relative increase in proliferation of splenocytes after co-culture with B16F10-SEA-TM cells, as compared with co-culture with B16F10 cells. (mean ± s.e.m., $n=3$, $^*P<0.05$ between pSEA-TM group and the others groups; Student's *t*-test). Lymphocyte proliferation from treated and control mice was determined by MTS assay. SEA, Staphylococcus enterotoxin.

SEA protein is a powerful immunostimulant that has been successfully used for antitumoral therapy, and studies are now under way to target tumor directly to limit its systemic side effects. For example, SEA proteins have been conjugated with a tumor-specific antibody (Fab-SEA) and injected into tumor-bearing mice, thus inducing tumor infiltration with T cells and tumor regression.[15–17,26] However, repeated injections of Fab-SEA proteins induced immune anergy that precluded further cytotoxicity and cytokine secretion.[18] In our experiment, repeated injections of pSEA-TM did not elicit detectable anergy of SEA-reactive spleen T cells, but rather an enhancement of this immune response as shown by the lymphocyte proliferation test (Figure 5). Also, cellular vaccine strategies with membrane–anchored SEA protein bearing B16 cells have been tested in mice. They induced significant tumor growth inhibition together with immune response and prolonged survival.[21,27] More generally, an immune response can be induced by artificially anchoring SAgs onto the tumor cell surface, even for MHC class II negative cells as this strategy can substitute for MHC class II presentation.[26] These approaches are attractive but require genetic manipulations of tumor cells *ex vivo*.

Direct *in vivo* transfections are easier to perform and repeatable. For example, adenovirus was used successfully to deliver transmembrane SEA in treating subcutaneous hepatocellular carcinoma in mice.[24] Although no side effects were reported, adenoviral vectors may induce strong immune responses, which may preclude iterative administrations.[28–31] Nonviral vectors may be more simple to use, easier to modify and relatively safe as compared with viral vectors. Here, we tested the cationic polymer PEI that has been widely and successfully used for both *in vitro* transfection and *in vivo* gene delivery through various routes including systemic delivery,[32] as well as intracerebral,[33] intraperitoneal,[34] intratumoral,[35] intramuscular[36] and intratracheal[37,38] routes.

The limiting parameter in using synthetic compounds for gene delivery is low transfection efficiency as compared with viral vectors. Here, we show that the addition of epinephrine to DNA/PEI complexes for intratumoral injections improves *in situ* transfection rates. Epinephrine is a local vasoconstrictor commonly added to local anesthetic drugs and has recently been used in association with intratumoral and intraperitoneal chemotherapy for enhancement of tumor penetration and antitumoral activity.[39,40] In our experiments, epinephrine greatly enhanced intratumoral transfection rate without any toxicity at the concentration used (1 mg/l).

Limited expression of transmembrane SAgs at the surface of tumor cells minimizes toxicity and stimulates the systemic antitumor immune response.[41] In our study, local expression of SEA did not induce any systemic toxicity but triggered local T cell infiltration and activation with antitumoral effects. The significant reduction in tumor growth obtained in controls using PEI as compared with the glucose treated group has already been reported with several synthetic vectors including PEI and polyamidoamine dendrimer.[42] Such an effect could be related to either immune activation or to the direct tumoral cytotoxicity of PEI.[43]

To conclude, this study demonstrates the efficacy and safety of iterative *in situ* pSEA-TM gene transfer using PEI in subcutaneous murine melanoma. Also, it indicates that epinephrine combination is a useful strategy to improve gene transfer when administered this way and that cationic polymers add their intrinsic antitumoral activity to the immuno-stimulating effect of tumor targeted SEA.

Acknowledgements

The authors are grateful to Franck Ménétrier for his technical assistance and to Charles Thomas, Véronique Laurens and Pierre Emmanuel Puig for helpful discussions. This study was supported by grants from the 'Ligue Nationale contre le Cancer' (commitees of Alsace and Côte d'Or to JC; commitee of Nièvre for BC; national label to ES group).

References

1 Diepgen TL, Mahler V. The epidemiology of skin cancer. *Br J Dermatol* 2002; **146**(Suppl 61): 1–6.
2 Stitz R, Kidd MR, Kenny LM, Howard AM. Skin cancer medicine in primary care: towards an agenda for quality health outcomes. *Med J Aust* 2006; **184**: 251.
3 Jemal A, Devesa SS, Hartge P, Tucker MA. Recent trends in cutaneous melanoma incidence among whites in the United States. *J Natl Cancer Inst* 2001; **93**: 678–683.

