ACCUMULATION OF HUMAN APOLIPOPROTEIN-E IN RAT PLASMA AFTER *IN VIVO* INTRAMUSCULAR INJECTION OF NAKED DNA

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Naked DNA was found to be incorporated and consistently expressed after *in vivo* direct injection into striated muscle. In addition to the local expression of muscle-related or exogenous proteins, intramuscular direct gene transfer may be a useful tool to deliver recombinant proteins into the blood stream. However, no direct demonstration of recombinant protein secretion from muscle to the circulation has been reported thus far. We have injected a naked plasmid DNA containing the human receptor-binding defective apo-E2 cDNA, under the control of CMV promoter, into the quadriceps of Yoshida rats, affected by hereditary hypercholesterolemia and altered LDL-receptor activity. Plasma accumulation of human apo-E2 was demonstrated for at least 45 days after injection. On the contrary, the expression of the normal human apo-E3, injected into the muscle of normal Wistar rats, was demonstrated only in the area of muscular injection and not in the blood plasma. Endogenous rat apo-E expression was not affected by the exogenous human apo-E2 production. Our results demonstrate the availability of intramuscular direct gene transfer as a safe and simple method for the chronic systemic delivery of recombinant proteins to the circulation, although further improvements are needed in order to enhance the efficiency and stability of expression.

Several reports have demonstrated that myofibers from mice (1), rats (2, 3), fishes (4), cats and non-human primates (5) have the ability to take up and express intramuscularly injected recombinant genes in the form of naked supercoiled plasmids. The injected DNA is maintained in an episomal, circular form and does not replicate (1). Gene expression was demonstrated for

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at least 2 months and up to 1 year (6). Persistence of the nonreplicating plasmid DNA was suggested to be related to the slow turnover rate of myofibers.

The encoded protein was identified at low level only in the site of muscular injection, but no sufficient gene expression to produce significant serum levels was demonstrated (1-7).

In some reports the expression of the recombinant functional protein was able to partially restore altered myofiber functions. In the MDX mice, the animal model of human Duchenne's muscular dystrophy, 1% of the myofibers in the site of injection expressed the human dystrophin gene and was partially cured (7). It was recently demonstrated that the direct intramuscular inoculation of recombinant genes encoding specific antigens can elicit humoral and cellular immune responses protecting the animals against subsequent antigenic challenges (8). The intramuscular repeated injections of the influenza virus nucleoprotein gene not only induced antibodies responses but also activated specific killer T-cells through the interaction of the encoded protein with the class I major histocompatibility protein displayed on the cell surface (9). Thus, direct gene transfer technique seems to be a simple and safe method with a broad possible application to gene therapy.

In this view, it would be important to determine if this technique is also applicable to the synthesis of proteins secreted in blood circulation. The complex of these observations does not seem to exclude the possibility that proteins encoded by injected genes may be secreted in the blood stream, even if the low efficiency of gene expression and protein synthesis, combined with the rapid plasma catabolism of most secreted proteins, may actually hinder the demonstration. In order to overcome these difficulties, we have choosen an experimental strategy that combines the injection of a mutant human apolipoprotein-E gene (apo E2) whose product shows a very low binding efficiency to the LDL-receptor (10), and an animal model, the Yoshida rat, that shows a complex hereditary alteration of lipid and cholesterol metabolism, combined with a reduction of LDL-receptor efficiency (11). This approach allowed us to generate a double block to the excretion and utilization of the recombinant human apo E, possibly inducing plasma accumulation of the human protein.

MATERIALS AND METHODS

Animals: Wistar and hypercholesterolemic Yoshida rats were obtained from Charles River Italia S.p.A. and fed a regular diet. For any of the experiments, a minimum of 4 animals received identical treatment and comprised an experimental group.

Plasmids: The cDNAs for both the normal human allele apo-E3 and the mutant human apo-E2 ($Arg_{158} \rightarrow Cys$) [generous gifts from S. Lauer, Gladstone Institute of Cardiovascular Disease, UCSF, San Francisco, CA (USA)] were subcloned under the control of CMV promoter, in a vector containing the homologous signal peptide sequence for secretion, the human growth hormon (hGH) terminator sequence, which includes the polyadenilation signal sequence, and the SV40 enhancer and origin of replication (21) (Fig. 1). CMV promoter was chosen because it determines a higher and more stable levels of gene expression following direct intramuscular injection of recombinant DNA (1). Plasmid DNA (pCMVE-3 and pCMV-E2 respectively) was prepared using Qiagen columns (Diagen) and was further purified by Geneclean columns (Bio 101 Inc.), according to the supplier's protocol. After purification, DNA was resuspended under laminar flow cabinet in sterile solutions.

