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Full Length Research Paper

Tissue distribution of intramuscularly and intratumouraly administered DNA plasmid harbouring apoptotic gene in mice

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This study investigated the bio-distribution and persistence of plasmid DNA following intramuscular and intratumoural administration in a mice model. Validated quantitative method (real-time qPCR) was used to quantify plasmid distribution in the tissue samples collected at 15 min, 1 h, 24 h and 1 week after administration of 100 µg (1.5 x 10^{13} copies) of naked plasmids. Plasmids remained in the circulating blood (3.6 ± 2.2 x 10^{2}copies/500 ng gDNA) and injected muscle (2.8 ± 1.1 x 10^{5} copies/500ng gDNA) for up to 1 week post administration. Plasmids were also detected in opposite muscle, lung, kidney, spleen, lymph nodes, liver and heart only 1 h post-injection or more. After 2 weeks of treatment, plasmids were retained solely in the tumor mass. These results suggest the presently used recombinant DNA plasmid was benefited with its early transgene expression characteristic which could release the anti-cancerous effect within short dwelling time.

Key words: DNA vaccine, recombinant plasmid, real-time PCR, biodistribution.

INTRODUCTION

Plasmid-based vectors appear to offer some advantages over viral vectors in the sense that they are less immunogenic, conveniently scalable, stably expressed in muscle, and not integrated into host genomic DNA (Ledwith et al., 2000; Martin et al., 1999, Vilalta et al., 2005; Wang et al., 2004). Plasmid DNA vaccines are being developed and provided several advantages for the treatment of cancer, autoimmune diseases or for the immunization of infectious diseases (Gurunathan et al., 2000, Meyer and Wagner, 2006). VP3/Apoptin protein encoded by the Chicken Anemia virus (CAV) possesses an inherent ability to specifically kill cancer cells. This virus-derived protein VP3 has a positively charged C-terminus that is reported to be tumor-selective, leaving normal cells intact (Visser et al., 2007). In tumor cells, VP3 binds as a multimeric complex to DNA and interacts with several nuclear targets, consequential in a G2/M phase arrest. Thereafter pro-apoptotic signal is transduced from the nucleus to cytoplasm by Nur77, which elicit a p53-independent mitochondrial death pathway (Los et al., 2009). This VP3 can induce apoptosis only in cancer or transformed cell and serve as anticancer therapy (Danen-Van et al., 1997; Maddika et al., 2006).

Inappropriate distribution of the plasmid DNA outside the treatment site, possibility of integration into the host cell chromosome and organ toxicity was the major concern in plasmid DNA as therapeutic vaccine (Brennan and Dougan, 2005). Biodistribution and general safety studies have been conducted in numerous animals such as mice (Eun et al., 2007; Ledwith et al., 2000; Liu et al., 2008; Martin et al., 1999; Vilalta et al., 2005; Wang et al., 2004; Zhang et al., 2005), rats (Gilchuk et al., 2006; Tuomela et al., 2005), rabbits (Gilchuk et al., 2006; Vilalta

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et al., 2005) swine (Gravier et al., 2007), and beef (Orság et al., 2008). However, little was known about biodistribution of naked DNA plasmid and the transgene after intratumoural injection. In the present study, a plasmid DNA harboring VP3 gene under stress inducible promoter (Glucose Regulate Protein; GRP78) was used to examine the tissue distribution of plasmid DNA vaccine following intramuscular and intratumoral administrations to mice.

MATERIALS AND METHODS

Plasmids preparation

pVIVO1-gfplacZ (InvivoGen, USA) is a dual system plasmid reporter vector consisting of CMVenhancer or SV40enhancer combined with tumor specific promoter. LacZ expression driven by CMVenhancer combined with GRP78 promoter while green fluorescent protein driven by SV40enhancer combined with GRP94 promoter. The pVIVO1-gfpVP3 which encoded apoptosis protein was generated by replacing lacZ gene at NcoI and NheI sites of pVIVO1-gfplacZ vector. The VP3 gene was synthesized from a local CAV isolate (accession no. AF030518 in GeneBank) as described by Mohd-Azmi et al., 1997. Plasmid DNA was produced by *E. coli* fermentation followed by EndoFree Maxi Prep Kit (Qiagen, Germany) to provided endotoxin free plasmid. The DNA quantification (A_{260}) and purity ($A_{260/280}$) was conducted using Biophotometer (Eppendorf, Germany). The concentration of stock plasmid preparation was 1 µg/µl and purity ($A_{260/280}$) was more than 1.8.

