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**Combination of Optison™ with ultrasound and electroporation increases albumin and thrompoietin transgene expression whilst elongation factor promoter prolongs its duration**

**Nagy Habib<sup>1\*</sup>, Roman Havlík<sup>2</sup>, Long Jiao<sup>1</sup>, Arpna Kiri<sup>3</sup>, Steen Jensen<sup>1</sup>, Joanna Nicholls<sup>1</sup>, Catherine Sarraf<sup>4</sup>, Geoffrey Goldspink<sup>3</sup>, Anthony Davies<sup>1</sup>, Ludek Slavík<sup>2</sup>, Chris Wood<sup>5</sup>, Michele Tiraby<sup>6</sup>, Daniel Drocourt<sup>7</sup>, Jean-Paul Reynes<sup>7</sup>, Claude Smadja<sup>8</sup>, Jean Gerard Tiraby<sup>6,7</sup>**

<sup>1</sup>Department of Surgical Oncology & Technology, Faculty of Medicine, Imperial College; <sup>2</sup>1st Department of Surgery and Department of Haemato-Oncology, Faculty of Medicine, Palacky University; <sup>3</sup>Basic Biomedical Science, Royal Free Hospital and University College Medical School; <sup>4</sup>Department of Biomedical Sciences, University of Westminster; <sup>5</sup>Bioenvision, New York; <sup>6</sup>InvivoGen, San Diego; <sup>7</sup>Cayla, Toulouse; <sup>8</sup>Hopital Antoine Beclere, Clamart.

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# Combination of Optison with ultrasound and electroporation increases albumin and thrombopoietin transgene expression whilst elongation factor promoter prolongs its duration

Research Article

Nagy Habib<sup>1\*</sup>, Roman Havlík<sup>2</sup>, Long Jiao<sup>1</sup>, Arpna Kiri<sup>3</sup>, Steen Jensen<sup>1</sup>, Joanna Nicholls<sup>1</sup>, Catherine Sarraf<sup>4</sup>, Geoffrey Goldspink<sup>3</sup>, Anthony Davies<sup>1</sup>, Ludek Slavík<sup>2</sup>, Chris Wood<sup>5</sup>, Michele Tiraby<sup>6</sup>, Daniel Drocourt<sup>7</sup>, Jean-Paul Reynes<sup>7</sup>, Claude Smadja<sup>8</sup>, Jean Gerard Tiraby<sup>6,7</sup>

<sup>1</sup>Department of Surgical Oncology & Technology, Faculty of Medicine, Imperial College, Hammersmith campus, Du Cane Road, London, W12 0NN, UK; <sup>2</sup>1<sup>st</sup> Department of Surgery and Department of Haemato-Oncology, Faculty of Medicine, Palacky University, I.P. Pavlova 6, 775 20 Olomouc, Czech Republic; <sup>3</sup>Basic Biomedical Science, Royal Free Hospital and University College Medical School, Rowland Hill Street, London, NW3 2QG UK; <sup>4</sup>Department of Biomedical Sciences, University of Westminster, 115 New Cavendish Street, London W1W 6UW, UK; <sup>5</sup>Bioenvision<sup>5</sup>, 509 Madison Avenue, Suite 404, New York, NY 10022, USA; <sup>6</sup>InvivoGen, 3950-A Sorrento Valley Boulevard, San Diego, California 92121, USA; <sup>7</sup>Cayla, 5 Rue Jean Rodier, Z.I Montaudran – B.P 4437, 31405 Toulouse cedex 4, France; <sup>8</sup>Hopital Antoine Beclere, 137 rue de la Porte de Trivaux, 92140 Clamart, France

**\*Correspondence:** Nagy Habib ChM FRCS, Head of Liver Surgery Section, Department of Surgical Oncology and Technology, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Hammersmith campus, Du Cane Road, London W12 0NN, UK; Tel: +44 20 8383 8574; Fax: +44 20 8383 3212; Email: nagy.habib@imperial.ac.uk

