Adenoviral delivery of the $\beta_2$-adrenoceptor gene in sepsis: a subcutaneous approach in rat for kidney protection

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ABSTRACT

Successful gene therapy requires gene delivery that is efficient, has an optimal route of administration and has biosafety. The aims of the present study were to evaluate the safety and applicability of the subcutaneous delivery route for adenoviral transgenes containing the human $\beta_2$-adrenoceptor (adeno-$\beta_2$-AR) and to investigate whether this approach prevented renal dysfunction in a rat model of endotoxaemic shock induced by LPS (lipopolysaccharide). Subcutaneous administration of adeno-$\beta_2$-AR (a total of $10^{10}$ viral particles) significantly increased $\beta$-AR density in the kidney, lung and liver, but was without effect on physiological and plasma biochemical parameters. Moreover, this dose of virus did not cause any of the potential toxic responses of viral administration, such as inflammation and tissue TNF (tumour necrosis factor)-$\alpha$ expression. Although the LPS challenge caused a decrease in glomerular filtration rate, fractional excretion of sodium and renal $\beta$-AR density in all groups, the reduction in renal function was significantly less in the rats given adeno-$\beta_2$-AR compared with non-treated rats. Thus, although further evaluation will be required, this initial study demonstrated that the subcutaneous injection of adeno-$\beta_2$-AR was efficient, comparatively non-pathogenic and potentially therapeutic to deal with acute renal failure associated with sepsis.

INTRODUCTION

In clinical trials with adenoviral vectors, route-dependent efficacy of gene therapy has been a significant concern [1]. Generally, direct injection into the target organ is more efficacious than systemic administration via intravenous or intramuscular routes. However, gene therapy aiming to overcome systemic inflammation, for example as occurs in sepsis, requires a suitable injection route for the viral gene vector such that it can effectively incorporate into the major target organs of sepsis, i.e. kidney, lung, liver and heart. An additional consideration of gene therapy using adenoviral vectors is the potential toxicity, which may cause inflammation of the tissues [2]. Therefore high transduction rates, biosafety of the gene product and minimally invasive administration are required to ensure an optimal route of gene delivery for a successful therapeutic strategy against sepsis.

Sepsis and its sequelae are still a major cause of morbidity and mortality within today’s intensive care

Key words: adenovirus, $\beta_2$-adrenoceptor, endotoxaemia, gene delivery, kidney, TNF (tumour necrosis factor).

Abbreviations: AR, adrenoceptor, adeno-$\beta_2$-AR, adenoviral constructs containing the human $\beta_2$-AR gene; BP, blood pressure; BPI, bactericidal/permeability-increasing protein; BUN, blood urea nitrogen; CPK, creatine phosphokinase; FEK, fractional excretion of potassium; FENA, fractional excretion of sodium; GFR, glomerular filtration rate; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; H&E, haematoxylin and eosin; HR, heart rate; IL, interleukin; i.p., intraperitoneally; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; PKA, cAMP-dependent protein kinase; TNF, tumour necrosis factor; t.v.p, total virus particles.

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units [3]. Patients with severe sepsis often experience multiple organ failure, including the kidney [4]. Recently, we reported in a rat model [5] that intraparenchymal injection of adenoviral constructs containing the human β2-AR (β2-adrenoceptor) gene (adeno-β2-AR) directly into the renal tissue effectively prevented the progressive renal damage associated with endotoxaemia. However, because gene delivery using intraparenchymal injections required major surgery, such an approach would be unlikely to be applicable in the clinical situation with septic patients. Since the goal of sepsis management is to reduce or control the detrimental systemic inflammatory responses to the infection, systemic delivery of gene therapy is of significant clinical interest as a potential therapeutic approach to manage the septic situation.

The subcutaneous route can be used as it does not require particular techniques or surgical manipulation and, therefore, it may be an option as a route of delivery of the vector and could be clinically relevant. However, it remains unclear whether subcutaneous delivery of adeno-β2-AR might be effective in providing sustained and therapeutic levels of the transgene in target organs. Furthermore, viral vectors have some negative characteristics, mainly that they can trigger inflammatory and immune responses with the possibility that adenovirus gene therapy may worsen the outcome of sepsis. With this in mind, the present study was undertaken to explore whether the utilization of the subcutaneous injection technique could potentiate β2-AR signalling systems in the kidney and, at the same time, to test the safety of gene product. Furthermore, we have sought to evaluate its efficacy in preventing endotoxin-induced renal dysfunction.

