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Plasmid Injection and Application of Electric Pulses Alter Endogenous mRNA and Protein Expression in B16.F10 Mouse Melanomas

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ORIGINAL ARTICLE

Plasmid injection and application of electric pulses alter endogenous mRNA and protein expression in B16.F10 mouse melanomas

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The application of electric pulses to tissues causes cell membrane destabilization, allowing exogenous molecules to enter the cells. This delivery technique can be used for plasmid gene therapy. Reporter gene expression after plasmid delivery with eight representative published protocols was compared in B16.F10 mouse melanoma tumors. This expression varied significantly based on the pulse parameters utilized for delivery. To observe the possible influence of plasmid injection and/or pulse application on endogenous gene expression, levels of stress-related mRNAs 4 and 24 h after delivery were determined by PCR array. Increases in mRNA levels for several inflammatory chemokines and cytokines were observed in response to plasmid injection, electric pulses alone or the combination. This upregulation was confirmed by individual real-time reverse transcription TaqMan PCR assays. Proteins were extracted at the same time points from identically treated tumors and inflammatory protein levels were assayed by enzyme-linked immunosorbent assay and by a custom multiplex bead array. Increases in inflammatory protein levels generally paralleled mRNA levels. Some differences were observed, which may have been due to differing expression kinetics. The observed upregulated expression of these cytokines and chemokines may aid or inhibit the therapeutic effectiveness of immune-based cancer gene therapies.
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Introduction

The use of *in vivo* electroporation (EP) as a therapeutic gene delivery approach has been successfully used in a variety of applications including cancer therapy and the regulation of protein levels to enhance or reduce protein function. For therapeutic cancer applications, plasmids evaluated for gene therapy encode the same types of genes or complementary DNAs tested using viral delivery methods, including immune modulators, cell cycle regulators, suicide genes, anti-angiogenic genes and genes encoding toxins or tumor antigens. Diverse delivery protocols varying in pulse parameters and in electrode configurations have been described.[1]

Many of the therapeutic studies of *in vivo* intratumor EP in experimental cancer models test the delivery of plasmids encoding immune modulators. These studies may demonstrate significant tumor regression, indicating that the delivered genes or complementary DNAs are potentially effective as antitumor agents. A limited number of these studies have demonstrated long-term, complete tumor regression, including studies delivering plasmids encoding interleukin (IL)-12,[2–6] interferon (IFN) α,[7,8] IL-15[9] and IL-21[10] as single agents. Complete tumor regression was observed after delivery of combinations of plasmids encoding IL-6 and IL-15,[11] granulocyte-macrophage colony-stimulating factor (GM-CSF) and B7.1,[12] or IL-12 and B7.1.[13] Intratumor EP of a plasmid encoding the human IL-12 complementary DNAs for melanomas has been successful therapeutically in a phase I clinical trial.[14] Intratumor delivery of a plasmid encoding the human IL-2 complementary DNA to melanomas has also reached clinical trials, although efficacy has not yet been reported.[15] These studies support the idea that immune modulators may be efficacious as cancer therapies.

Inflammation induced by the combination of plasmid delivery and electric pulses has been described in several tissues, most commonly muscle. Local inflammatory responses have been observed between 24 h and 7 days after plasmid injection[16] or delivery of pulses alone in rat,[17] mouse[18–22] and pig[23] muscle. The combination of vector plasmid and pulses may induce higher levels of inflammation than plasmid alone.[20,24,25] In skin,

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no significant histological changes were observed up to 5–7 days after delivery of pulses alone.[26] In another study, minimal to mild inflammation was observed.[19] Tumors have also been studied. In B16.F10 mouse melanomas, a strong infiltration of polymorphonuclear cells, monocytes and some lymphocytes was observed 24 h after vector plasmid delivery.[27] In the RM4 rat bladder cancer model, macrophages were observed in the tumor periphery 3 days after electrically mediated plasmid delivery.[28]

When plasmid DNA is present, the observed inflammation may be due in part to the induction of an inflammatory response to CpG motif DNA. The mammalian TLR9 receptor recognizes double-stranded DNA that is not CpG methylated as a danger signal.[29] As plasmid DNA is produced bacterially and is not CpG methylated, an inflammatory immune response may be produced in response to its introduction, particularly to B and plasmacytoid pre-dendritic cells. Secreted immune modulators may include IFNγ, IL-1β, IL-6, IL-8, IL-10, IL-12, IL-18, tumor necrosis factor (TNF) α, IFN-γ-inducible protein 10 (IP-10), macrophage inflammatory protein (MIP)-1β and GM-CSF. This inflammatory response can be reduced or eliminated by deleting CpG motifs from the plasmid.[30]

Approximately 50 different EP protocols for *in vivo* plasmid delivery to tumors have been described. These studies have differed in the electrode for delivery, tumor type, animal model, reporter protein and the time point chosen for assessment. No direct comparison of reporter gene expression after plasmid delivery using these protocols has been reported. One purpose of this study is to clarify the relationship between EP parameters and transgene expression by using a single commercially prepared reporter plasmid in a single tumor model at a single time point. In addition, mRNA and protein levels of endogenous genes regulated by intratumor plasmid vector DNA injection, by the delivery of two different types of electric pulses to the tumor, and to the combination will be quantified. Endogenous genes regulated by delivery may be important considerations when designing therapeutic antitumor protocols.

