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Prolonged hypotensive effect of human tissue kallikrein gene delivery and recombinant enzyme administration in spontaneous hypertension rats

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Abbreviations: BSA, bovine serum albumin; CMV, cytomegalovirus; FCS, fetal calf serum; IFA, indirect immunofluorescent assay; K1, tissue kallikrein; KLK1, tissue kallikrein gene; PEG, polyethylene glycol; PEI, polyethytenimine; SHR, spontaneous hypertension rat

Abstract

To evaluate the feasibility of treating hypertension by human tissue kallikrein gene (KLK1) delivery and by enzyme (rK1) administration, two recombinant vectors expressing KLK1 cDNA were constructed for gene delivery (pcDNA-KLK1) and recombinant enzyme preparation (pOV-KLK1). Expression of the pcDNA-KLK1 vector in COS-1 cells was confirmed by immunofluorescence and in spontaneous hypertension rats (SHR) by enzymatic detection. Following intramuscular or intravenous injection with the pcDNA-KLK1 vector, systolic pressure of SHR was significantly decreased, which lasted for 20 d to two months depending on dose, route and/or time of injection. Egg white containing recombinant hK1 was prepared by injection of egg-laying hens with the oviduct-specific expression vector pOV-KLK1 and administered into SHR via oral gavage. Following administration, systolic pressure of the SHR was decreased to that of normal rats, which lasted for 3-5 d depending on the dosage used. These data suggest that both *hKLK1* gene delivery and recombinant enzyme administration can be used as alternative strategies for treating human hypertension.

Keywords: gene delivery; human tissue kallikrein; hypotensive effect; recombinant enzyme administration

Introduction

Human tissue kallikrein (EC 3.4.21.35; KLK1 gene, hK1 protein), a glycoprotein of the serine proteinase superfamily, proteolizes kininogen to release kinin, which plays important roles in blood pressure regulation, electrolyte and glucose transport, and renal function (Yayama *et al*., 1998). The relationship between hK1 and blood pressure was revealed as early as 1934 (Elliot *et al*., 1934). Extensive clinical studies have shown that hK1 levels in urine are associated with the risk of essential hypertension (Margolius *et al*., 1971; Zinner *et al*., 1978).

Hypertension is a multifactorial and multigenetic chronic disease. Although the treatment of hypertension by conventional drug therapy seems to be successful, but only 20-35% patients have their blood pressure controlled (Burt *et al*., 1995). The limitation of drug therapy includes its excessive taken- frequency and side effects on patients. Recently, more attention has been paid to gene therapy for hypertension, which would produce a long-lasting effect without taking pills daily. The tested genes include those coding for atrial natriuretic peptide (Lin *et al*., 1997a), endothelial nitric oxide synthase (Lin *et al*., 1997b) and hKLK1 (Wang *et al*., 1995), among which hKLK1 gene has been proved to be more potential to achieve hypotensive effect. Here we present additional evidence that both hKLK1 gene delivery and rK1 administration can be used as alternative strategies for treating human hypertension.

Materials and Methods

Vector construction

The recombinant vectors used in this study contain hKLK1 cDNA (Shi *et al*., 2003) under the control of CMV promoter (pcDNA-KLK1) or the 5'- and 3'-regulatory regions of chicken ovalbumin gene (pOV-KLK1, Gao *et al*., 2003).

Animals

SHR and normal SD rats from Shanghai Experimental Animal Center were housed in isolators at 20°C and allowed normal rat chow and tap water. Egg-dropping hens from Institute of Poultry Science, Chinese Academy of Agricultural Science were housed in hen-

coops, and standard food pellets and tap water were provided *ad libitum*.

Cell transfection

pcDNA-KLK1 plasmid was prepared and purified using Wizard Clean-up Kit (Promega) according to manufacturer's instructions. 50,000 COS-1 cells were seeded into each well of 24-well plates (Nunc) and cultured at 37°C in a humidified 5% CO_2-containing atmosphere in MEM supplemented with 10% fetal calf serum (FCS, HyClone), 100 ㎎/ml streptomycin and 100 U/ml penicillin. Immediately before transfection, cells were rinsed with MEM and 0.5 ml MEM supplemented with 5% FCS was added into each well. The transfection was performed according to previously described method (Boussif *et al*., 1995) with 2 ㎎ of plasmid DNA and 4 ㎖ of 10 mM 25 kDa polyethylenimine (PEI) for each transfection. After 3 h of incubation, the medium was replaced with normal medium supplemented with 10% FCS. On the following day, the trasnfection was repeated once.