**MATERIALS AND METHODS**

**Reagents**

Adeno-Expression Vector kit was obtained from Takara Biomedicals. 125I-Cyanopindolol was obtained from PerkinElmer Life Science. [α-35P]dCTP and cAMP ELISA kits were supplied by Amersham Biosciences. The GF/C filter was from Whatman. Rat TNF (tumour necrosis factor)-α, IL (interleukin)-1β and IL-6 ELISA kits were obtained from Biosource International. SpotchemTMII was purchased from Arkray. Unless stated, all other reagents were from Sigma.

**Construction of recombinant adenovirus**

Adeno-β2-AR was a kind gift from Dr Walter J. Koch (Center for Translational Medicine, Jefferson Medical College, Philadelphia, PA 19107, U.S.A.) and Dr Robert J. Lefkowitz (Howard Hughes Medical Institute, Durham, NC 27710, U.S.A.). These adenoviruses were a replication-deficient first-generation type V adenovirus with deletions of the E1 and E3 genes, as described previously [6]. Virus isolates of adeno-β2-AR were plaque-purified and propagated in HEK 293 cells (a gift from Dr Ikuo Wada, Department of Biochemistry, Sapporo Medical University, Sapporo, Japan), isolated, concentrated and the titre determined using an Adenovirus Expression Vector kit.

**Rat preparation and protocols**

All procedures and protocols were approved by the Teikyo University Guide for the Care and Use of Laboratory Animals. Four-week old Wistar rats were fed a standard laboratory diet (126 mmol/l sodium and 118 mmol/l potassium per kg of food) and had free access to water. After a 7-day acclimatization period, rats were anaesthetized with pentobarbitone [50 mg/kg, i.p. (intraperitoneally)] and a 50 μl sample of the virus [1015 t.v.p. (total virus particles)] was injected subcutaneously into the back of the rats using a 25-gauge needle attached to a 1 ml syringe. Control rats were injected subcutaneously with an equal volume of PBS. During the 3 weeks after injection of adeno-β2-AR, swelling and nodes at the injection site were not observed. Adeno-β2-AR-treated and control rats were approx. 130–150 g in weight, and there was no significant difference between the groups. Systolic BP (blood pressure) and HR (heart rate) were monitored by means of a tail cuff sphygmomanometer, using an automated system with a photoelectric sensor (KN-201-1; Natsume Seisakusho). To estimate β-AR density in kidney, lung, liver and heart, groups of rats were killed at 1, 2 and 3 weeks after the administration of adenoovirus or PBS.

To induce acute renal failure in the rats, LPS (lipopolysaccharide; *Escherichia coli* O127:B8; 10 mg/kg of body weight) was injected i.p. into rats 2 weeks after the administration of the adenoviral vector. Control rats were injected i.p. with an equal volume of PBS. At this time, animals were housed in metabolic cages for 24 h to collect urine samples. Thereafter the animals were given an overdose of pentobarbitone, blood samples were collected and kidneys, lungs, liver and heart were removed, weighed and immediately frozen at −70°C.

**β2-AR binding assay**

Membrane fractions from all tissues were extracted following the method described by Lefkowitz et al. [7,8] with minor modifications. Membrane preparations (25 μg) were incubated with 125I-cyanopindolol (15–315 pmol/l) in binding buffer [75 mmol/l Tris/HCl (pH 7.4), 12.5 mmol/l MgCl2 and 2 mmol/l EDTA] either alone or with 20 μmol/l alprenanol, which was used for determination of non-specific binding. The incubation was carried out at 37 °C for 1 h in a total volume of 500 μl, followed by rapid filtration on GF/C filters and three washings with 750 μl of ice-cold binding buffer. β-AR density (Bmax) was determined using linear regression analysis of saturation isotherm data, linearly transformed
to give a Scatchard plot (Prizm4.0; GraphPad Software). Receptor density (measured in femtomoles) was normalized to mg of membrane protein. The protein concentration was assayed using a microprotein determination kit.