4 Komenaka I, Hoerig H, Kaufman HL. Immunotherapy for melanoma. *Clin Dermatol* 2004; **22**: 251–265.
5 Baskar S. Gene-modified tumor cells as cellular vaccine. *Cancer Immunol Immunother* 1996; **43**: 165–173.
6 Liu M, Acres B, Balloul JM, Bizouarne N, Paul S, Slos P *et al*. Gene-based vaccines and immunotherapeutics. *Proc Natl Acad Sci USA* 2004; **101**(Suppl 2): 14567–14571.
7 Kyte JA, Kvalheim G, Lislerud K, thor Straten P, Dueland S, Aamdal S *et al*. T cell responses in melanoma patients after vaccination with tumor-mRNA transfected dendritic cells. *Cancer Immunol Immunother* 2007; **56**: 659–675.
8 Hill HC, Conway Jr TF, Sabel MS, Jong YS, Mathiowitz E, Bankert RB *et al*. Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. *Cancer Res* 2002; **62**: 7254–7263.
9 Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM *et al*. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; **314**: 126–129.
10 Dohlsten M, Kalland T, Gunnarsson P, Antonsson P, Molander A, Olsson J *et al*. Man-made superantigens: tumor-selective agents for T-cell-based therapy. *Adv Drug Deliv Rev* 1998; **31**: 131–142.
11 Kominsky SL, Torres BA, Hobeika AC, Lake FA, Johnson HM. Superantigen enhanced protection against a weak tumor-specific melanoma antigen: implications for prophylactic vaccination against cancer. *Int J Cancer* 2001; **94**: 834–841.
12 Torres BA, Kominsky S, Perrin GQ, Hobeika AC, Johnson HM. Superantigens: the good, the bad, and the ugly. *Exp Biol Med* 2001; **226**: 164–176.
13 Baker MD, Acharya KR. Superantigens: structure-function relationships. *Int J Med Microbiol* 2004; **293**: 529–537.
14 Dohlsten M, Abrahmsén L, Björk P, Lando PA, Hedlund G, Forsberg G *et al*. Monoclonal antibody-superantigen fusion proteins: tumor-specific agents for T-cell-based tumor therapy. *Proc Natl Acad Sci USA* 1994; **91**: 8945–8949.
15 Dohlsten M, Hansson J, Ohlsson L, Litton M, Kalland T. Antibody-targeted superantigens are potent inducers of tumor-infiltrating T lymphocytes *in vivo*. *Proc Natl Acad Sci USA* 1995; **92**: 9791–9795.
16 Hansson J, Ohlsson L, Persson R, Andersson G, Ilbäck NG, Litton MJ *et al*. Genetically engineered superantigens as tolerable antitumor agents. *Proc Natl Acad Sci USA* 1997; **94**: 2489–2494.
17 Søgaard M, Ohlsson L, Kristensson K, Rosendahl A, Sjoberg A, Forsberg G *et al*. Treatment with tumor-reactive Fab-IL-2 and Fab-staphylococcal enterotoxin A fusion proteins leads to sustained T cell activation, and long-term survival of mice with established tumors. *Int J Oncol* 1999; **15**: 873–882.
18 Rosendahl A, Kristensson K, Carlsson M, Skartved NJ, Riesbeck K, Søgaard M *et al*. Long-term survival and complete cures of B16 melanoma-carrying animals after therapy with tumor-targeted IL-2 and SEA. *Int J Cancer* 1999; **81**: 156–163.
19 Schrayer DP, Kouttab N, Hearing VJ, Wanebo HJ. Synergistic effect of interleukin-2 and a vaccine of irradiated melanoma cells transfected to secrete staphylococcal enterotoxin A. *Clin Exp Metastasis* 2002; **19**: 43–53.
20 Huang C, Yu H, Wang Q, Yang G, Ma W, Xia D *et al*. A novel anticancer approach: SEA-anchored tumor cells expressing heat shock protein 70 onto the surface elicit strong anticancer efficacy. *Immunol Lett* 2005; **101**: 71–80.
21 Si SY, Hu PZ, Huang YY, Yc J, Huang Y, Li ZS *et al*. Tumor cells with B7.1 and transmembrane anchored staphylococcal enterotoxin A generate effective antitumor immunity. *Biochem Biophys Res Commun* 2006; **347**: 208–214.
22 Dow SW, Elmslie RE, Willson AP, Roche L, Gorman C, Potter TA. *In vivo* tumor transfection with superantigen plus cytokine genes induces tumor regression and prolongs survival in dogs with malignant melanoma. *J Clin Invest* 1998; **101**: 2406–2414.
23 Li ZS, Yang XW, Chen Z, Dong HL, Ye J, Qu P *et al*. *In vivo* tumor co-transfection with superantigen and CD80 induces systemic
immunity without tolerance and prolongs survival in mice with hepatocellular carcinoma. *Cancer Biol Ther* 2004; **3**: 660–666.
24 Si S, Sun Y, Li Z, Ge W, Zhang X, Hu P *et al*. Gene therapy by membrane-expressed superantigen for alpha-fetoprotein-producing hepatocellular carcinoma. *Gene Ther* 2006; **13**: 1603–1610.
25 Carlsson G, Gullberg B, Hafstrom L. Estimation of liver tumor volume using different formulas—an experimental study in rats. *J Cancer Res Clin Oncol* 1983; **105**: 20–23.
26 Dohlsten M, Hedlund G, Akerblom E, Lando PA, Kalland T. Monoclonal antibody-targeted superantigens: a different class of antitumor agents. *Proc Natl Acad Sci USA* 1991; **88**: 9287–9291.
27 Ma W, Yu H, Wang Q, Jin H, Solheim J, Labhasetwar V. A novel approach for cancer immunotherapy: tumor cells with anchored superantigen SEA generate effective antitumor immunity. *J Clin Immunol* 2004; **24**: 294–301.
28 Liu Q, Muruve DA. Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther* 2003; **10**: 935–940.
29 Sun JY, Anand-Jawa V, Chatterjee S, Wong KK. Immune responses to adeno-associated virus and its recombinant vectors. *Gene Ther* 2003; **10**: 964–976.
30 Vorburger SA, Hunt KK. Adenoviral gene therapy. *Oncologist* 2002; **7**: 46–59.
31 Muruve DA. The innate immune response to adenovirus vectors. *Hum Gene Ther* 2004; **15**: 1157–1166.
32 Kircheis R, Wightman L, Schreiber A, Robitza R, Rössler V, Kursa M *et al*. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther* 2001; **8**: 28–40.
33 Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA* 1995; **92**: 7297–7301.
34 Louis MH, Dutoit S, Denoux Y, Erbacher P, Deslandes E, Behr JP *et al*. Intraperitoneal linear polyethylenimine (L-PEI)-mediated gene delivery to ovarian carcinoma nodes in mice. *Cancer Gene Ther* 2006; **13**: 367–374.
35 Dolivet G, Merlin JL, Barberi-Heyob M, Ramacci C, Erbacher P, Parache RM *et al*. *In vivo* growth inhibitory effect of iterative wild-type p53 gene transfer in human head and neck carcinoma xenografts using glucosylated polyethylenimine nonviral vector. *Cancer Gene Ther* 2002; **9**: 708–714.
36 Williams JH, Sirsi SR, Latta DR, Lutz GJ. Induction of dystrophin expression by exon skipping in mdx mice following