Materials and methods

Tumor and animal model
All procedures were approved by the Animal Use and Care Committee of the University of South Florida College of Medicine. In all, 10^6 B16.F10 (ATCC CR6475) mouse melanoma cells in 50 µl phosphate-buffered saline were injected subcutaneously in the left flank of female 7- to 8-week-old C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME). Tumors were allowed to grow 8 days to a diameter of approximately 4 mm before plasmid delivery.

Plasmid DNA preparation
gWizLuc, which encodes the luciferase gene driven by the cytomegalovirus (CMV) promoter, and gWizBlank, a plasmid vector that does not encode a transgene, were commercially prepared (Aldevron, Fargo, ND) and sus-pended to a concentration of $1\,\mu g\,\mu l^{-1}$ in sterile injectable saline. Endotoxin levels were $<0.1\,EU\,\mu g^{-1}$ plasmid.

EP delivery
Mice were anesthetized using a mixture of 2.5% isoflurane and 97.5% O_2. Tumors were injected with 50 µl plasmid DNA and pulsed immediately as described in each figure at a frequency of 1 Hz using a T820 Electrosquare porator (BTX, Holliston, MA). Delivery was performed with caliper electrodes moistened with electrode paste or with a six-needle array per each published protocol. An autoswitcher was used for delivery with the six-needle array. At the time points indicated after delivery, mice were humanely killed, tumors removed and snap frozen on dry ice.

Reverse transcription PCR assays
For PCR assays, RNA was extracted from tumors using Trizol (Invitrogen, Carlsbad, CA), purified using RNeasy columns (Qiagen, Valencia, CA) and quantified RNA from four tumors per group was pooled. mRNA levels were determined using Mouse Stress and Toxicity SYBR PCR arrays (SABiosciences, Frederick, MD) after reverse transcription per manufacturer's instructions. Relative quantification was performed by comparison with the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase using the $\Delta\Delta C_t$ method. TaqMan one-step RT-PCR was performed for GM-CSF, Cxcl10, IL-1α, IL-1β, IL-6, β-actin and glyceraldehyde 3-phosphate dehydrogenase to confirm changes in mRNA levels (TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA). As untreated tumors did not express GM-CSF endogenously, total lung RNA was used as a positive control.

Protein assays
For luciferase quantification, the tumors were weighed, homogenized and enzyme activity in extracts was normalized to the tumor weight.[31] For enzyme-linked immunosorbent assays (ELISAs) and multiplex bead arrays, tumors were homogenized in phosphate-buffered saline containing protease inhibitors. Homogenates were centrifuged to remove cell debris. Bicinchoninic acid assays (Pierce Biotechnology, Rockford, IL) were performed and samples normalized to $1\,mg\,ml^{-1}$ total protein. ELISAs were performed per manufacturer's instructions (R&D Systems, Minneapolis, MN). A custom multiplex bead array including mouse IL-1α, IL-1β, IL-4, IL-6, IL-10, MIP-1α, MIP-1β, TNFα and GM-CSF was constructed and samples assayed using a Bio-Plex System (Bio-Rad, Hercules, CA) per manufacturer's instructions.

Statistical analysis
Analysis of reporter gene expression after electrically mediated plasmid delivery to B16.F10 mouse melanomas was completed using analysis of variance. A *post hoc* Dunnett's test was used to adjust for multiple comparisons with one control group (injection of gWizLuc without EP). Analysis of cytokine protein expression was completed using analysis of variance. A *post hoc* Fisher's

Least Significant Difference test was used to adjust for multiple comparisons.

Results

Reporter gene expression

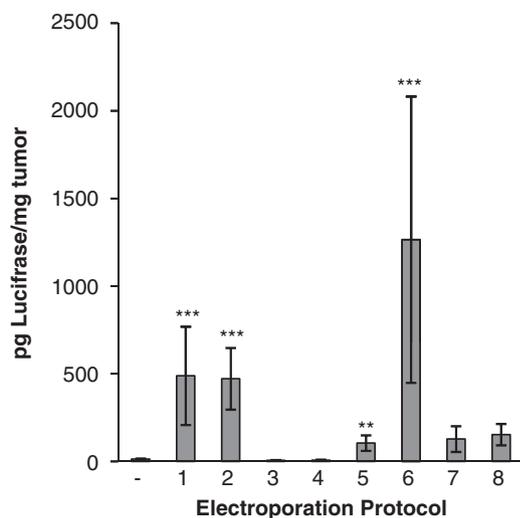Eight published EP protocols for intratumor delivery were compared in the subcutaneous B16. F10 mouse

Figure 1 Reporter gene expression 24 h after electrically mediated plasmid delivery to B16.F10 mouse melanomas. In all, 50 μg gWizLuc (Aldevron) was delivered to melanomas using various EP protocols: 1, ten 50 ms 800 V cm^{-1} pulses with a caliper electrode;[59] 2, eight 20 ms 500 V cm^{-1} pulses with a caliper electrode;[54] 3, eight 50 ms 66 V cm^{-1} pulses with a six-needle array;[5] 4, six 50 ms 167 V cm^{-1} pulses with a caliper electrode;[60] 5, six 0.1 ms 1300 V cm pulses with a six-needle array;[2] 6, one 100 ms 667 V cm^{-1} pulse with a caliper electrode;[32] 7, two 20 ms 450 V cm^{-1} pulses with a caliper electrode;[3] 8, five 10 ms 400 V cm^{-1} pulses with a caliper electrode.[55] Activity was normalized to tumor weight. **$P < 0.01$, ***$P < 0.001$ with respect to injection alone, $n = 12$–18 per group. Values represent mean ± standard error.

melanoma model using a plasmid-encoding luciferase as a reporter (gWizLuc, Aldevron). These protocols varied with respect to electrode, and pulse amplitude, length and number. At 24 h, luciferase expression was determined (Figure 1). Using this specific reporter and time point, four delivery protocols produced expression that was significantly higher than expression from plasmid injection. Two protocols suggested increased expression, while two protocols exhibited no substantial expression above plasmid injection alone.