**Biochemical measurements**

Plasma and urine creatinine levels were determined using a creatinine assay kit, according to the protocols specified by the manufacturer. GFR (glomerular filtration rate; ml·min⁻¹·100 g⁻¹ of body weight) was expressed as a creatinine clearance rate. Plasma and urinary sodium or potassium concentrations were measured using a spectrophotometer (Hitachi 1170).

FENa (fractional excretion of sodium) was calculated from PNa and UNa (serum and urine sodium respectively) and PCr and UCr (serum and urine creatinine respectively) using the equation:

\[
\text{FENa} = \frac{(\text{UNa} / \text{PNa}) \times (\text{PCr} / \text{UCr}) \times 100}{100}
\]

Urinary and renal cAMP levels were estimated using a commercially available ELISA kit in which the assay was based on the competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP-specific antibody. For measurement of renal cAMP levels, frozen kidney samples were lysed using a liquid-phase extraction method [9], and the supernatants were taken for the analysis of renal cAMP levels, according to the manufacturer’s instructions. Renal cAMP levels were expressed as pmol/g of kidney. Plasma concentrations of TNF-α IL-1β and IL-6 were estimated using an ELISA kit, according to the manufacturer’s instructions. Plasma concentrations of BUN (blood urea nitrogen), GOT (glutamic-oxaloacetic transaminase), GPT (glutamic-pyruvic transaminase), LDH (lactate dehydrogenase) and CPK (creatinine phosphokinase) were assayed with enzymatic methods. A pathological assessment of kidney was made by adenovirus was based upon the results of serological comparisons. The unpaired Student t test was used for comparisons between adeno-β2-AR and control rats. The scores for microscopic grading were evaluated statistically using the non-parametric Kruskal-Wallis test. Results are expressed as means ± S.E.M.

**Assay of TNF-α mRNA**

mRNA levels were estimated using Northern blot hybridization analysis as described in our previous study [13]. For Northern blot hybridization, the 546 bp cDNA for TNF-α [13] and the 420 bp Hinf I fragment of human β-actin (National Children’s Research Centre, Tokyo, Japan) were labelled using the oligolabelling method in the presence of [α-³²P]dCTP and were used as a hybridization probe. All mRNA samples (10 μg) were applied to a Biodyne A membrane, hybridized simultaneously and exposed for the same time. The β-actin cDNA probe was used as a loading control after the TNF-α probe was stripped from the membrane.

**Statistics**

Statistical analysis was undertaken using ANOVA, followed by a Bonferroni and Dunnett test for multiple comparisons. The unpaired Student t test was used for comparisons between adeno-β2-AR and control rats. The scores for microscopic grading were evaluated statistically using the non-parametric Kruskal-Wallis test. Results are expressed as means ± S.E.M.

**RESULTS**

**β-AR density**

β-AR density levels were measured in kidney, lung, liver and heart at 1, 2 and 3 weeks after subcutaneous gene delivery (Figure 1). Administration of adeno-β2-AR resulted in over-expression of β2-AR in kidney, lung and liver. In kidney and lung, peak levels were reached at 2 weeks and, in the liver, β-AR density reached a peak level at 1 week and had returned to basal levels by 3 weeks. By contrast, β-AR density in the heart did not change at any time point. The renal cAMP content (determined from both right and left kidneys) in the adeno-β2-AR-treated rats was significantly increased (P < 0.05) 2 weeks after the injection of adeno-β2-AR.
Physiological and biochemical analysis
Subcutaneous administration of adeno-β2-AR had no effect on body weight, BP or HR compared with control rats (Table 1). Furthermore, plasma biochemistry (BUN, GOT, GPT, LDH and CPK) and cytokine (TNF-α, IL-1β and IL-6) levels 2 weeks after delivery of adeno-β2-AR were also unchanged compared with control rats (Table 1). Rat sera before injection of the adeno-β2-AR were all negative for antibodies to human adenovirus. These data suggest that the gene delivery did not initiate systemic inflammation or organ dysfunction at least in relation to kidney, lung, liver and heart.