**Key words:** Gene therapy, liver failure, hypoalbuminaemia, thrombocytopenia

**Abbreviations:** thrombopoietin, (TPO); granulocyte colony-stimulating factor, (G-CSF); human thrombopoietin, (hTPO); vascular endothelial growth factor, (VEGF); locus control regions, (LCRs);  $\beta$ -globin LCR, (bLCR);

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## Summary

Hypoalbuminaemia and thrombocytopenia are two clinical problems frequently encountered in patients with chronic liver failure or cancer following treatment with chemotherapy. The current study was designed to assess the magnitude and duration of thrombopoietin and albumin transgene expression hoping to increase the production of albumin and platelets. Immunocompetent and immunocompromised (nude) mice were injected intramuscularly with plasmids expressing either human serum albumin or human thrombopoietin. The therapeutic expression cassette of the plasmids was driven by either CMV or elongation factor 1-alpha promoters respectively. In order to achieve muscle specific expression both gene constructs included the myosin light chain enhancer. The experiment was conducted in a group of mice which were injected with the transgene plasmid either in normal saline or plasmid followed by electroporation, ultrasound, optison and a combination of all three to increase transgene expression. The result showed that plasmids with the CMV promoter induced the highest transgenic expression lasting for one week whilst plasmids with the elongation factor 1-alpha promoter produced a weaker expression lasting for a longer and more stable duration of expression up to 3 months in both immunocompetent and nude mice. The combination of electroporation and ultrasound with Optison provided the highest transgene expression. We concluded that it would be possible to increase albumin and platelets production by an intramuscular injection of plasmids expressing human albumin and thrombopoietin. A combination of electroporation and ultrasound with Optison can increase their expression.

## I. Introduction

Albumin produced exclusively by liver is the most important factor in regulation of plasma volume and tissue fluid balance. It also binds to and transports a range of

lipid and lipid-soluble substances. Hypoalbuminaemia commonly secondary to chronic liver disease can result in clinically ascites and peripheral oedema.

Thrombopoietin (TPO) is a cytokine produced by liver and involved in the regulation of platelets production. The receptor for TPO is c-Mpl. The proto-oncogene *c-mpl* is a member of cytokine receptors superfamily with a similar sequence to erythropoietin and granulocyte colony-stimulating factor (G-CSF) receptors (Souyri et al, 1990; Gisselbrecht and Souyri, 1992). Expression of *c-mpl* in normal mice appears to be restricted to haematopoietic stem cells, megakaryocytes, and platelets although low levels of expression have been detected in endothelial cells (Gisselbrecht and Souyri, 1992; Methia et al, 1990). The TPO level decreases significantly in chronic liver diseases.

Skeletal muscle has previously been shown to express the encoded proteins in quantities sufficiently enough to generate immune responses following its introduction of naked plasmid DNA (Wolff et al, 1990; Ulmer et al, 1993; Hasan et al, 1999). The potential usefulness of skeletal muscle for local expression (Miller et al, 1995; Alila et al, 1997; Novo et al, 1997) and systemic delivery of therapeutic proteins has been explored (Raz et al, 1993; Raz et al, 1995; Fazio et al, 1994; Ma et al, 1995; Levy et al, 1996; Tripathy et al, 1996; Tokui et al, 1997; Aihara and Miyazaki, 1998; Anwer et al, 1998; Nitta et al, 1998; Horton et al, 1999). Although muscle lacks the cellular machinery necessary for regulated protein secretion, muscle cells appear to be able to utilise a constitutive secretory pathway to release heterologous proteins.

Direct *in vivo* transfer of the gene encoding erythropoietin via transduction by plasmid DNA (Rizzuto et al, 2000; Maruyama et al, 2001; Vilalta et al, 2001), or adenoviral and adeno-associated vectors (Osada et al, 1999; Bohl et al, 2000; Maione et al, 2000; Rudich et al, 2000; Yan et al, 2000), has shown to be effective in replacing repeated systemic administrations of recombinant human erythropoietin in the management of anaemia due to decreased red blood cell production (Rudich et al, 2000; Maione et al, 2000; Osada et al, 1999).