Endogenous mRNA levels

To determine whether *in vivo* EP can effect endogenous gene expression, two very different pulse protocols from Table 1 were compared. One protocol included six relatively short (100 μs) pulses at a field strength of 1300 V cm^{-1} using a six-needle array electrode (EP5).[2] Delivery with this protocol produced low-level and reproducible reporter gene expression. A second protocol varied markedly in that a single relatively long (100 ms) pulse at 667 V cm^{-1} was delivered with a caliper electrode (EP6).[32] Delivery with this protocol produced high levels of reporter gene expression.

Gene expression was observed after delivery of saline or plasmid vector DNA with each pulse protocol and compared with untreated tumors and tumors injected with plasmid DNA without EP. At 4 and 24 h after delivery, mice were killed and tumors excised. Pooled RNA from tumors in each experimental group ($n = 4$) was subjected to two-step real-time SYBR RT-PCR performed using Mouse Stress and Toxicity SYBR PCR arrays (SABiosciences). The gene groups contained in the array included oxidative or metabolic stress, heat shock, proliferation and carcinogenesis, growth arrest and senescence, necrosis or apoptosis, and inflammation. Each array was then repeated with a second group of four tumors. The fold changes in expression were determined by comparison with the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase.

No downregulated mRNA levels were observed. The inflammation group contained the highest number

Table 1 Mean fold changes in mRNA expression by reverse transcription and PCR array with respect to untreated control tumors after plasmid delivery to B16.F10 mouse melanomas

	Time point (h)	Ccl21b	Ccl3/MIP-1α	Ccl4/MIP-1β	Cxcl10/IP-10	IL-1α	IL-1β	IL-6	IL-18	iNos	Serpine1/PAI-1
gWizBlank only	4	2.2	111.4	512.0	19.0	26.0	76.1	8.9	35.5	48.5	7.7
	24	15.5	14.4	46.9	6.5	4.8	7.5	1.3	73.5	12.0	11.7
Saline+EP5	4	11.7	2.4	2.5	2.8	6.1	6.7	3.4	1.5	3.1	18.4
	24	4.4	3.7	2.5	2.8	3.4	3.3	1.6	6.6	3.1	15.7
Saline+EP6	4	11.5	7.1	31.5	25.6	23.8	62.9	36.1	6.8	15.2	102.2
	24	3.7	2.7	2.7	2.1	3.4	7.5	1.7	5.9	1.5	22.6
gWizBlank+EP5	4	1.7	4.3	6.3	46.8	10.2	16.6	32.0	2.8	17.8	1.9
	24	13.7	10.4	29.3	11.1	3.5	7.3	1.7	26.5	18.7	40.1
gWizBlank+EP6	4	−2.0	6.1	81.6	28.8	11.3	71.0	28.8	10.2	9.8	132.5
	24	1.1	3.2	28.8	3.4	1.5	7.7	−2.1	6.3	6.5	16.6

Abbreviations: IL, interleukin; IP-10, inducible protein 10; MIP, macrophage inflammatory protein; PAI-1, plasminogen activator inhibitor-1.
EP5, six 100 μs 1300 V cm^{-1} pulses delivered with a six-needle array electrode; EP6, one 100 ms 667 V cm^{-1} pulse delivered with caliper electrodes.

Table 2 Fold changes in mRNA expression by reverse transcription and TaqMan PCR with respect to untreated control tumors after plasmid delivery to B16.F10 mouse melanomas

	Time point (h)	Cxcl10/IP-10	IL-1α	IL-1β	IL-6	Serpine1/PAI-1
gWizBlank only	4	24.8	7.7	7.6	39.0	0.7
	24	3.2	1.0	1.1	1.2	0.3
Saline+EP5	4	1.4	1.5	2.0	3.6	1.2
	24	2.0	0.2	1.7	2.4	0.9
Saline+EP6	4	13.7	14.8	68.6	128.0	2.5
	24	0.3	2.0	6.5	2.3	0.8
gWizBlank+EP5	4	35.8	4.5	7.9	26.0	1.1
	24	2.1	2.7	4.2	3.9	1.1
gWizBlank+EP6	4	21.4	14.4	40.0	128.0	1.7
	24	2.6	3.2	9.1	5.6	1.0

Abbreviations: IL, interleukin; IP-10, inducible protein 10; PAI-1, plasminogen activator inhibitor-1.

Table 3 Cytokine expression measured by ELISA after plasmid delivery to B16.F10 mouse melanomas (mean ± s.d.)