Histological findings and TNF-α mRNA
To estimate immunological reactions and organ injury in response to adenoviral vector delivery, we performed a morphological analysis of H&E-stained kidney (Figures 2A and 2B), lung (Figures 2C and 2D), liver (Figures 2E and 2F) and heart (Figures 2G and 2H) tissue from adeno-β2-AR rats 2 weeks after gene delivery compared with control rats. Microscopically, adeno-β2-AR-treated rats had little, if any, cellular infiltration in these organs compared with control rats (Figure 2). Microscope grading in liver was 0.8 ± 0.3 in both control and adeno-β2-AR-treated rats, whereas in kidney, lung and heart this was 0 in both groups. No significant
Acute renal failure and gene therapy

Figure 2  Cross-section of kidney (A and B), lung (C and D), liver (E and F) and heart (G and H) 2 weeks after injection of adeno-β_2-AR

Rats were injected with 10^{10} t.v.p of adeno-β_2-AR. The section of heart was taken at the mid-ventricular level. Sections were stained with H&E. No obvious histological abnormalities and cellular infiltration in the kidney, lung, liver, and heart were observed in adeno-β_2-AR-treated rats. In A, C, E and G, the scale bars represent 100 μm. In B, D, F and H, the scale bars represent 50 μm.

No difference was observed between control and adeno-β_2-AR-treated rats. Moreover, myocardial inflammation was not obvious following delivery of adeno-β_2-AR (Figure 2G and 2H). No difference in terms of TNF-α mRNA expression in kidney, lung, liver and heart was observed between adeno-β_2-AR-treated and control rats (Figure 3).

Renal function in adeno-β_2-AR-treated rats following sepsis

As shown in Figures 4(A) and 4(B), GFR and FENa were similar in the control and adeno-β_2-AR-treated rats. Injection of LPS in the control rats significantly (P < 0.05) decreased both GFR and FENa, whereas it had no effect in adeno-β_2-AR-treated rats. FENa (fractional excretion of potassium) levels after delivery of adeno-β_2-AR were unchanged compared with control rats and were not changed by the injection of LPS (results not shown). β-AR density measured in the right kidney from control rats was significantly decreased (P < 0.05) 24 h after the LPS challenge (Figure 4C). Renal β-AR density in adeno-β_2-AR-treated rats, although higher than the control rats under basal conditions, was also significantly decreased (P < 0.05) by the injection of LPS (Figure 4C).

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Protection from endotoxaemia by subcutaneous injection of adeno-β2-AR

Injection of LPS decreased the body weight in both control and the adeno-β2-AR-treated rats (a weight loss of approx. 20–30 g). BP in both control and adeno-β2-AR-treated rats was significantly decreased \((P < 0.05)\) 6 h after the LPS challenge (from \(116 \pm 4\) to \(75 \pm 8\) mmHg in control rats, and from \(111 \pm 3\) to \(81 \pm 3\) mmHg in adeno-β2-AR-treated rats; both \(n = 4\)). Four out of ten (40%) of the control rats treated with vehicle (PBS) died within 16 h following the LPS challenge. By contrast, the mortality rate of rats receiving adeno-β2-AR gene transfer was approx. 10% (one out of 11). Importantly, LPS significantly increased \((P < 0.05)\) the plasma level of TNF-α, IL-1β and IL-6 in control rats, but the increase in TNF-α was inhibited by prior treatment with adeno-β2-AR (Figure 4D). On the other hand, adeno-β2-AR had no effect on plasma levels of either IL-1β (214 ± 4 pg/ml in control rats; and 154 ± 7 pg/ml in adeno-β2-AR-treated rats; both \(n = 5\)) or IL-6 (184 ± 3 pg/ml in control rats; and 144 ± 2 pg/ml in adeno-β2-AR-treated rats; both \(n = 5\)).

DISCUSSION

Preclinical studies of gene therapy must be performed in order to validate the feasibility of the approach, the safety of the gene product and to determine the appropriate dose range for administration in humans. In a previous

Figure 3 Representative Northern blots for renal TNF-α and β-actin mRNA at 2 weeks after subcutaneous administration of adeno-β2-AR

RNA was extracted from the kidney (right), lung (right), liver and heart (ventricle) of each rat. mRNA levels (arbitrary units) in each group are means ± S.E.M. (\(n = 5\)), and are expressed as the ratio of TNF-α to β-actin mRNA. A, adeno-β2-AR group; C, control group.