Indirect immunofluorescent assay (IFA)

At 48 h after transfection, the cells were rinsed three times with cold phosphate buffered saline (PBS) (pH 7.4) and fixed for 5 min in cold acetone/ethanol mixture (v/v = 3:2). Following blocking at 37°C for 2 h with PBS containing 0.5% bovine serum albumin (BSA), cells were washed three times with PBS containing 0.1% Tween-20. Then, anti-hK1 monoclonal antibody (Zhang *et al*., 2003) diluted in the blocking buffer was added to each well and incubation was continued at 37°C for 1 h. After three times of washing, 1,000-fold diluted anti-mouse FITC conjugate (Sigma) was added to each well and incubation was continued for additional 45 min. Following four times of washing, the cells were observed under fluorescent microscope.

In vivo expression of hKLK1 cDNA

1.0 mg of pcDNA-KLK1 or pcDNA3 was diluted in 1.0 ml of 5% glucose solution and injected intra-quadriceps or via tail vein into each SHR. At 3 d after injection, animals were sacrificed and different tissues were collected for detecting hK1 activity using published method (Shanghai Medical Publishing House, 1980).

Preparation of rhK1

The human KLK1 cDNA was subcloned into the oviduct-specific expression vector pOV and the resulting vector is called pOV-KLK1. The vector was prepared by polyethylene glycol precipitation (Sambrook *et al*., 2001) and diluted with 5% glucose solution to a final concentration of 10 mg/ml. 1 mg of the vector was mixed with 2 ml of 10 mM 25 kD PEI and vortexed briefly. After incubation for 15 min at room temperature, the mixture was injected into each egg-dropping hen *via* wing vein. On the following day, the injection was repeated once. From the second day of last injection, eggs were collected and hK1 activity in the egg white was detected using the above method.

Vector injection

Before injection, normal SD rats and SHR were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body weight for systolic pressure measurement using photoelectic tail-cuff device (Natsume Co). The purified pcDNA-KLK1 vector was diluted in 0.5 M sodium phosphate to a final concentration of 1 mg/ml and each animal was injected intravenously or intra-quadriceps with different doses. At different time points of post-injection, systolic pressure of each animal was measured. For some animals, vector injection was repeated once when the systolic pressure returned to the original level following first injection.

Oral administration of rhK1

The rhKLK1-containing egg white was diluted in PBS to 40 U/ml, 80 U/ml or 120 U/ml and administrated into each 15-week-old SHR *via* oral gavage. Normal egg white diluted in PBS was used as the control. At different time points of post-administration, systolic pressure was measured using the above method.

Result

Immunofluorescence

To test whether the vector can drive hKLK1 cDNA expression in eukaryotic cells, COS-1 cells were mock-transfected or transfected with pcDNA-KLK1

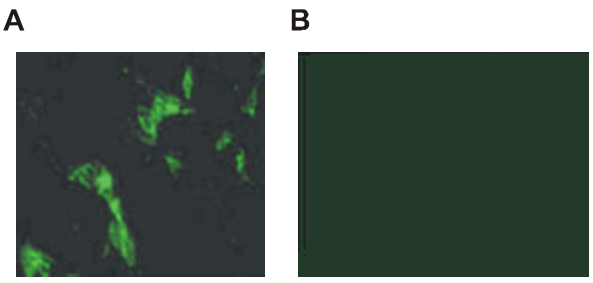

Figure 1. Expression of KLK1 cDNA in COS-1 cells detected by immunofluorescence. (A) pcDNA-KLK1-transfected cells. (B) mock-transfected cells.

plasmid and submitted to indirect immunofluorescence (IFA). At 48 h after transfection, specific expression of hK1 was detected by hK1-specific monoclonal antibody in pcDNA-KLK1-transfected cells but not in mock-transfected cells (Figure 1).