Detection of tissue distribution

Intramuscular injection of plasmids

Forty-eight BALB/c mice (male and female, 7-week old, obtained from Institute Medical Research, Malaysia) were fed on standard rodent diet. All experimental procedures were approved by the Animal Care and Use Committee of Faculty Veterinary Medicine, Universiti Putra Malaysia. The first group of 24 mice received a single intramuscular administration of 100 µg pVIVO1-gfpVP3 (1.59 x 10^{13} copies) in 100 µL of endotoxin-free PBS via a 29-gauge needle in the right quadriceps. While the second group of 24 mice was injected with 100 µL of endotoxin-free PBS per quadriceps served as untreated controls and sacrificed at the same time. The mice were observed for any adverse reaction before sacrification at all time points.

Necropsy and sample storage

Three male and three female mice were randomly selected and sacrificed at 15 min, 1 h, 1 day, and 1 week after plasmids injection for blood and vital organ samplings. The control group sacrificed at the same time points. The delay between sacrifice and organ sampling was kept as short as possible in all animals. Blood samples were collected into 1.5 ml microtube and incubated at 37°C for 30 min. Sera were obtained after centrifugation at 3000 X g, 4°C, for 10 min and stored at -80°C until DNA isolation.

Multiple tissues were removed namely spleen, liver, heart, kidney, lung, lymph nodes, the injected and uninjected quadriceps muscle. The different organs were sampled directly inside the animal, to avoid contamination. The injected muscle was removed last to avoid contaminating with other tissues. All tissues were rinsed in PBS, snap-frozen in liquid nitrogen and stored at -80°C until DNA isolation.

DNA isolation from tissues

Genomic DNA (gDNA) isolation was performed on 50 mg of tissue samples using DNAzol (Molecular Research Centre, Inc) and 300 µl sera using DNAzol BD (Molecular Research Centre, Inc) according to the manufacturer's protocol. Final gDNA pellets were dissolved in 200 µl dH_2O and the concentration and purity determined by $A_{260/280}$ Biophotometer. The DNA samples were adjusted to 50 ng/µl and stored at -30°C prior to analysis.

Real-time qPCR analysis

The Primer3 software (http://frodo.wi.mit.edu) was used to design primers that amplify specific VP3 sequence from pVIVO1-gfpVP3, generating 120 bp amplicon. The primer sequences were: sense 5'-GAACGCTCTCCAACTAGATA-3' and antisense 5'-GTAATTCCAGTTATACCAAT-3'. Real-time qPCR was performed in the ABI 7000 SDS (Applied Biosystems). 500ng of gDNA template was amplified in triplicate in optical-grade 96 well plates. The 20 µl final volume containing 1X KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems), 200 nM of each primer and 500 ng gDNA. All reactions were normalized using ROX reference dye at a final concentration of 500nM. The optimal PCR cycling conditions were: 2 min at 50°C, enzyme activation at 95°C for 10 min, 40 cycles of PCR consisting of a 15 sec denaturing step at 95°C and 1 min annealing/extension step at 60°C were performed. The amount of plasmids in the samples was determined by comparing with a ten-fold standard of the pVIVO1-gfpVP3 ranging from 10^8-10^2 copies. The plasmid copy numbers for each mouse group were expressed as plasmid copies per 500 ng of gDNA.