In this study the feasibility of expressing TPO and albumin after appropriate plasmid injection into mouse skeletal muscle are investigated by giving constructs containing the human albumin gene and human TPO gene respectively both into immunocompetent and immunocompromised animals. This provides an alternative extra-hepatic site for promoting and enhancing expression of proposed therapeutic genes for TPO or albumin production.

The aim of this study was to develop a system that can be utilized clinically for increasing albumin levels to treat patients with hypoalbuminaemia, and thrombopoietin (TPO) to treat thrombocytopaenia by using the CMV and the elongation factor 1- $\alpha$  (EF1) promoters and the myosin lightchain (Souyri et al, 1990; Methia et al, 1990) enhancer (Rosenthal et al, 1989). The inclusion of the latter in the gene constructs has been shown to not only increase expression, but also to result in muscle specific expression (Novo et al, 1997). In this way the site of therapeutic protein secretion was limited to a secure environment. Electroporation, ultrasonic cavitation and potentiation with Optison (Mallinckrodt Medical Ltd.,

Northampton, UK) were used to augment the magnitude of TPO and albumin gene expression (Yamashita et al, 2002).

## II. Materials and methods

### A. Plasmids

Plasmids were constructed to express either a.) human thrombopoietin (hTPO), or b.) human albumin (hAlb) genes. Two different promoters were used to drive the gene cassettes; either the CMV promoters (pGT123TPO and pGT123hAlb) or the EF1 promoters (pDERMhTPO and pDERMhAlb).

PGTalb and the pGThTPO were based on the pGT vector produced by InvivoGen (San Diego, CA, USA) with CMV promoter and myosin lightchain 1 and 3 MLC sequence enhancer. The pDERMhTPO and pDERMhAlb had EF1 promoter and a myosin enhancer. The transgene in the pDERM plasmids was preceded by 5'UTR Glut1. Both plasmids were grown in *E.coli*.

Quality control data showed OD 260/280:1.91-% supercoiled DNA: >95%-RNA was not detectable on agarose gel-toxin assay <0.1 EU/ $\mu$ g plasmid, less than 2% protein. Sterility test showed no colony forming units in a bioburden assay.

### B. Murine models

Immunocompetent (Balb C) and immunocompromised nude (CD-1) mice were used. BALB/c mice were bred in the animal facility of the Palacky University, Olomouc, Czech Republic. Female mice aged 10 - 14 weeks and weighing 20 - 24g were used in the experiments. They were maintained in a 12 hours light/dark cycle at a temperature of 20 - 24 °C and fed with standard diet and water ad libitum in accordance with the National Institutes of Health guidelines for animal care. Immunocompromised CD-1 mice were maintained under pathogen free conditions at the same institution and under the same regimen. Female CD-1 mice aged 8-10 weeks and weighing 17-21g were used in the experiment.

### C. Plasmid administration

Plasmids were injected intramuscularly in the tibialis anterior of both hind-legs at a dose of 50 $\mu$ g of plasmid per mouse in 50 $\mu$ l of saline.

### D. Transfection

The following methods were employed in order to increase gene uptake and expression after intramuscular plasmid administration:

#### 1. Electroporation

Electric pulses were delivered using pulse generator Electro Square Porator™ ECM 830 (BTX, Genetronics Inc., San Diego, CA, USA). The pulses were delivered using a 2-needle array inserted into the muscle. The distance between electrodes (needles) was 5 mm. The following protocol was used: 8 pulses, voltage 200V/cm, 40 ms duration of the pulse.

#### 2. Ultrasound

Ultrasound machine, Sonitron 1000 (Rich-Mar Corporation, Inola, Oklahoma, USA) was used. The probe was applied at the side of injection. Ultrasound with 1MHz frequency was applied for 2 minutes using 2 Watts of power and operated at 20% of duty cycle.

Optison (Mallinckrodt Medical Ltd., Northampton, UK) was added to potentiate cavitation of ultrasound. When Optison

was used, it was added to the plasmid solution at a ratio of 2:5. The plasmid in saline and Optison (28%) were injected together.