In vivo expression of pcDNA-KLK1

To demonstrate whether pcDNA-KLK1 vector can express enzymatic activity *in vivo*, 3 SHR were injected intramuscularly or intravenously with 1 mg of pcDNA3 (control) or pcDNA-KLK1 and different tissues were collected for hK1 activity detection at 3 d after injection. Compared to control SHR, significant increase in enzymatic activity was detected in all tissues tested (Figure 2).

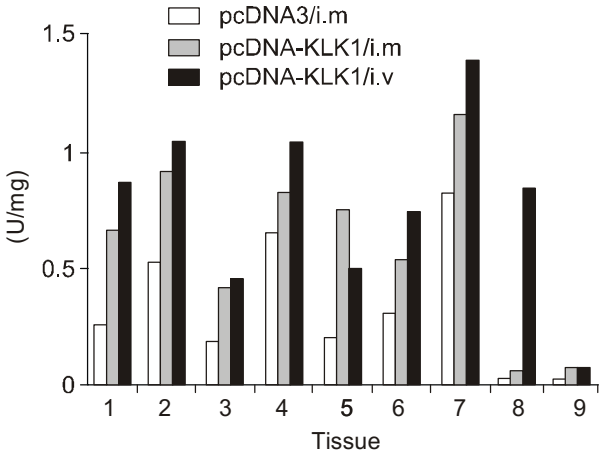

Figure 2. *In vivo* expression of KLK1 cDNA in SHR. 1-9 represent enzymatic activity in the heart, liver, brain, kidney, muscle, lung, spleen, blood and urine of pcDNA-KLK1-injected SHR, respectively.

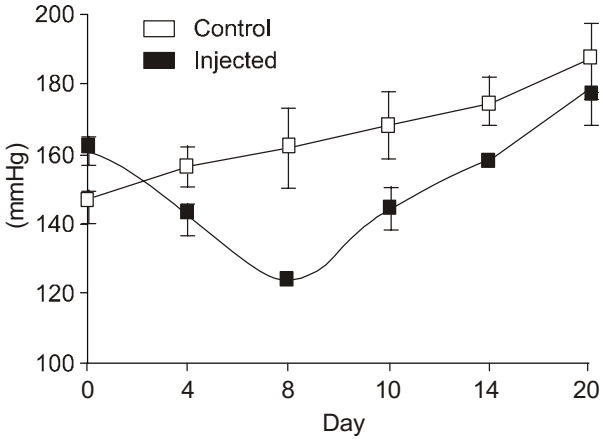

Figure 3. Hypotensive effect of intramuscular vector injection in SHR (pilot experiment). Empty box presents the control SHR and solid box SHR injected with pcDNA-KLK1 plasmid.

Pilot gene delivery

To test whether vector injection had hypotensive effect, 5 SHR were injected intramuscularly with 1 mg of pcDNA-KLK1 plasmid and systolic pressure was measured before and after injection. Significant decrease in blood pressure was evident at 4 d and reached lowest level (125 mmHg) at 8 d after injection. Thereafter, the decreased blood pressure returned gradually and to the level (180 mmHg) of the control SHR at 3 weeks after injection (Figure 3).

Dose- and route-dependent effects of vector injection

To test whether hypotensive effect of vector injection is dose-and/or route-dependent, different doses of pcDNA-KLK1 plasmid ranging from 200 mg to 1 mg were injected intra-quadriceps or intravenously into SHR (n = 2) and systolic pressure of each animal was