	Time point (h)	IL-1β (pg mg^{-1} total protein)	IL-6 (pg mg^{-1} total protein)
Untreated		4.5 ± 5.9	21.2 ± 9.8
gWizBlank only	4	112.5 ± 50.0	245.9 ± 126.8
	24	1.9 ± 2.2	32.1 ± 14.5
Saline+EP5	4	12.0 ± 5.8	32.6 ± 9.9
	24	3.0 ± 3.7	10.1 ± 8.7
Saline+EP6	4	57.6 ± 24.5	141.2 ± 106.5
	24	7.8 ± 4.7	10.1 ± 8.8
gWizBlank+EP5	4	219.0 ± 126.4***	1644.7 ± 868.7***
	24	8.6 ± 5.9	45.0 ± 3.5
gWizBlank+EP6	4	156.3 ± 111.7*	996.8 ± 865.0**
	24	10.0 ± 7.0	49.2 ± 20.8

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IL, interleukin.
$*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

of upregulated mRNA levels across the delivery groups. Within this group, mRNAs for Ccl21b, Ccl3/MIP-1α, Ccl4/MIP-1β, Cxcl10/IP-10, IL-1α, IL-1β, IL-6, IL-18, iNos and Serpine1/plasminogen activator inhibitor-1 (PAI-1) were upregulated in response to at least one delivery condition, while mRNA levels for Csf2/GM-CSF, Lta/TNFβ, MIF and nuclear factor κ-B1 were unchanged.

In response to plasmid DNA injection alone, the array results indicated that most of the mRNA levels were upregulated by 4 h after delivery. This is likely a response to CpG motif DNA. By 24 h, expression was considerably reduced. Saline injection followed by EP protocol EP5 alone appeared to induce upregulation for Ccl21b and Serpine1/PAI-1, while saline injection with protocol EP6 appeared to upregulate Ccl21b, Cxcl10/IP-10, IL-1α, IL-1β, IL-6, iNos and Serpine1/PAI-1 to higher levels. The detected increases in relative levels of mRNAs were tested by a one-step TaqMan RT-PCR for a subset of targets, including IP-10, IL-1α, IL-1β, IL-6 and Serpine1 (Table 2). The specific PCR quantifications for IP-10,

IL-1α, IL-1β, and IL-6 in general tended to support the increases in mRNA levels detected by the PCR array. However, increased levels of Serpine1 observed with the PCR array were the not confirmed with the individual PCR.

Endogenous protein levels

To determine whether the observed changes in mRNA levels resulted in concomitant changes in protein levels, a subset of proteins was assayed using ELISAs and multiplex bead arrays. For these experiments, deliveries were performed and samples taken as described for the RNA experiments. For each delivery group, four tumors were assayed individually.

Using ELISAs for mouse IL-1β and IL-6, low endogenous levels of each cytokine were present in untreated tumors (Table 3). Four hours after delivery, IL-1β protein levels in tumors from the group receiving plasmid injection tended to be higher than the levels in untreated tumors. Protein levels in tumors receiving pulse protocol EP5 with saline also increased, but this increase appeared to be greater after delivery of saline with pulse protocol EP6. In groups receiving the combination of plasmid DNA and either EP protocol, levels were significantly higher than in untreated tumors. At 4 h, IL-6 protein levels also increased after plasmid injection, but this increase was not statistically greater than untreated tumors. Similar to IL-1β, the increase in IL-6 levels tended to be higher after saline delivery with protocol EP6 than with EP5. With the combination of plasmid and EP, these levels were statistically discernable with either EP protocol. For both cytokines, increased levels were reduced by 24 h.

Multiplex bead arrays were performed for mouse MIP-1α, MIP-1β, IL-1α, IL-1β and IL-6 per manufacturer's instructions (Table 4). Three cytokines that may be upregulated in response to CpG motif DNA (IL-4, IL-10 and TNFα) were also tested. At the tested time points, IL-4 and TNFα were not detected in untreated tumors and were undetected or detected at insignificant levels in the delivery groups (data not shown). Detectable endogenous levels of the remaining protein targets, particularly IL-1α and MIP-1α, were present in untreated

Table 4 Cytokine expression measured by multiplex bead array after plasmid delivery to B16.F10 mouse melanomas (mean ± s.d.)

	Time point (h)	Ccl3/MIP-1α (pg mg^{-1} total protein)	Ccl4/MIP-1β (pg mg^{-1} total protein)	IL-1α (pg mg^{-1} total protein)	IL-1β (pg mg^{-1} total protein)	IL-6 (pg mg^{-1} total protein)	IL-10 (pg mg^{-1} total protein)
Untreated		67.9 ± 27.6	10.9 ± 6.6	44.2 ± 25.6	11.1 ± 10.0	2.5 ± 2.5	2.4 ± 1.2
gWizBlank only	4	453.4 ± 38.1**	130.7 ± 159.5	214.0 ± 115.3***	104.3 ± 31.8	47.3 ± 18.3	27.8 ± 16.0**
	24	422.5 ± 136.5*	159.8 ± 120.1	32.4 ± 8.7	14.2 ± 4.9	4.6 ± 2.5	13.5 ± 3.1
Saline+EP5	4	30.0 ± 26.0	4.3 ± 3.8	151.8 ± 44.4*	13.9 ± 7.4	6.3 ± 2.1	2.2 ± 2.0
	24	157.7 ± 121.2	18.2 ± 14.9	8.3 ± 1.7	3.1 ± 3.6	0.7 ± 0.9	4.3 ± 1.7
Saline+EP6	4	43.4 ± 18.8	3.0 ± 5.2	73.1 ± 36.0	34.9 ± 12.4	61.1 ± 65.1	1.5 ± 0.5
	24	28.5 ± 28.0	0.7 ± 1.2	8.4 ± 4.0	7.6 ± 10.0	1.4 ± 1.5	0.4 ± 0.7
gWizBlank+EP5	4	874.8 ± 339.3***	369.8 ± 221.1***	114.3 ± 41.2	221.6 ± 130.2***	544.1 ± 202.3***	47.7 ± 19.1***
	24	430.4 ± 139.4**	119.7 ± 56.5	10.0 ± 6.2	8.0 ± 7.3	6.4 ± 2.2	8.0 ± 4.1
gWizBlank+EP6	4	398.2 ± 111.0*	206.4 ± 110.8	100.5 ± 73.8	110.0 ± 79.1	299.0 ± 312.4*	24.1 ± 3.4**
	24	226.2 ± 61.4	75.6 ± 44.9	13.3 ± 10.2	6.1 ± 7.2	7.1 ± 3.4	9.7 ± 2.8