### 3. Anaesthesia

Plasmid delivery, as well as ultrasound and electroporation application, was administered under general anaesthesia. A combination of Hypnorm (fentanyl/fluanisone) and Hypnovel (midazolam) (F. Hoffmann/La Roche Ltd., Basel, Switzerland). Hypnorm, Hypnovel and sterile water for injection were mixed 1:1:2. Animals were injected in the peritoneal cavity with 0.005 ml of this solution per 1g of mouse weight. After taking blood samples animals were sacrificed.

### E. Experimental design

Control animals (group 1, n=36) were injected with normal saline or with plasmid only. Plasmid TPO or plasmid albumin in normal saline were injected into group 2 mice (n=36). A further group (group 3, n=36) of animals received each plasmid in normal saline followed by electroporation, and group 4 animals (n=36) received plasmid in saline followed by ultrasound. In group 5 (n=36) was plasmid application followed by both, ultrasound and immediately after ultrasound with electroporation and group 6 (n=36) mice received plasmid in saline mixed with Optison and as in group 5 in these animals was plasmid application followed by electroporation and ultrasound.

All mice were given a once only injection and 0.5 ml of blood was withdrawn. Then animals were sacrificed at 1, 2, 3 and 4 weeks, and at 3 and 6 months.

Plasmids used were pGT123hTPO with CMV promoter plus MLC enhancer, pDERMhTPO with EF1 promoter plus MLC enhancer, pGT123hAlbumin with CMV promoter plus MLC enhancer and pDERMhAlbumin with EF1 promoter.

### F. Levels of hTPO

For the study of serum TPO the ELISA TPO (Human TPO Quantikine Colorimetric Sandwich ELISA, R&D Systems Europe Ltd., Abingdon, UK) assay was used. This employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TPO was pre-coated on to the microplate. Standards and samples were pipetted into the wells and the immobilised antibody bound any TPO present. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for TPO was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, substrate solution was added to the wells and colour developed in proportion to the amount of TPO bound in the

initial step. The colour development was stopped and intensity of colour was measured.

### G. Levels of hAlb

All reagents, including microtitre plates and Human Albumin ELISA (E80-129, Bethyl Laboratories Inc., Montgomery, USA) quantitation kit, were obtained from Bethyl Laboratories Inc., Montgomery, Texas 77356, USA. All incubations were performed at room temperature. Microwells were first incubated for 1 hr with 100 µl of affinity purified human albumin capture antibody diluted 1/100 in carbonate buffer (0.05 M sodium carbonate, pH 9.6). After incubation, the capture antibody was removed and the wells were washed twice with Tween 20, 0.05% in TBS-Tris buffered saline, (50 mM Tris, 0.14 M NaCl, pH 8.0). Wells were then incubated with 200 µl of postcoat solution (TBS containing 1% BSA-bovine serum albumin) for 30 minutes. After incubation wells were washed two times as above.

Standards and samples were diluted in sample diluent (TBS, containing 0.05% Tween 20, 1% BSA) and transferred to predetermined wells and incubated for 1hr. Wells were then washed twice and 100 µl of HRP (horseradish peroxidase)-conjugated human albumin detection antibody, diluted 1/35000, was added. After incubation for 1hr the wells were washed 3 times.

Albumin was detected by addition of 100 µl of TMB (3,3', 5,5'-tetramethyl benzidine) substrate. The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and the optical density was measured at 450 nm.

### H. Statistical analyses

Standard Student's T-test was used for statistical analyses of measurements to compare the control with each injected group.

## III. Results

### A. Plasmids with the CMV promoter

pGT123hTPO administered with ultrasound and electroporation to nude mice resulted in a steep rise in serum hTPO expression at week 1 (1010 ± 1440 pg/ml, mean ± SD,  $p < 0.05$ ), but the level decreased to 58 pg/ml at week 2, 54 pg/ml at week 3 and 30 pg/ml at week 4 (**Table 1**). However, there was no statistically significant change in platelet count when compared with the control.