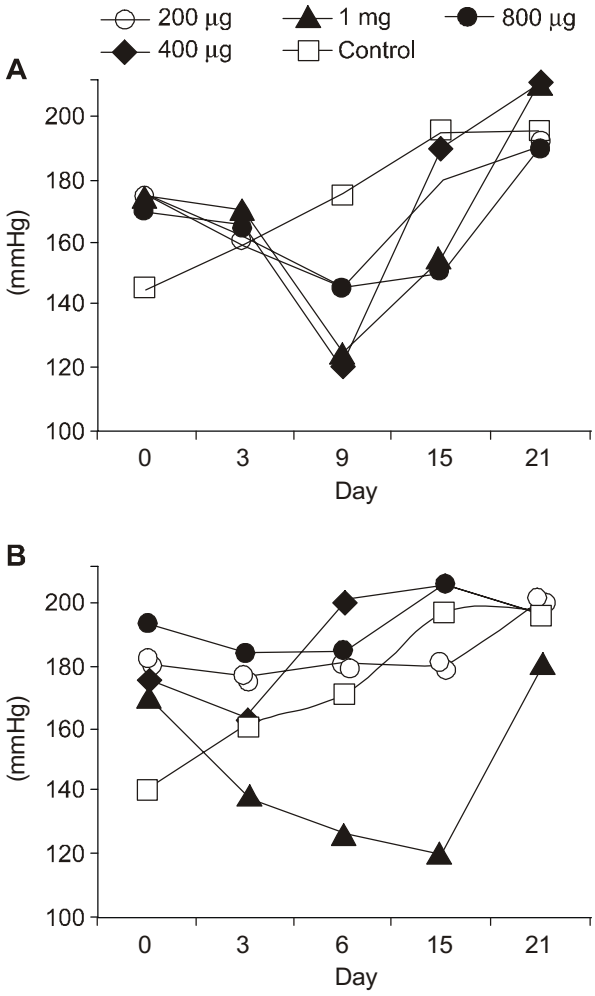

Figure 4. Dose- and route-dependent hypotensive effect of vector injection in SHR. (A) intramuscular injection. (B) intravenous injection.

measured before and after injection. As Figure 4A shows, decrease in blood pressure was evident at 3 d after injection in all vector-injected animals via intramuscular route. The blood pressure reached its lowest level in a dose-dependent manner at 9 d and returned gradually to that of the control rat at 21 d after injection. In the case of intravenous delivery, similar pattern of blood pressure decrease can be seen at 3 d after injection, but the decrease in 1-mg group was much more significant than that in other groups. Similarly, the hypotensive effect also lasted for about 3 weeks (Figure 4B).

Hypotensive effect of repetitive gene delivery

Since hK1 differs considerably from rat K1 in amino acid sequence, expression of human KLK1 cDNA in immune competent SHR, in principle, could induce immune response against the expressed protein, which could compromise the outcome of repetitive gene delivery. To address this question, the vector injection was repeated once with 1 mg of pcDNA-KLK1 when the decreased blood pressure returned to the level of control animal following the first injection. As Figure 5 shows, secondary injection caused even faster decrease in blood pressure, which lasted for 2 months.

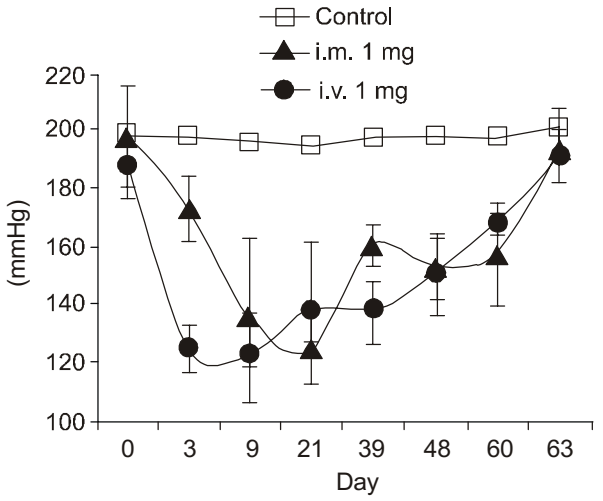

Figure 5. Hypotensive effect of the secondary vector injection in SHR.

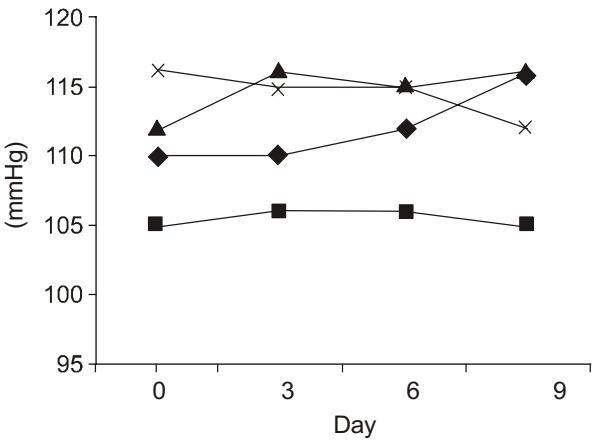

Figure 6. Hypotensive effect of vector injection in normal rats. 4 SD rats were injected intramuscularly with 1 mg of pcDNA-KLK1 plasmid.