Detection of GFP distribution on tumor-bearing model

Tumor induction and treatment

Murine colon cancers were established by injecting 5-6 week old BALB/c mice with 1.0 x 10^6 CT26 cells in 100 µl PBS in the right flank. Seven days later, when the tumors were palpable, mice were randomly divided into 2 groups (n=3 animals/group). Treatment was given when the tumor volume ranged between 80-100 mm^3 with 100 µg pVIVO1-gfpVP3 in 100 µl endotoxin-free PBS. Treatment with 100 µl endotoxin-free PBS served as control. The mice in all groups were sacrificed 14 days post treatment.

GFP distribution

Samples of the subcutaneous tumor, liver, heart, spleen, and kidney were harvested 14 days post treatment. All samples were fixed immediately in 10% formalin overnight at 4°C and embedded in paraffin. Serial sagittal sections in 5 µm thickness were made and viewed under florescence microscope (Eclipse TE 2000-S, Nikon).

Conventional PCR analysis of tumors and organs

Genomic DNA was isolated from treated tumors, untreated tumors and organs according to the method described in this study. PCR assay was done on gDNA to distinguish the biodistribution of plasmid DNA in tumor-bearing model. Two pairs of primers were designed, GFP sense (5'- GCG GAA TTC ATG AGC AAG GGA

GAA-3'), and antisense (5'- GAG TCT AGA TTT ACT TGT ACA GCT-3'); pVIVO1-gfpVP3 sense (5'- GCG CAG CCA TGG ATG AAC GCT AAT CAA -3'), and antisense (5'- CGC CCG GGC AGC TTA CAG TCT TAT ACG-3'). Briefly, the assay was performed in a reaction volume of 20 µl according to the following condition: 1X PCR buffer, 1 mM $MgSO_4$, 0.2 µmol/L deoxynucleotide triphosphates, 0.15 µmol/L of each primers, 5 units Taq DNA polymerase (BioLabs, New England), and 50 ng DNA sample. The PCR was performed on a PTC-200 gradient cycler (MJ Research Inc., Minnesota, USA) using the following profile: Pre incubation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 1 s; a final extension step at 72°C for 10 min was included. The PCR products were separated on a 1% agarose gel stained with 0.5 µg/ml of ethidium bromide, and visualized under UV illumination. The expected size of the PCR products was 710 bp and 390 respectively.

Statistical analysis

Statistical significance of data was evaluated using Student's t-test between two groups. A p-value of less than 0.05 was considered significant.

RESULTS

Post-administration observation

After the intramuscular injection of pVIVO1-gfpVP3, all mice survived throughout the duration of experiment. No abnormal clinical signs were recorded during the study, neither in apparent changes nor loss of body weight. No changes in the autonomic and central nervous system were detected due to normal behavior of the mice observed. At necropsy, no gross pathological changes were observed especially at the site of injection indicating that pVIVO1-gfpVP3 was well tolerated.

Validation of the real-time qPCR method

The distribution and persistence of pVIVO1-gfpVP3 in tissue was determined using real-time qPCR methodology designated to recognize specific location of the VP3 sequence. The methodology comprised specificity, sensitivity and linearity in the detection of pVIVO1-gfpVP3. Mouse genomic DNA was used to assess the specificity of the method. Four concentrations (10^2, 10^3, 10^4, 10^5 copies) of plasmid DNA in dH_2O and mouse gDNA were amplified together.

Neither inhibition due to sample matrix (gDNA) nor possible impurities co-extracted with the gDNA were observed. The linear quantification range (LQL) was demonstrated within 10^2-10^8 copies of pVIVO1-gfpVP3 (Figure 1). The dilution series generated linear equation ($y = -3.119x + 36.54$) with a correlation coefficient (R^2) greater than 0.992. The limit of detection was obtained at 31 copies/500 ng gDNA. Using the slope from the linear equation, the overall efficiency of the assay was estimated to be 99%.