Abbreviations: IL, interleukin; MIP, macrophage inflammatory protein.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

tumors. After plasmid injection, levels of MIP-1α, IL-1α and IL-10 reached statistical significance. When saline was delivered with EP5 pulses, only IL-1α levels increased significantly at 4 h. No other statistically significant increases in protein levels to pulses alone were observed. However, with the combination of EP5 and plasmid DNA, significant increases in IL-1β, IL-6, MIP-1α and MIP-1β proteins were observed at 4 h. When plasmid DNA was combined with EP6, MIP-1α, IL-6 and IL-10 were significantly increased at 4 h.

Discussion

Using reporter gene expression 24 h after electrically mediated delivery, the level of transgene expression varied up to 92-fold over plasmid injection alone after delivery with different EP protocols. For some groups, no statistical increase in reporter gene expression was observed after delivery. For these protocols, reporter gene expression may not be detectable at this time point. Alternatively, the reduction in transmembrane potential necessary for membrane permeability may not have been reached. These results clearly indicate that the level of therapeutic transgene expression can be controlled by the EP protocol with which the plasmid is delivered. Although the expression levels varied significantly, it is difficult to make any generalizations concerning the relative usefulness of each pulse type. The therapeutic gene delivered and its desired level of expression should influence the pulsing protocol used for delivery, along with other methods used in the control of transgene expression, such as the use of alternative plasmid promoters.

Increases in levels of several inflammatory mRNAs were observed particularly after plasmid injection, application of pulse protocol EP6 or the combination of plasmid DNA with either pulse protocol. Although changes in mRNA levels were confirmed using individual

PCRs, particularly at >10-fold increase on the array, the precise fold change in expression was not necessarily confirmed. Lower levels of upregulation were not necessarily reproducible, particularly at the 24-h time point. This may be due to the differences in specific sequence targets and detection chemistries between the two PCR detection methods.

Although proteins tended to be upregulated when the mRNA levels were upregulated, the specific levels did not necessarily correlate. It appeared that any manipulation of the tumor-induced short-term IL-1α expression. For IL-1β, the array very closely reproduced the results of the ELISA assay. Interestingly, while the pattern of expression was similar for IL-6, the multiplex quantified levels were clearly lower than the ELISA quantified levels. After the array was performed, the ELISA was repeated on the same samples and the ELISA values were confirmed. This discrepancy between the two protein assays may be due to differing target affinities of the antibodies used in the respective assays. However, each assay indicated that IL-6 in particular was upregulated synergistically in response to combination delivery.

IL-10 levels were significantly increased at 4 h after any delivery containing plasmid DNA, indicating that a CpG motif DNA effect was responsible. The expression of the chemokines MIP-1α and MIP-1β tended to be longer than cytokines expression, continuing through 4 and 24 h. Although MIP-1α, MIP-1β, IL-1β, IL-6 and IL-10 protein levels were increased after delivery by both EP protocols, expression tended to be higher after plasmid delivery with EP5 than with EP6. This conflicts with the observed mRNA levels, in which detected mRNA was much higher after combination delivery with EP6 than with EP5. These discrepancies may be due to the particular time points chosen for the assays; changes in protein levels may occur at different time points. Also, protein levels may not necessarily directly depend on mRNA levels. For example, both mRNA and protein stability may affect protein levels[33] and protein synthesis may be regulated.[34]

In a study of pulse delivery alone in mouse muscle, increased levels of several mRNAs encoding proinflammatory modulators, including MIP-1α, MIP-1β, IP-10, IL-6, iNos and GM-CSF were observed by microarray 2 and 4 h after delivery.[21] With the exception of GM-CSF, the data presented here confirm this upregulation in a tumor model at 4 h. As in tumors, the cytokine mRNA levels decreased to background by 24 h. However, a difference in chemokine mRNA levels was observed. In muscle, these levels decreased to background more quickly than in tumor tissue. In a study of EP delivery of a low concentration of plasmid DNA to rodent muscle, no changes in expression of mRNAs involved in an inflammatory response were observed by microarray analysis at time points of 4 h, 48 h and 3 weeks. However, an infiltration of mononuclear cells was observed histologically at 48 h,[35] suggesting the presence of an inflammatory response. Cytokine protein expression after EP of plasmid to mouse lung in bronchial alveolar lavage fluid was tested 24 h after delivery. No upregulation of IL-6, TNFα, IFNγ or IL-12 was observed.[36] The IL-6 results presented here confirm this observation, since by 24 h all observed increases in mRNA and protein levels had decreased to insignificance. In an array analysis of EP pulses to a human melanoma cell line, no significant increase in inflammatory gene expression was observed.[37] This is not unexpected because of the absence of immune cells in this *in vitro* study.