Hypotensive effect of vector injection in normal rats

To demonstrate whether the gene delivery has hypotensive effect in normal rats, each normal SD rat (n = 4) was injected intramuscularly with 1 mg of pcDNA-KLK1 plasmid and its blood pressure was measured at three-day interval for 9 d. As Figure 6 shows, no significant change in blood pressure was evident during the experimental period.

Hypotensive effect of orally administered rhK1

To further demonstrate hypotensive effect of hK1 protein in SHR, 1.0 ml of egg white containing 40 U, 80 U or 120 U of rhKLK1 was orally administrated to each 15-week-old SHR with blood pressure over 190 mmHg. At 7 h post-administration, systolic pressure of the three animals decreased significantly and down to 130 mmHg on the following day. The hypotensive effect persisted for 3-5 d in a dose-dependent manner, whereas blood pressure of normal egg white-administered SHR remained unchanged (Figure 7).

Discussion

Conventional drug therapy for treating human hyper-

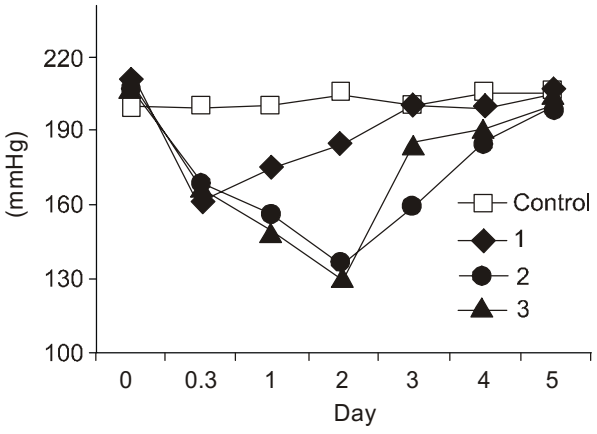

Figure 7. Hypotensive effect of orally administered rhK1 on SHR. Control: 1 ml of diluted normal egg white; 1-3: 1 ml of egg white containing 40, 80 or 120 units of rhK1.

tension is short-lasting (normally $<$24 h) with considerable side effects. Gene therapy, viral-vector mediated gene therapy in particular, has the advantage of long-lasting effect (Huard *et al*., 1995). However, viral vector-mediated gene transfer has considerable safety concern and unwanted immune responses, both of which are difficult to tack in the near future. Non-viral gene delivery has the disadvantage of low levels of gene expression and thus relatively short-lasting therapeutic effect, but it has been recognized to be relatively safe. This was further supported by our experiments, in which no overt side effects and significant immune responses were observed during the experimental periods.

Our data further suggest that non-vial delivery of *KLK1* gene is a feasible way of treating human hypertension (Chao *et al*., 1996; 1997). In both pilot and formal experiments, systemic delivery of pcDNA3-KLK1 plasmid into SHR caused significant decrease in systolic pressure, which lasted for up to 3 weeks in a dose-dependent manner following first injection. Although the two delivery routes had similar hypotensive effect in SHR, larger doses were needed for intravenous transfer to obtain meaningful hypotensive effect, but such effect was more significant and long-lasting than that of intramuscular delivery.

Although hK1 is significantly different from rat K1 at the level amino acid sequence, first injection of SHR with pcDNA-KLK1 plasmid did not lead to significant immune response to the expressed protein. This was supported by the secondary gene delivery, in which blood pressure decrease was even deeper and longer than that caused by first transfer. This is very important for non-viral gene therapy for treating human hypertension, which is a lifelong disease and repetitive gene delivery is needed.

Interestingly, gene transfer of SHR with our vector caused systolic pressure decrease only to the levels of control animals with no further decrease observed even at large doses. Even more interestingly, the vector transfer had no significant hypotensive effect in normal SD rats. Although its reason is unclear, the phenomenon is clinically beneficial and warrants further investigation.

In addition, oral administration of rhK1-containing egg white not only supports the claim that *in vivo* expressed hK1 has hypotensive effect in SHR, but also indicates that a practical strategy is available for large-scale production of rhK1 for treating human hypertension.

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