intramuscular injection of antisense oligonucleotides complexed with PEG-PEI copolymers. *Mol Ther* 2006; **14**: 88–96.
37 Gautam A, Waldrep JC, Kleinerman ES, Xu B, Fung YK, T'Ang A *et al*. Aerosol gene therapy for metastatic lung cancer using PEI-p53 complexes. *Methods Mol Med* 2003; **75**: 607–618.
38 Zou SM, Erbacher P, Remy JS, Behr JP. Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse. *J Gene Med* 2000; **2**: 128–134.
39 Duvillard C, Benoit L, Moretto P, Beltramo JL, Brunet-Lecomte P, Correia M *et al*. Epinephrine enhances penetration and anti-cancer activity of local cisplatin on rat sub-cutaneous and peritoneal tumors. *Int J Cancer* 1999; **81**: 779–784.
40 Duvillard C, Romanet P, Cosmidis A, Beaudouin N, Chauffert B. Phase 2 study of intratumoral cisplatin and epinephrine treatment for locally recurrent head and neck tumors. *Ann Otol Rhinol Laryngol* 2004; **113**: 229–233.
41 Wahlsten JL, Mills CD, Ramakrishnan S. Antitumor response elicited by a superantigen-transmembrane sequence fusion protein anchored onto tumor cells. *J Immunol* 1998; **161**: 6761–6767.
42 Dufes C, Keith WN, Bilsland A, Proutski I, Uchegbu IF, Schatzlein AG. Synthetic anticancer gene medicine exploits intrinsic antitumor activity of cationic vector to cure established tumors. *Cancer Res* 2005; **65**: 8079–8084.
43 Moghimi SM, Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A. A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy. *Mol Ther* 2005; **11**: 990–995.