Intramuscular injection of naked DNA: All procedures were carried out on the quadriceps muscle of 2 months old normal Wistar or hypercholesterolemic Yoshida rats under mild sodium pentobarbital anesthesia (11). A 1.0 cm incision in the skin was made so that the underlying muscle might be directly visualized. Plasmid DNA was prepared in aliquots of 100 μg in 25 μl of sterile 20% sucrose solution and injected by a 27-gauge needle and a 1 ml syringe. The skin was sutured by stainless steel clips (Ethicon, Proximate plus PPW15). Sham injected control rats were treated with 20% sterile sucrose solution. DNA and sham (20% sucrose solution) injections were administered in only one or both legs.

Preparation of protein extracts: Approximately 0.5 cm^3 of muscle around the site of injection was excised after 15 and 45 days, finely minced by a razor, placed into a 1.5 ml microcentrifuge tube containing 100 μl of lysis solution (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 50 mM EDTA) and several protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin), ground with a pestle and vortexed overnight at 4°C. The dissolved muscle suspension was cleared by centrifugation. Supernatant was frozen in liquid nitrogen and stored at -80°C. Blood samples were obtained from tail vein bleeding (approximately 300 μl per animal). Blood was left at room temperature until serum and cells were completely separated. Serum was then recovered, supplemented with protease inhibitors, quickly frozen in liquid nitrogen and stored at -80°C in aliquots.

Western blot analysis: Protein samples were subjected to SDS-polyacrylamide gel electrophoresis, using a 12% gel. Proteins were transferred from gel to nitrocellulose membranes, which were incubated with either polyclonal rabbit anti-human apo-E antiserum or anti-rat apo-E antiserum. As secondary antibody, goat anti-rabbit antibodies, labelled with ^{125}I, were used.

Measurements of lipids and lipoproteins: 1 μl of plasma was subjected to agarose gel electrophoresis for 35 minutes at 100 Volts. Gel was dried and stained with Red Fat 7B. Cholesterol and triglycerides were measured enzymatically using commercially available kits, according to manufacturer's protocols (Boehringher Mannheim).

RESULTS

Initial experiments were performed to determine whether the intramuscular injection of the normal human apolipoprotein-E3 cDNA could allow muscular expression, secretion and identification of the recombinant protein in the blood plasma of normal rats. Two plasmids

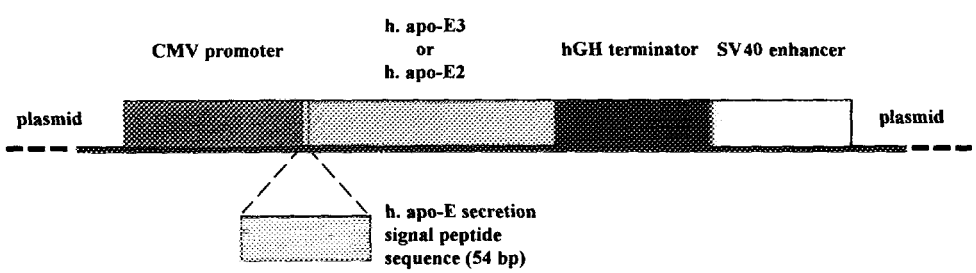

Fig. 1. Apo-E cDNA constructs used for intramuscular direct injection. CMV (cytomegalovirus) promoter; the cDNAs for both the normal human allele apo-E3 (pCMV-E3) and the mutant, receptor-binding defective, human apo-E2 ($Arg_{158} \rightarrow Cys$) (pCMV-E2), which include the human apo-E signal peptide sequence for secretion; hGH terminator: human growth hormone terminator sequence, which includes the polyadenilation signal sequence; SV40 enhancer: SV40 origin of DNA replication and early region enhancer sequences.

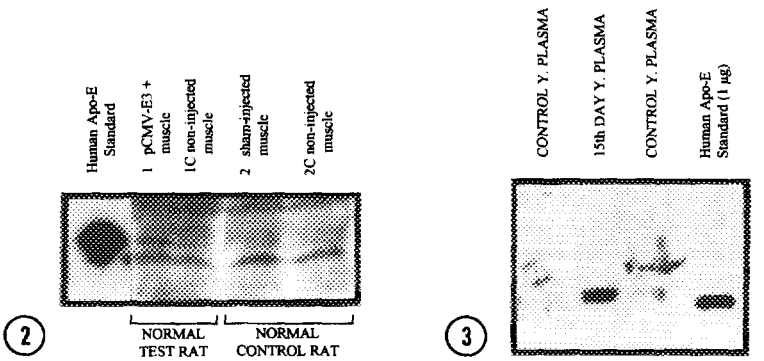

Fig. 2. Western blot analysis of protein extracts from injected and non-injected muscles of normal Wistar rats. After SDS-polyacrylamide gel electrophoresis and electrotransfer the nitrocellulose filter was incubated with polyclonal rabbit anti-human apo-E antiserum. As secondary antibody, goat anti-rabbit ^{125}I-labelled antibodies were used. Protein extracts from pCMV-E3 injected muscle (second lane); controlateral non-injected muscle from the same animal (third lane); muscles of two control rats, one sham-injected (fourth lane) and one non-injected (fifth lane). First lane, 1 µg human apo-E standard control sample.