Tissue distribution of the plasmids post intramuscular injection

The differential plasmid distribution in tissues was detected using real-time qPCR, following intramuscular administration at various time points (Table 1). No plasmids detection in PBS-injected mice. Plasmid DNA from injected muscle spread to other tissues via blood circulation. The majority of the plasmids resided at the injected site, up to 1 week post-injection. Rely on real-time qPCR detection, maximum concentration of the plasmid DNA level was $3.1 \pm 0.4 \times 10^9$ copies/500 ng gDNA at 15 min post-injection and continuously degraded to $2.8 \pm 1.1 \times 10^5$ copies/500 ng gDNA after 1 week. Upon 1 h post-injection, approximately 20% of the concentration ($6.2 \pm 0.6 \times 10^8$ copies/500ng gDNA) was present and gradually reduced to less than 1% over 24 h period (Figure 2a).

Blood plasmid concentration post intramuscular injection showed the highest quantity of $2.8 \pm 0.09 \times 10^4$ copies/500 ng gDNA at earliest 1hour detection. Within 24 h administration, pVIVO1-gfpVP3 degraded rapidly, reduced to almost 20-fold at $1.3 \pm 0.8 \times 10^3$ copies/500 ng gDNA. At least, $3.6 \pm 2.2 \times 10^2$ copies/500 ng gDNA of plasmid can be detected in blood circulation one week post-injection (Figure 2b). Beside the injection site and blood sera, highest concentration of plasmid was detected in the spleen at 24 h post-injection (72 ± 4.7 copies/500 ng gDNA). The level of pVIVO1-gfpVP3 decreased rapidly and gone undetected after 24 h in lymph nodes and kidney (Table 1). Significant amount of pVIVO1-gfpVP3 was detected in the spleen and heart after 1 week administration. However, the amounts were extremely low compared to the site of injection.

GFP distribution of the plasmids post intratumoral injection

The GFP protein expression was detected in the tumor and all vital organs (liver, kidney, heart and spleen) 14 days post-treatment (Figure 3). Neither tumors nor organs from control mice treated with PBS, showed GFP expression. After 14-days of treatment, subcutaneous tumor, kidney, heart, spleen, lung and liver were harvested from CT26 tumor-bearing mice. No pVIVO1-gfpVP3 sequence-related PCR products could be amplified. Plasmids were only detected in tumor tissue but not in distant organs tested (Figure 4).

DISCUSSION

Investigation on biodistribution, persistence and general safety of DNA vaccine on animal model is mandatory