The CMV promoter is commonly used for gene therapy because it induces high levels of transcription in many tissues. *In vitro*, transcription from this promoter is cytokine regulated, particularly by IFNγ and TNFα,[38–43] although other cytokine regulators have been described. In mice, regulation of CMV promoter expression has been correlated to serum TNFα[44] and IL-12 levels.[45] Lung expression has been correlated to TNFα, IL-12 and IFNγ expression in bronchoalveolar lavage fluid.[46] The effect on expression may be dependent on the tissue type.[45] In this study, low but consistent levels of TNFα were observed in all tumor groups at the tested time points, limiting any conclusions concerning differential regulation in this model. IL-1β upregulates CMV-driven transcription[42,43] in some cell types. Although the combination of plasmid with EP5 induced higher levels of IL-1β than EP6, reporter gene expression driven by the CMV promoter was considerably lower. This does not support IL-1β upregulation of the promoter, however, there are many additional variables that would affect transcription. IL-10 has been observed to downregulate CMV-regulated transcription.[43] In the case of IL-10, EP5 induces higher levels of IL-10 than EP6, and this downregulation could account for the reduced luciferase activity. Once again, there are many additional variables to be considered.

Inflammation induced by EP acts as a vaccine adjuvant after muscle delivery of antigen-encoding plasmids.[21,23,47–50] Changes in CD3+ peripheral blood lymphocyte levels have also been observed in macaques after plasmid DNA immunization in combination with EP.[51] It is possible that the induction of these immune modulators can also increase the effectiveness of cancer immunotherapies by the induction of a specific antitumor immune response.[52] This endogenous cytokine induction may enhance natural killer cell activation, producing a direct antitumor effect. Antigen-presenting cells could potentially be stimulated, which would aid in the induction of an immune response by the protein expressed from the delivered therapeutic gene. The electric pulses may induce tumor cell damage that may make tumor antigens available to the immune response. Interestingly, an observable[8,9,53–57] or statistically significant[27,58] effect on tumor growth has been observed after EP delivery of non-coding control plasmids when compared with untreated tumors. This effect may be due to the induction of gene expression, including the immune modulators described here. It is also possible that some induced cytokines may work in opposition to the induction of a specific immune response, for example, the suppressive cytokine IL-10. The production of appreciable levels of these immune modulators should be considered when designing any EP-based antitumor therapy.

Conflict of interest

RH has ownership interest in RMR Technologies and owns stock and stock options of Inovio Biomedical Corporation.

Acknowledgements

References

1 Heller LC, Heller R. *In vivo* electroporation for gene therapy. *Hum Gene Ther* 2006; **17**: 890–897.

2 Lucas ML, Heller L, Coppola D, Heller R. IL-12 plasmid delivery by *in vivo* electroporation for the successful treatment of established subcutaneous B16.F10 melanoma. *Mol Ther* 2002; **5**: 668–675.

3 Li S, Zhang X, Xia X. Regression of tumor growth and induction of long-term antitumor memory by interleukin 12 electro-gene therapy. *J Natl Cancer Inst* 2002; **94**: 762–768.

4 Lucas ML, Heller R. IL-12 gene therapy using an electrically mediated nonviral approach reduces metastatic growth of melanoma. *DNA Cell Biol* 2003; **22**: 755–763.

5 Goto T, Nishi T, Kobayashi O, Tamura T, Dev SB, Takeshima H *et al*. Combination electro-gene therapy using herpes virus thymidine kinase and interleukin-12 expression plasmids is highly efficient against murine carcinomas *in vivo*. *Mol Ther* 2004; **10**: 929–937.

6 Pavlin D, Cemazar M, Kamensek U, Tozon N, Pogacnik A, Sersa G. Local and systemic antitumor effect of intratumoral and peritumoral IL-12 electrogene therapy on murine sarcoma. *Cancer Biol Ther* 2009; **8**: 2114–2122.

7 Li S, Zhang X, Xia X, Zhou L, Breau R, Suen J *et al*. Intramuscular electroporation delivery of IFN-alpha gene

therapy for inhibition of tumor growth located at a distant site. *Gene Ther* 2001; **8**: 400–407.

8 Heller LC, Ingram SF, Lucas ML, Gilbert RA, Heller R. Effect of electrically mediated intratumor and intramuscular delivery of a plasmid encoding IFN alpha on visible B16 mouse melanomas. *Technol Cancer Res Treat* 2002; **1**: 205–209.

9 Ugen KE, Kutzler MA, Marrero B, Westover J, Coppola D, Weiner DB *et al*. Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-15-expressing plasmid followed by *in vivo* electroporation. *Cancer Gene Ther* 2006; **13**: 969–974.

10 Hanari N, Matsubara H, Hoshino I, Akutsu Y, Nishimori T, Murakami K *et al*. Combinatory gene therapy with electrotransfer of midkine promoter-HSV-TK and interleukin-21. *Anticancer Res* 2007; **27**(4B): 2305–2310.

11 Chou PC, Chuang TF, Jan TR, Gion HC, Huang YC, Lei HJ *et al*. Effects of immunotherapy of IL-6 and IL-15 plasmids on transmissible venereal tumor in beagles. *Vet Immunol Immunopathol* 2009; **130**: 25–34.

12 Collins CG, Tangney M, Larkin JO, Casey G, Whelan MC, Cashman J *et al*. Local gene therapy of solid tumors with GM-CSF and B7-1 eradicates both treated and distal tumors. *Cancer Gene Ther* 2006; **13**: 1061–1071.

13 Liu J, Xia X, Torrero M, Barrett R, Shillitoe EJ, Li S. The mechanism of exogenous B7.1-enhanced IL-12-mediated complete regression of tumors by a single electroporation delivery. *Int J Cancer* 2006; **119**: 2113–2118.