**Table 1.** Human thrombopoietin levels in serum of treated animals. Immunocompromised mice were injected intramuscularly with 50 µg of plasmid pGT123hTPO containing TPO with CMV promoter. Results (in ng/ml) are shown as mean ± SD.  $p$  value was calculated against control group each week.

	Week 1 (n=6)	Week 2 (n=6)	Week 3 (n=6)	Week 4 (n=6)
Simple plasmid injection I.M (Group 2, n=36)	68.7 ± 53 ( $p < 0.05$ )	56.3 ± 32 (NS)	39 ± 13 (NS)	61 ± 18 (NS)
I.M. injection plus electroporation (Group 3, n=36)	175.3 ± 261 (NS)	52.5 ± 18 (NS)	183.7 ± 248 ( $p < 0.05$ )	8 ± 11 (NS)
I.M. injection plus ultrasound (Group 4, n=36)	430 ± 523 ( $p > 0.05$ )	37.3 ± 43 (NS)	17.7 ± 17 (NS)	15.3 ± 19 (NS)
I.M. injection plus ultrasound and electroporation (Group 5, n=36)	1010 ± 1440 ( $p < 0.05$ )	58 ± 74 (NS)	54 ± 62 (NS)	30 ± 14 (NS)
Control (Group 1, n=36)	6 ± 8	35 ± 49	10 ± 14	14 ± 20

I.M., intramuscular

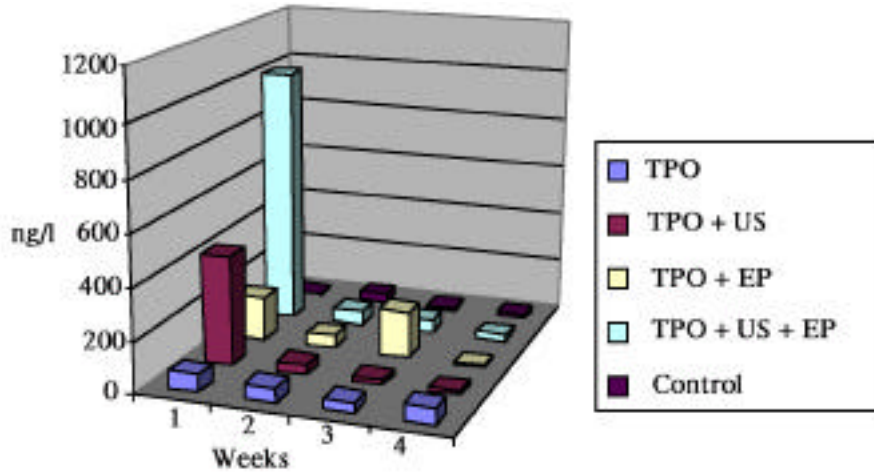
Group 1 – control group mice were injected with pGT123hAlb plasmid intramuscularly.

Group 2 – treated with intramuscular plasmid injection of 50 µg per mouse into both tibialis anterior muscles.

Group 3 – treated with intramuscular plasmid injection of 50 µg per mouse followed by electroporation (8 pulses, 200 V/min, duration of pulse 40 ms).

Group 4 – treated with intramuscular plasmid injection of 50 µg per mouse followed by ultrasounds application (2 min., 2 W, 1 MHz).

Group 5 – treated with intramuscular plasmid injection of 50 µg per mouse followed by both ultrasound 2 min., 2 W, 1 MHz) and electroporation (8 pulses, 200 V/min, duration of pulse 40 ms).



**Table 2. Human albumin levels in serum of treated animals.** Immunocompetent mice were injected with 50 µg of plasmid pGT123hAlb, containing human albumin. Results (in ng/ml) are shown as mean ± SD. *p*value was calculated against control group each week.