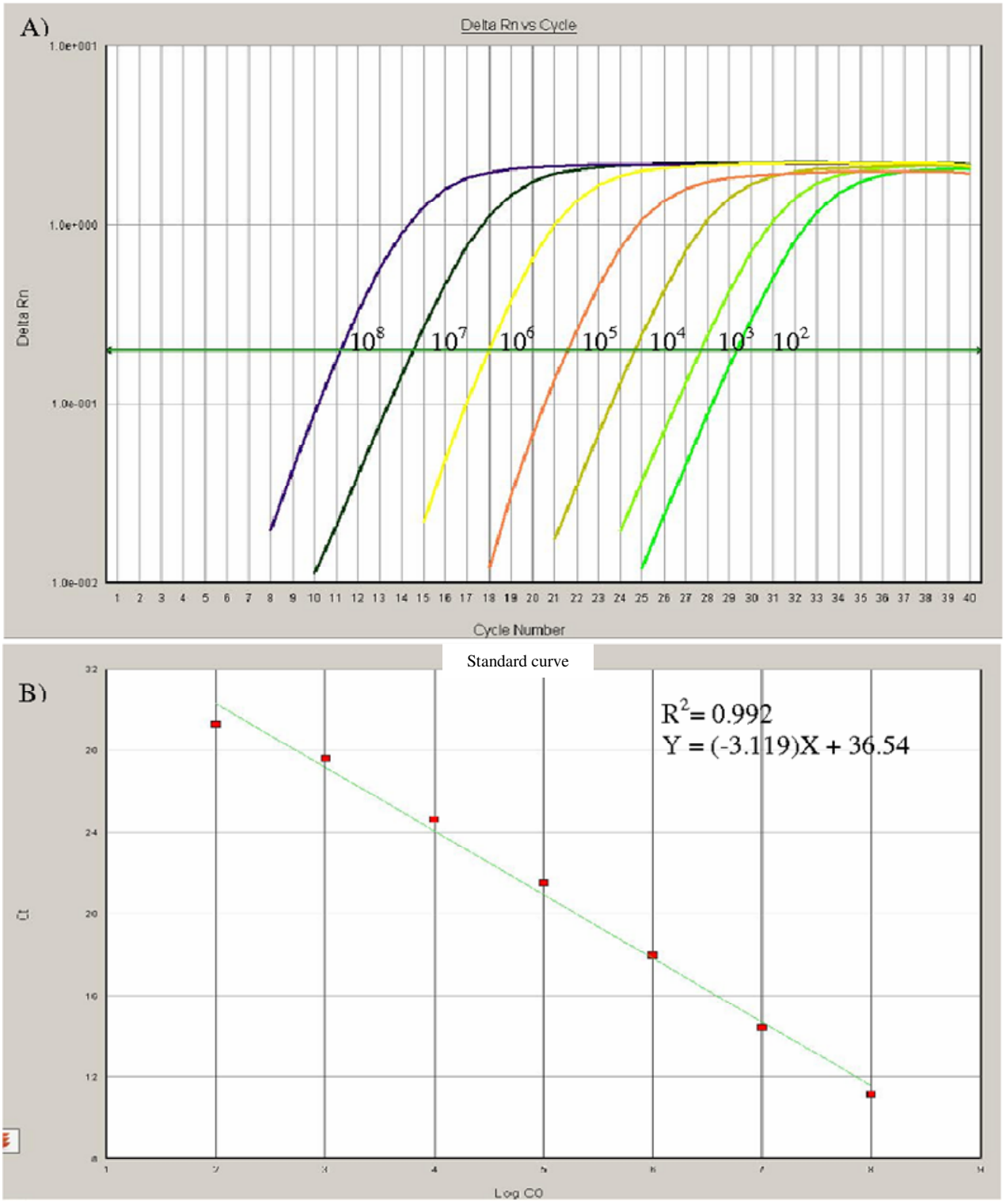

Figure 1. Real-time qPCR standard curve of plasmid containing VP3. A) Dilution series of pVIVO1-VP3 (10^8 - 10^2 copies) was amplified using 1 µg/µl stock. B) Calibration curve was generated using Ct value versus log copy number (C0). Squares represent Ct values recorded from three independent pVIVO1-VP3 dilutions. The straight line represents linear regression analysis with correlation coefficient (R^2), 0.992.

Table 1. Tissue distribution in mice receiving a single intramuscular injection of pVIVO1-gfpVP3 (copies/500ng gDNA)[a].

Time point	15 min		1 h		24 h		1 week		2 weeks	
Groups[b]	**pVIVO1-gfpVP3**	**PBS**	**pVIVO1-gfpVP3**	**PBS**	**pVIVO1-gfpVP3**	**PBS**	**pVIVO1-gfpVP3**	**PBS**	**pVIVO1-gfpVP3**	**PBS**
Blood	83 ± 12[c]	0/6	$2.8 \pm 0.09 \times 10^4$	0/6	$1.3 \pm 0.8 \times 10^3$	0/6	$3.6 \pm 2.2 \times 10^2$	0/6	0/3[f]	0/3
Injected muscle	$3.1 \pm 0.4 \times 10^9$	0/6	$6.2 \pm 0.6 \times 10^8$	0/6	$3.8 \pm 1.5 \times 10^5$	0/6	$2.8 \pm 1.1 \times 10^5$	0/6	0/3	0/3
Opposite muscle	20 ± 11	0/6	36 ± 0.6	0/6	32 ± 27	0/6	0/6	0/6	0/3	0/3
Spleen	10 ± 7	0/6	54 ± 7.8	0/6	72 ± 4.7	0/6	55 ± 5.3	0/6	0/3	0/3
Lymph nodes	0/6[f]	0/6	73 ± 0.8	0/6	0/6	0/6	0/6	0/6	0/3	0/3
Kidney	0/6	0/6	17 ± 1.5	0/6	0/6	0/6	0/6	0/6	0/3	0/3
Heart	91 ± 5.0	0/6	87 ± 4.9	0/6	36 ± 3	0/6	30 ± 7	0/6	0/3	0/3
Liver	11 ± 0.4	0/6	43 ± 7.1	0/6	50 ± 2.8	0/6	0/6	0/6	0/3	0/3
Lung	28 ± 8	0/6	64 ± 6.3	0/6	19 ± 12	0/6	0/6	0/6	0/3	0/3
Tumor[d]	n/s		n/s		n/s		n/s		3/3[e]	0/3