14 Daud AI, DeConti RC, Andrews S, Urbas P, Riker AI, Sondak VK *et al*. Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *J Clin Oncol* 2008; **26**: 5896–5903.

15 Richards J, Gonzalez R, Schwarzenberger P, Whitman E, Stardal K, Westhoff C *et al*. Phase I trial of IL-2 plasmid DNA with electroporation in metastatic melanoma. *J Clin Oncol* 25[18S]; **8578**: 2007.

16 McMahon JM, Wells KE, Bamfo JE, Cartwright MA, Wells DJ. Inflammatory responses following direct injection of plasmid DNA into skeletal muscle. *Gene Ther* 1998; **5**: 1283–1290.

17 Lee RC, River LP, Pan FS, Ji L, Wollmann RL. Surfactant-induced sealing of electropermeabilized skeletal muscle membranes *in vivo*. *Proc Natl Acad Sci USA* 1992; **89**: 4524–4528.

18 Lefesvre P, Attema J, van Bekkum D. A comparison of efficacy and toxicity between electroporation and adenoviral gene transfer. *BMC Mol Biol* 2002; **3**: 12.

19 Rabussay DP, Nanda GS, Goldfarb PM. Enhancing the effectiveness of drug-based cancer therapy by electroporation (electropermeabilization). *Technol Cancer Res Treat* 2002; **1**: 71–82.

20 Ahlen G, Soderholm J, Tjelle T, Kjeken R, Frelin L, Hoglund U *et al*. *In vivo* electroporation enhances the immunogenicity of hepatitis C virus nonstructural 3/4A DNA by increased local DNA uptake, protein expression, inflammation, and infiltration of CD3 + T cells. *J Immunol* 2007; **179**: 4741–4753.

21 Peng B, Zhao Y, Xu L, Xu Y. Electric pulses applied prior to intramuscular DNA vaccination greatly improve the vaccine immunogenicity. *Vaccine* 2007; **25**: 2064–2073.

22 Hojman P, Gissel H, Andre F, Cournil-Henrionnet C, Eriksen J, Gehl J *et al*. Physiological effects of high and low voltage pulse combinations for gene electrotransfer in muscle. *Hum Gene Ther* 2008; **19**: 1249–1260.

23 Babiuk S, Baca-Estrada ME, Foldvari M, Middleton DM, Rabussay D, Widera G *et al*. Increased gene expression and

inflammatory cell infiltration caused by electroporation are both important for improving the efficacy of DNA vaccines. *J Biotechnol* 2004; **110**: 1–10.

24 Hartikka J, Sukhu L, Buchner C, Hazard D, Bozoukova V, Margalith M *et al*. Electroporation-facilitated delivery of plasmid DNA in skeletal muscle: plasmid dependence of muscle damage and effect of poloxamer 188. *Mol Ther* 2001; **4**: 407–415.

25 Tevz G, Pavlin D, Kamensek U, Kranjc S, Mesojednik S, Coer A *et al*. Gene electrotransfer into murine skeletal muscle: a systematic analysis of parameters for long-term gene expression. *Technol Cancer Res Treat* 2008; **7**: 91–101.

26 Dujardin N, Staes E, Kalia Y, Clarys P, Guy R, Preat V. *In vivo* assessment of skin electroporation using square wave pulses. *J Contr Release* 2002; **79**: 219–227.

27 Heller L, Coppola D. Electrically mediated delivery of vector plasmid DNA elicits an antitumor effect. *Gene Ther* 2002; **9**: 1321–1325.

28 Shibata MA, Horiguchi T, Morimoto J, Otsuki Y. Massive apoptotic cell death in chemically induced rat urinary bladder carcinomas following *in situ* HSVtk electrogene transfer. *J Gene Med* 2003; **5**: 219–231.

29 Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 2002; **20**: 709–760.

30 Yew NS, Zhao H, Wu IH, Song A, Tousignant JD, Przybylska M *et al*. Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol Ther* 2000; **1**: 255–262.

31 Heller L, Jaroszeski MJ, Coppola D, Pottinger C, Gilbert R, Heller R. Electrically mediated plasmid DNA delivery to hepatocellular carcinomas *in vivo*. *Gene Ther* 2000; **7**: 826–829.

32 Cichon T, Jamrozy L, Glogowska J, Missol-Kolka E, Szala S. Electrotransfer of gene encoding endostatin into normal and neoplastic mouse tissues: inhibition of primary tumor growth and metastatic spread. *Cancer Gene Ther* 2002; **9**: 771–777.

33 Hargrove JL, Schmidt FH. The role of mRNA and protein stability in gene expression. *FASEB J* 1989; **3**: 2360–2370.

34 Merrick WC. Mechanism and regulation of eukaryotic protein synthesis. *Microbiol Rev* 1992; **56**: 291–315.

35 Hojman P, Zibert JR, Gissel H, Eriksen J, Gehl J. Gene expression profiles in skeletal muscle after gene electrotransfer. *BMC Mol Biol* 2007; **8**: 56.

36 Zhou R, Norton JE, Zhang N, Dean DA. Electroporation-mediated transfer of plasmids to the lung results in reduced TLR9 signaling and inflammation. *Gene Ther* 2007; **14**: 775–780.

37 Mlakar V, Todorovic V, Cemazar M, Glavac D, Sersa G. Electric pulses used in electrochemotherapy and electrogene therapy do not significantly change the expression profile of genes involved in the development of cancer in malignant melanoma cells. *BMC Cancer* 2009; **9**: 299.