Figure 4 GFR (A), FENa (B), renal β-AR density (C) and plasma TNF-α (D) at 24 h after PBS or LPS injection in control or adeno-β2-AR-treated rats

LPS was injected at 10 mg/kg of body weight i.p. Adeno-β2-AR-treated rats were used on the day 14 after subcutaneous delivery of \(10^{10}\) t.u.p of adeno-β2-AR.

\*\(P < 0.05\) compared with control rats without LPS treatment.

\[P < 0.05\] compared with adeno-β2-AR-treated rats without LPS treatment.

\§\(P < 0.05\) compared with control rats with LPS treatment. \(n = 5\).

LPS(−), no LPS injection; LPS(+), LPS injection; cont, control; β2-AR, adeno-β2-AR treated.

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study [5], we demonstrated that direct injection of adeno-β2-AR into cortical areas of the kidney raised β-AR levels and successfully protected the kidney against the LPS-induced increase in TNF-α and reduction in renal function. It was also evident from this study [5] that, although there had been a local injection of the vector into the left kidney, there was also a raised β-AR content in the right kidney, suggesting that the vector had spread further into other organs of the body. The question arose as to whether a simpler approach, that of subcutaneous injection, might be sufficient. The present study demonstrates that adeno-β2-AR (10^{10} t.v.p) delivery via the subcutaneous route was effective in providing sustained levels of the transgene in the target organ (at least a doubling of the endogenous content) for at least 3 weeks. Interestingly, a similar magnitude and pattern of changes in AR content were also observed in lung, but to a lesser extent in liver and heart. Importantly, it was evident from the physiological parameters, i.e. body weight, BP and plasma biochemistry, that these were very comparable in the control and viral-vector-treated rats and suggested that the vector had no untoward effect at least over the 3 week time frame. Moreover, histological evaluation of kidney, lung, liver and heart showed that a 10^{10} t.v.p dose of adeno-β2-AR did not produce any evidence of cellular deterioration or toxicity using this approach. It has been reported that adenoviral gene therapy induces a pro-inflammatory response in lung and liver characterized by increased TNF-α expression [14,15]. However, in the conditions of the present study, there was no elevation in TNF-α mRNA levels as a consequence of adeno-β2-AR administration. Furthermore, the data show that the plasma cytokine levels are not increased in the adeno-β2-AR-treated rats. Together, these findings provide support for the view that the approach utilized in the present study did not initiate any inflammatory responses at least over the 3 week observation period. Nevertheless, the possibility remains that, over a longer time frame, administration of adenoviral vectors into the body might carry a greater risk of malignancy due to their ability to randomly integrate into the target genome. Furthermore, it remains unclear whether the immune responses to adeno-β2-AR may cause inflammation in the tissues of rats positive for adenoviral antibody prior to administration. An important outcome is that further basic study will be required to improve and evaluate the technical aspects of gene therapy using adenoviral vectors.

Endotoxaemia caused by Gram-negative bacteria in animal models can result in sepsis and deterioration in organ function, which includes kidney damage leading to renal failure [16,17]. A number of investigators have evaluated the effect of adenoviral vector gene transfer in animal models to overcome the consequences of sepsis. Alexander et al. [18] demonstrated that adenoviral gene transfer of human BPI (bactericidal/permeability-increasing protein) inhibited the effect of a non-lethal dose of LPS on cytokine responses and improved the survival of mice subject to lethal septic shock. On the basis of these results, they suggested that the human BPI gene transfer had the potential of being used as a therapeutic agent for septic conditions. In addition, Minter et al. [19] reported that adenoviral expression of the anti-inflammatory cytokine IL-10 could be successfully used in the treatment of two acute inflammatory disease situations, necrotizing pancreatitis and multi-system organ failure. However, whether these in vivo gene therapies prevented the progression of organ dysfunction associated with endotoxaemia remains unclear. Previously, we delivered adeno-β2-AR into the rat kidney by means of intraparenchymal injections and found that renal over-expression of β2-ARs was effective in preventing LPS (non-lethal dose)-induced renal injury [5]. Furthermore, this issue was also investigated in the present study, which provides experimental evidence demonstrating that subcutaneous delivery of adeno-β2-AR gene had the ability to prevent progressive renal dysfunction, as demonstrated by the deterioration in renal haemodynamics (GFR) and sodium handling (FENa) resulting from LPS (sublethal dose)-induced endotoxaemia. It was of concern that, although administration of adeno-β2-AR effectively supported renal function, gene therapy was not able to protect all of the endotoxaemic animals. Therefore gene therapy and adjuvant therapies with recombinant human activated protein C and early administration of empirical antibiotic therapy may be also required to improve the survival of the patients with sepsis [20].