a. The copies of detected plasmid DNAs pVIVO1-gfpVP3 in 500 ng gDNA isolated from tissues or fluid; **b.** Mice were treated intramuscularly in the right quadriceps with 100 µl PBS or 100 µg pVIVO1-gfpVP3 in 100 µl PBS; c. Result shown mean copies/500 ng gDNA of six individual's ± standard deviation; d. Mice were treated intra-tumoral with 100 µl PBS or 10 µg pVIVO1-gfpVP3 in 100 µl PBS.
e. Positive tissues analyzed using conventional PCR; f. Positive detection per mice.
n/s- no sacrification

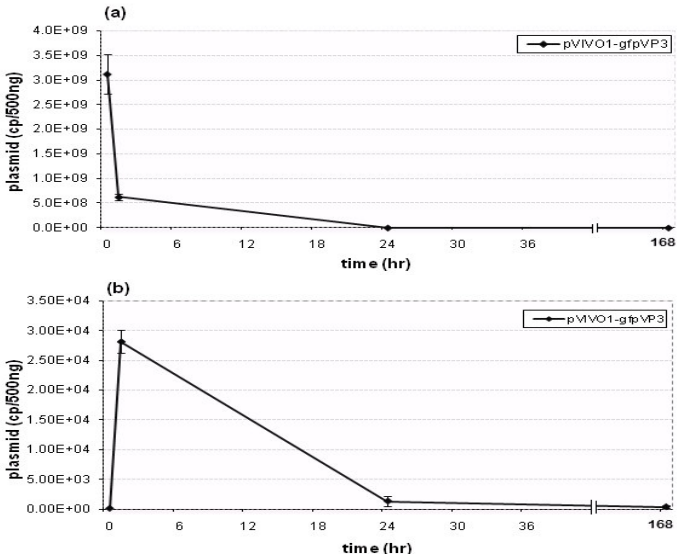

Figure 2. (a) Plasmid levels in the mice muscle after the intramuscular administration of 100 µg of pVIVO1-gfpVP3 in 100 µl PBS. (b) Plasmid levels in the blood after the intramuscular administration of 100 µg of pVIVO1-gfpVP3 in 100 µl PBS. Data represents the plasmid levels from six mice at different time-point, mean ± std Dev.