38 Gribaudo G, Ravaglia S, Caliendo A, Cavallo R, Gariglio M, Martinotti MG *et al*. Interferons inhibit onset of murine cytomegalovirus immediate-early gene transcription. *Virology* 1993; **197**: 303–311.

39 Prosch S, Staak K, Stein J, Liebenthal C, Stamminger T, Volk HD *et al*. Stimulation of the human cytomegalovirus IE enhancer/promoter in HL-60 cells by TNFalpha is mediated via induction of NF-kappaB. *Virology* 1995; **208**: 197–206.

40 Harms JS, Splitter GA. Interferon-gamma inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter. *Hum Gene Ther* 1995; **6**: 1291–1297.

41 Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* 1997; **8**: 2019–2029.

42 Kline JN, Hunninghake GM, He B, Monick MM, Hunninghake GW. Synergistic activation of the human cytomegalovirus major immediate early promoter by prostaglandin E2 and cytokines. *Exp Lung Res* 1998; **24**: 3–14.

43 Ritter T, Brandt C, Prosch S, Vergopoulos A, Vogt K, Kolls J et al. Stimulatory and inhibitory action of cytokines on the regulation of hCMV-IE promoter activity in human endothelial cells. *Cytokine* 2000; **12**: 1163–1170.

44 Kako K, Nishikawa M, Yoshida H, Takakura Y. Effects of inflammatory response on *in vivo* transgene expression by plasmid DNA in mice. *J Pharm Sci* 2008; **97**: 3074–3083.

45 de Wolf HK, Johansson N, Thong AT, Snel CJ, Mastrobattista E, Hennink WE et al. Plasmid CpG depletion improves degree and duration of tumor gene expression after intravenous administration of polyplexes. *Pharm Res* 2008; **25**: 1654–1662.

46 Hyde SC, Pringle IA, Abdullah S, Lawton AE, Davies LA, Varathalingam A et al. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. *Nat Biotechnol* 2008; **26**: 549–551.

47 Drabick JJ, Glasspool-Malone J, King A, Malone RW. Cutaneous transfection and immune responses to intradermal nucleic acid vaccination are significantly enhanced by *in vivo* electropermeabilization. *Mol Ther* 2001; **3**: 249–255.

48 Scheerlinck JPY, Karlis J, Tjelle TE, Presidente PJA, Mathiesen I, Newton SE. *In vivo* electroporation improves immune responses to DNA vaccination in sheep. *Vaccine* 2004; **22**: 1820–1825.

49 LeBlanc R, Vasquez Y, Hannaman D, Kumar N. Markedly enhanced immunogenicity of a Pfs25 DNA-based malaria transmission-blocking vaccine by *in vivo* electroporation. *Vaccine* 2008; **26**: 185–192.

50 Rosati M, Valentin A, Jalah R, Patel V, von Gegerfelt A, Bergamaschi C et al. Increased immune responses in rhesus macaques by DNA vaccination combined with electroporation. *Vaccine* 2008; **26**: 5223–5229.

51 Luckay A, Sidhu MK, Kjeken R, Megati S, Chong SY, Roopchand V et al. Effect of plasmid DNA vaccine design and *in vivo* electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *J Virol* 2007; **81**: 5257–5269.

52 Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science* 1996; **272**: 50–53.

53 Heller L, Pottinger C, Jaroszeski MJ, Gilbert R, Heller R. *In vivo* electroporation of plasmids encoding GM-CSF or interleukin-2 into existing B16 melanomas combined with electrochemotherapy induces longterm antitumour immunity. *Melanoma Res* 2000; **10**: 577–583.

54 Slack A, Bovenzi V, Bigey P, Ivanov MA, Ramchandani S, Bhattacharya S et al. Antisense MBD2 gene therapy inhibits tumorigenesis. *J Gene Med* 2002; **4**: 381–389.

55 Elez R, Piiper A, Kronenberger B, Kock M, Brendel M, Hermann E et al. Tumor regression by combination antisense therapy against Plk1 and Bcl-2. *Oncogene* 2003; **22**: 69–80.

56 Mccray AN, Ugen KE, Muthumani K, Kim JJ, Weiner DB, Heller R. Complete regression of established subcutaneous B16 murine melanoma tumors after delivery of an HIV-1 Vpr-expressing plasmid by *in vivo* electroporation. *Mol Ther* 2006; **14**: 647–655.

57 Prud'homme GJ, Glinka Y, Khan AS, Draghia-Akli R. Electroporation-enhanced nonviral gene transfer for the prevention or treatment of immunological, endocrine and neoplastic diseases. *Curr Gene Ther* 2006; **6**: 243–273.

58 Deharvengt S, Rejiba S, Wack S, Aprahamian M, Hajri A. Efficient electrogene therapy for pancreatic adenocarcinoma treatment using the bacterial purine nucleoside phosphorylase suicide gene with fludarabine. *Int J Oncol* 2007; **30**: 1397–1406.

59 Rols MP, Delteil C, Golzio M, Dumond P, Cros S, Teissie J. *In vivo* electrically mediated protein and gene transfer in murine melanoma. *Nat Biotechnol* 1998; **16**: 168–171.

60 Lohr F, Lo DY, Zaharoff DA, Hu K, Zhang X, Li Y et al. Effective tumor therapy with plasmid-encoded cytokines combined with *in vivo* electroporation. *Cancer Res* 2001; **61**: 3281–3284.