There have been a number of studies reporting that a decreased expression or function of β2-ARs is involved in the pathogenesis of endotoxaemia development [21–23]. The present study provides further evidence to show that administration of LPS, as a model of sepsis, caused a decrease in GFR, together with a decrease in the number of β2-AR-binding sites in the rat kidney. The provision of extra receptors to replace those lost as a consequence of sepsis partially overcame the acute renal failure associated with sepsis and opens up this approach as a novel therapeutic strategy. The question arises as to the mechanisms involved in the β2-AR-mediated protective effect on renal function during LPS-induced endotoxic shock. One of the mechanisms that may be implicated following β2-AR activation is that of an anti-inflammatory action [24–26], whereby cytokine production is suppressed. Importantly, the administration of adeno-β2-AR, which markedly elevated β2-AR density in the kidney, was found to attenuate the stimulation of renal TNF-α gene expression associated with endotoxaemia [5]. Furthermore, elevation of plasma TNF-α levels by LPS was suppressed in the adeno-β2-AR-treated rats, which suggested that the renal inflammatory response and dysfunction could be influenced indirectly by β2-AR over-expression in non-renal tissues, such as liver and lung. It is likely that the protective effect of β2-AR
activation was exerted through the intracellular cAMP/ 
PKA (cAMP-dependent protein kinase) pathway, as 
previous reports found that cAMP/PKA activation was 
important in preventing the development of acute renal 
failure [27,28]. Indeed, it was observed that the fall in 
cAMP level was correlated with the decrease in 
GFR caused by LPS [5]. In the aden-β2-AR-treated rats, 
an increase in the renal content of cAMP may play an 
important role in the protection against the development 
of acute renal failure.

In the present study, aden-β2-AR rats were unable to 
inhibit LPS-induced IL-1β release despite the suppression 
of plasma TNF-α levels. Some evidence indicates 
that transcription of the gene encoding IL-1β or TNF-α is 
blocked or increased respectively, by β2-AR agonists [29– 
31]. Thus the difference may reflect different regulation 
of these cytokines by β2-AR activation. There is a 
discrepancy in Figure 1(A) and Figure 4(C) of the present 
study regarding the renal β2-AR density. The discrepancy 
could be due to a difference in the experimental protocols. 
Rats used for Figure 1(A) were housed in groups of 
four for 14 days after administration of the adenoviral 
vector under standard temperature, humidity and light 
conditions and were killed quickly after an overdose of 
pentobarbitone. On the other hand, although rats used 
for Figure 4(C) were also housed in groups of two or 
three for 14 days, each rat was subsequently placed for 
24 h into an individual metabolic cage. This individual 
housing may have resulted in an acute stress inducing a 
reduction in renal β2-AR density [32,33].

In summary, the present study demonstrates that the 
subcutaneous delivery of a 1010 t.v.p. dose of aden- 
β2-AR was relatively safe over a short time frame and 
yielded prolonged transgene expression. Furthermore, 
the presence of enhanced levels of β2-ARs in kidney 
appeared to maintain renal function following LPS- 
induced endotoxaemic challenge. These findings suggest 
that administration of aden-β2-AR may be a potential 
prophylactic/therapeutic approach in patients at high risk 
of developing acute renal failure or patients who suffer 
sepsis but have not yet developed renal failure.

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