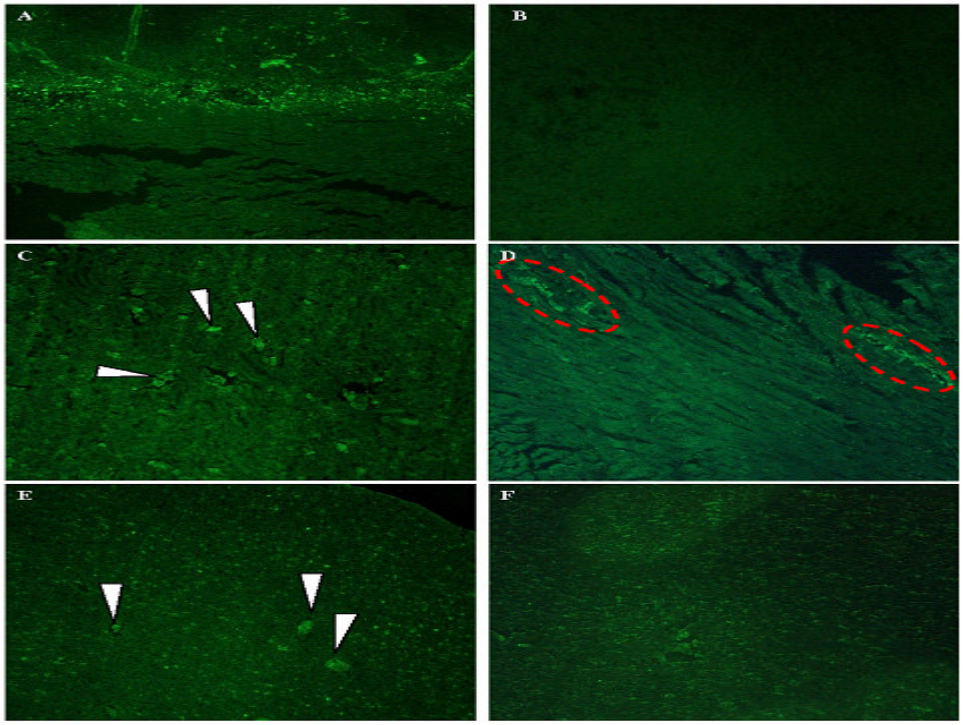

Figure 3. GFP protein expression on tumor (A) and control (B). GFP distributed to all organs: kidney (C), heart (D), liver (E), and spleen (F), mostly in blood vessels (arrows and circle) 14 days post-treatment. (Magnification at 100x).

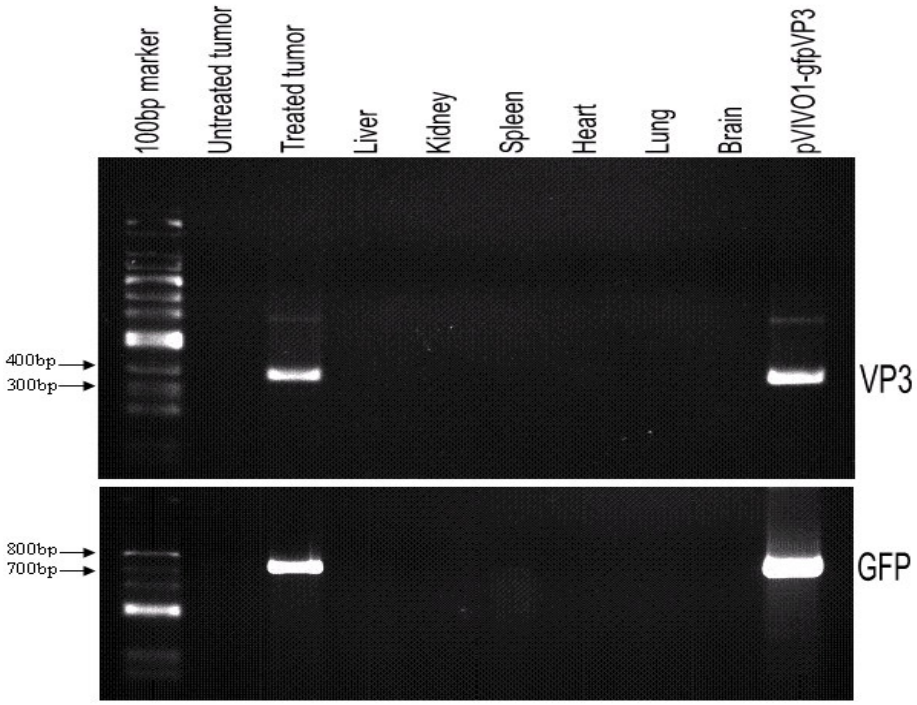

Figure 4. Analysis of pVIVO1-gfpVP3 distribution in tumor-bearing mice by PCR of VP3 and GFP transgene. Genomic DNA was extracted from subcutaneous tumor and various distant organs 14-days post treatment. VP3 and GFP amplification generated 390bp and 710bp amplicon respectively.

before entering the preclinical trials (FDA, 2007; Brennan and Dougan, 2005). In comparison to normal tissue, CMVenh or SV40enh combined with stress-inducing promoter had a greater expression in hypoxic tumor tissue (Lee, 2001). However, plasmid DNA usually degraded rapidly before delivery of the therapeutics gene due to extracellular and intracellular barrier (Ogris and Wagner, 2002). The production of proinflammatory cytokines from host such as TNF-α and IFN- γ in response to stimulatory CpG motifs against promoter or gene product may play a significant role in the loss of expression following gene transfer (Qin et al., 1997). In pVIVO1-gfpVP3, all CpG motifs have been removed by chemically synthesizing the gene. Therefore, the existence of plasmid DNA in the blood circulation is essential for therapeutic properties.