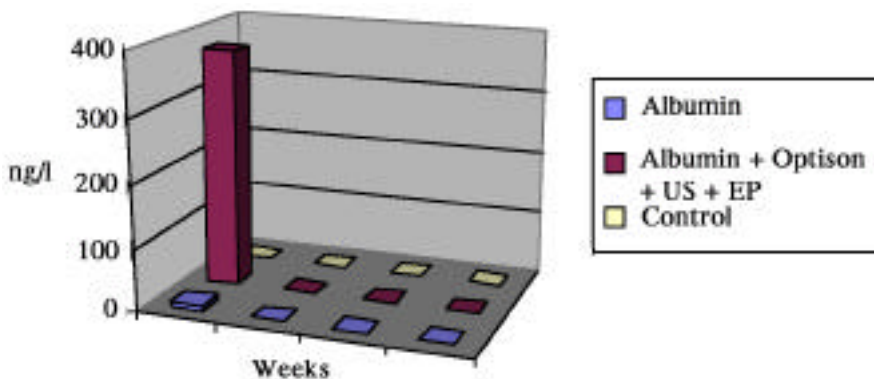
	Week 1 (n=6)	Week 2 (n=6)	Week 3 (n=6)	Week 4 (n=6)
I.M. injection of pGT123hAlb (Group 2, n=36)	9 ± 11 ( <i>p</i> <0.0001)	0	0	0
I.M. injection of pGT123hAlb with Optison™, ultrasound and electroporation (Group 6, n=36)	377 ± 100 ( <i>p</i> <0.0001)	0	0	0
Control (Group 1, n=36)	0	0	0	0

Group 1 – treated with intramuscular plasmid injection of 50 µg per mouse into both tibialis anterior muscles.

Group 2 – treated with intramuscular injection of pGT123hTPO plasmid.

Group 6 – treated with intramuscular injection of 50 µg of plasmid mixed with 20 µl Optison per mouse, followed by ultrasound (2 min., 2 W, 1 MHz) and electroporation (8 pulses, 200 V/min, duration of pulse 40 ms).

Zero means that albumin concentration was below the detectable level of ELISA test.



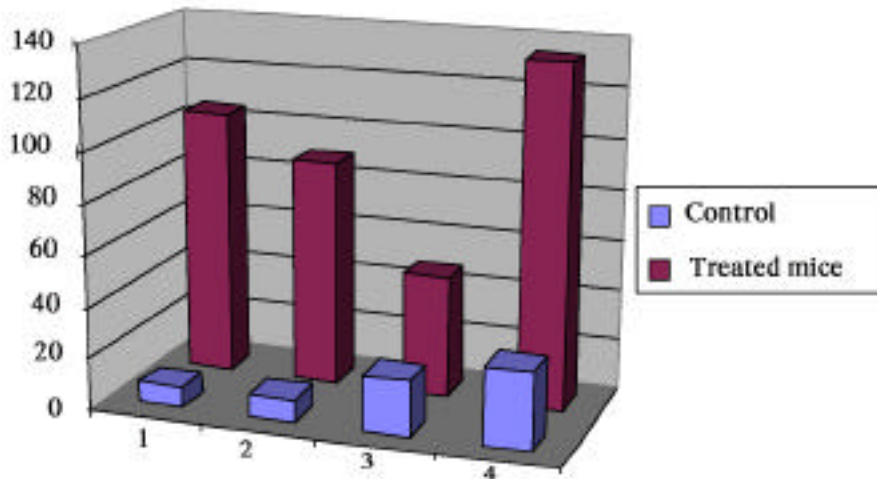
**Table 3. Serum levels of human thrombopoietin in mice, treated with pDERMhTPO plasmids.** 50µg pDERMhTPO and 20 µl Optison was injected per mouse. Ultrasound (2 min., 2 W, 1MHz) and electroporation (8 pulses, 200 V/min, duration of pulse 40 ms) were applied at the site of injection. Control mice were injected with pDERMhAlb plasmid which does not contain human TPO. Results (in pg/ml) are shown as mean ± SD. *p* value was calculated against control group on each occasion.

		2 weeks	1 month	3 months	6 months
Balb/C Mice (immunocompetent)	Treated Group 6, (n=36)	105 ± 107 ( <i>p</i> <0.03)	89.3 ± 122 ( <i>p</i> <0.05)	47.8 ± 77 ( <i>NS</i> )	134 ± 87 ( <i>p</i> <0.01)
	Control Group 1, (n=36)	8.3 ± 19	8.8 ± 19	21.7 ± 32	31 ± 26
CD 1 Mice (immunocompromised)	Treated Group 6, (n=36)	168 ± 78 ( <i>p</i> <0.002)	70.2 ± 54 ( <i>p</i> <0.01)	70.8 ± 75 ( <i>p</i> <0.04)	250 ± 179 ( <i>p</i> <0.01)
	Control Group 1, (n=36)	23 ± 49	7.3 ± 18	8.8 ± 11	30 ± 28

Group 1 – treated with intramuscular plasmid injection of 50 µg per mouse into both tibialis anterior muscles.

Group 6 – treated with intramuscular injection of 50 µg of plasmid mixed with 20 µl Optison per mouse, followed by ultrasound (2 min., 2 W, 1 MHz) and electroporation (8 pulses, 200 V/min, duration of pulse 40 ms).

Serum levels of human thrombopoietin  
(at 2 weeks, 1, 3, and 6 months) in ng/l in B  
treated with pDERMhTPO plasmids



The same procedure was performed with plasmid pGT123hAlb. Here, transgene expression was only detectable in the serum when the echo-contrast agent was added to the plasmid solution and a combination of electroporation and ultrasound were employed. Then the serum level of hAlb in immunocompetent mice was significantly increased at one week ( $377 \mu\text{g} \pm 100$ , mean ± SD, *p* < 0.0001) (Table 2).

The procedures indicated that transgene expression for serum hTPO or hAlb was highest with the combination of electroporation, ultrasound and Optison, used concomitantly.

### B. Plasmids with EF1 promoter

hTPO levels rose to 105 pg/ml (*p* < 0.03) in immunocompetent and 168 pg/ml (*p* < 0.002) in nude mice at week 2. Although this was less than expression seen with the CMV promoter, the duration of expression lasted for 6 months in both classes of mouse. Again there was no statistically significant change in platelet count throughout the experiments when compared with the

control (Table 3).

### IV. Discussion

This study has shown that both CMV and EF1 promoter MLC enhancer based plasmids expressed human TPO and human albumin in the serum of both immunocompetent and immunocompromised mice. The duration of expression was greater with the CMV promoter compared to the EF1 promoter, but expression was short-lived and the level had dramatically decreased by the second week after administration of the plasmid. The EF1 promoter had weaker expression than with CMV, but continued until the sixth month.

Expression was essentially the same whether the plasmids were injected in immunocompetent or immunocompromised animals. This showed that expression was not related to the immune status of the animal, but to the type of promoter used. The duration of expression is of clinical importance as the effect of recombinant human albumin lasts only 2 or 3 days and patients require repeat administrations two or three times

per week. Similarly, patients with thrombocytopenia and bleeding receive repeated platelet transfusions every 12 to 24 hours.

Other studies have elegantly shown the feasibility of an intramuscular gene therapy approach. Baumgartner and colleagues (Baumgartner et al, 1998) showed clinical improvement of blood flow in patients with peripheral vascular disease by injection of plasmid encoding vascular endothelial growth factor (VEGF). This promoted collateral vessel development in patients with critical limb ischaemia. Wang et al. (1998) demonstrated the induction of antigen specific cytotoxic T lymphocytes in humans by a malarial DNA vaccine.

It is advantageous to incorporate the muscle specific MLC enhancer in plasmids used with naked DNA or viral vectors as it increases expression in mature muscle fibres (Novo et al, 1997) and results in muscle specific expression (Rosenthal et al 1989) minimising the theoretical risk of possible harm in the event of integration and activation in germ cells.