Real-time qPCR method was developed in this study to quantify plasmid harboring VP3 in the tissues and sera following intramuscular injection. The fate of pVIVO1-gfpVP3 was evaluated following intramuscular administration. This administration route represented the intended application route in clinical study. The result showed that plasmid DNA in the i.m injected mice distributed extensively to the vital organs such as lung, liver and kidney 1 h post-administration. However, a low possibility of toxicity was anticipated due to rapid metabolization of the plasmid in these organs within 1 week. According to previous reports (Delépine et al., 2003; Zhang et al., 2005), over a half of the plasmid administered were degraded and excreted through urine and feces. Less than 1% ($3.8 \pm 1.5 \times 10^5$) of plasmids remained at the injection site after 24 h of administration. This study was in accordance with previous study where naked plasmid DNA were rapidly degraded over 99% in 24 h (Orság et al., 2008), over 95% in 90 min (Eun et al., 2007) and over 95% in 24 h (Zhang et al., 2005) following intramuscular administration. Aside from rapid degradation due to extracellular nuclease, the short plasmid's dwelling time was sufficient for the cellular uptake and VP3 expression. Previous study showed that VP3 expression and nuclear localization can be detected as early as 48 h post-transfection and trigger apoptosis less than 72 h (Natesan et al., 2006; Noteborn et al., 1994).

Naked plasmid DNA administration through i.v was observed as quick distributed and rapidly degraded with half life around 1.34 min (Kim et al., 2003), 4.5 min (Zhou et al., 2009), and 6.6 min (Osaka et al., 1996) in mouse model. In contrast, $2.8 \pm 1.1 \times 10^5$ copies/500 ng gDNA still can be detected at the injected muscle after 1 week administration. Long-term persistence of plasmid DNA only at the injection site was reported up to 2 years after application but the risk of mutation due to genome integration was negligible (Armengol et al., 2004).

The GFP protein expression observed in the tumor and all vital organs (liver, kidney, heart and spleen) at 14 days post-treatment was abundance in the tumor due to stress inducible promoter effect. Aggressive tumors often suffer from insufficient blood supply resulting in areas of acidosis, nutrient deprivation and hypoxia, which activate the GRP78 promoter (Lee, 2001). The GFP expression in blood vessel was expected due to the enhanced permeability and retention (EPR) effect commonly seen in solid tumors (Maeda et al., 2000). These indicated the possibility of leaky vasculature between tumor and entire body which promotes metastases. Even though after plasmid DNA degraded, the capability of low molecular weight GFP protein to stay in the blood circulation through blood diffusion give promise for the use of transgene as an anticancer therapy (Noguchi et al., 1998). No discernible effect was observed on histopathology, clinical chemistry, or haematology as VP3 transgene induces apoptosis in tumour cells selectively (Maddika et al., 2006; Pietersen et al., 1999; Visser et al., 2007). In order for plasmid DNA to become therapeutics agent, a certain degree of persistence within the body is required. For tumor treatment, a long persistence in the circulating blood is needed to create a passive accumulation in the tumor tissue (Greish, 2007). However, if the persistence was too long it might induce adverse reactions such as the risk of genome integration.

Conclusions

It was established that pVIVO1-gfpVP3 was extensively distributed and rapidly degraded in vital organs but stayed longer in the muscle and tumor tissue. The recombinant DNA plasmid used benefited from its early transgene expression characteristic releasing the anti-cancerous effect within a short dwelling time. A further evaluation on pharmacokinetics following administration is however needed to develop a strategy to improve gene therapy trials for this plasmid DNA vaccine.

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