Various techniques have been devised to resolve the problems related to low transfection efficiency, and safety in gene therapy. Therapeutic ultrasound was developed to induce cell membrane permeability. Ultrasound irradiation has been shown to increase the transfection efficiency of naked plasmid DNA into skeletal muscle cells especially with the use of Optison. Electron microscopy demonstrated the transient formation of holes (less than 5µm) in the cell surface with this system, which might explain the rapid migration of the transgene into the cells. Taniyama et al. (2002) have shown that transfection of hepatocyte growth factor plasmid by the ultrasound-Optison method could be useful for safe clinical gene therapy to treat peripheral arterial disease without a viral vector.

Electroporation is another gene delivery method for non-viral gene therapy in multiple tissues (Heller et al, 1996; Mir et al, 1999). Several groups have observed a 2 - 3 log increase in the level of gene expression after transfection of reported gene in the muscle by electroporation (Heller et al, 1996; Mir et al, 1999). Li et al. (2002), have shown regression of tumours by interferon electroporation gene therapy in mice.

One of the major problems using non-viral delivery approach is the short duration of transgene expression. For effective gene therapy of chronic disease, sustained transgene expression at therapeutic levels is required. The loss of transgene expression may be due to loss of the vector, transcriptional silencing of the transgene promoter, loss of the transfected cell through cell turnover, or the generation of an immune response to the transgene product or the transfected cell itself. The majority of pre-clinical and clinical studies have used strong viral promoters such as the CMV enhancer/promoter to achieve high levels of gene expression. Gill et al., (2001) have shown transient transgene expression with CMV, RSV and SV40 promoters. However, this showed that the use of the promoters from the human polyubiquitin C (UbC) and the EF1 genes resulted in sustained gene expression in the mouse lung. The UbC promoter directed high level reporter activity, which was maintained for up to 8 weeks

and was still detectable six months after a single administration. The results of our study using EF1 promoter support their findings.

Using a different system, Chow et al. showed long-term tissue specific gene expression using locus control region within replicating episomal plasmid and cosmid vectors (Chow et al, 2002). Locus control regions (LCRs) are transcriptional regulatory elements, which process a dominant chromatin remodelling and transcriptional activating capability conferring full physiological levels of expression on a gene linked in cis, when integrated into the host cell genome. Using the human  $\beta$ -globin LCR (bLCR) as a model Chow et al., showed that this class of control element can drive high levels of tissue specific gene expression in stable infected cultured cells from within an Epstein-Barr virus-based plasmid (Chow et al, 2002). LCRs are tissue-specific regulatory elements, which possess powerful chromatin remodelling capability and are able to establish and maintain a transcriptional competent, open chromatin domain, which leads to a sustained level of transgene expression. Therefore, inclusion of such elements is able to counteract the cell's selective gene silencing mechanism.

Another reason for the transient expression of plasmid DNA vectors could be due to immunostimulatory properties of plasmid DNA. Bacterial DNA and bacterially derived pDNA have the expected mathematical frequency of CpG dinucleotides and the DNA is predominantly unmethylated (Kreig 1999). Recognition of these differences by the host leads to a pleiotropic inflammatory response that includes the activation of B cells, monocytes, macrophages, dendritic cells, and natural killer cells (Krieg et al, 1995; Klinman et al, 1996; Ballas et al, 1996; Sparwasser et al, 1997; Sparwasser et al 1998).

Yew et al, (2002) have shown that CpG-depleted plasmid DNA vectors have enhanced safety and long term gene expression *in vivo*. This was achieved by reduction of CpG content of pDNA vectors by replacing large portion of vector with synthetic, non-CpG sequence.

The longevity of expression was related to sequence changes made within the CMV enhancer/promoter that somehow rendered the promoter insensitive to inactivation over time (Yew et al, 2002). Other promoters appear to have greater longevity, such as the ubiquitin promoter or liver-specific promoters, may be more resistant to any possible inflammation-induced inactivating factors (Qin et al, 1997; Herweijer et al 2001; Yew et al, 2001)

In this study the ultrasound option with Optison and electroporation increases expression significantly from ng/l or pg/ml up to µg/l or ng/ml. However, the 2 - 3 log increase was not sufficient to reach clinical efficacy. Probably 3 - 6 log increase is needed to achieve clinical efficacy.

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Nagy Habib ChM FRCS