Fig. 3. Western blot analysis of plasma collected at 15 days after injection of pCMV-E2 plasmid in hypercholesterolemic Yoshida rats. 1 µl of plasma was directly analyzed by SDS-polyacrylamide gel electrophoresis and subsequent Western blotting. Human apo-E was detected as described in Fig. 2. Plasma from injected Yoshida rats (second lane); non-injected control Yoshida rat plasma (first and third lane). 1 µg human apo-E standard control (fourth lane).

containing respectively the normal apo-E3 cDNA (pCMV-E3) and the ligand-defective apo-E2 cDNA (pCMV-E2) (Fig.1) were generated. Both constructs contained the CMV promoter, the apo-E signal peptide sequence for protein secretion and the human growth hormon (hGH) terminator sequence. Fifteen days after the injection of 100 µg of pCMV-E3 plasmid DNA, in 25 µl of 20% sterile sucrose solution, into the quadriceps of 2 months old Wistar rats, muscular extracts and blood plasma were analyzed by SDS-polyacrylamide gel electrophoresis and subsequent Western blotting (Fig 2). For immunostaining of the blot, a polyclonal antibody was used that showed partial cross-reaction with rat apo-E. Human recombinant apo-E3 produced in the injected muscle was very similar in electrophoretic migration to human apo-E standard control sample (1 µg loaded on standard lane, Fig. 2). Controlateral non-injected muscle and the muscles of two control rats, one sham-injected with 25 µl of 20% sucrose solution and one non-injected, did not show the presence of the human protein but only faint cross-reactivity with an endogenous rat protein. Immunoblot analysis was also performed on sera obtained from the same Wistar rats whose quadriceps were injected with pCMV-E3 and that were demonstrated to locally produce the recombinant apo-E. No human apo-E3 was identified after SDS-polyacrylamide gel electrophoresis of 1 µl total plasma and western blotting using the same anti-human apo-E antiserum (data not shown).

This result could have been determined either by a low efficiency of gene expression and protein synthesis and/or by a complex of hindrances in protein secretion from muscle cells to blood circulation, particularly the short half life of apo-E in plasma, due to its receptor-

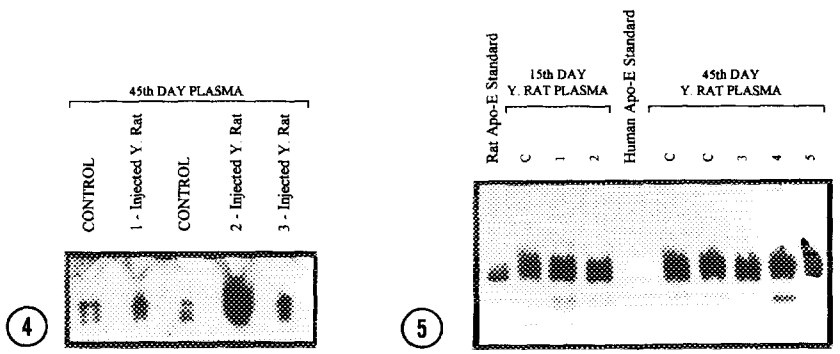

Fig. 4. Western blot analysis of plasma collected at 45 days from injection of 100 μg pCMV-E2 plasmid in hypercholesterolemic Yoshida rats. Plasma from different injected animals (lanes 2, 4, 5); lanes 1 and 3, non-injected rat control plasma.

Fig. 5. Western blot analysis of endogenous rat apo-E plasma levels in injected and control Yoshida rats at 15 and 45 days. Blot was immunostained using polyclonal anti-rat apo-E antibodies. Samples 1-5, 1 μl of plasma from injected Yoshida rats; C, 1 μl of plasma from non-injected Yoshida control rats. No cross-reaction to 1 μg human apo-E standard control (lane 5) was observed with anti-rat apo-E antibodies. First lane, 1 μg rat apo-E control standard.

mediated clearance. To test this hypothesis we have designed an experimental strategy that generated a double block to the excretion and utilization of the exogenous apo-E. 100 μg of pCMV-E2, coding for the receptor-binding defective human apo-E2, were injected into quadriceps of Yoshida rats (11). These rats are a spontaneous model of hyperlipemia showing plasma cholesterol levels twice as high as normal rats, hypertriglyceridemia, overproduction of lipoproteins by the liver and, in particular, decreased expression of lipoprotein receptor by the liver (11). Western blotting analysis of sera collected at 15 and 45 days from injection was performed following the same procedure as described above. Plasma accumulation of human apo-E was clearly demonstrated at 15 days as compared to non-injected control rats (Fig.3). According to densitometric scanning of the immunostaining signal, we calculated approximately 0.5 mg/dl of the exogenous human apo-E in the plasma of injected Yoshida rats. At 45 days the plasma levels of human apo-E were generally both lower and more variable among different animals than at 15 days, but still clearly detectable (Fig. 4).

In order both to confirm the specificity of the immunostaining signal for human apo-E, and to detect any alteration in the expression of the endogenous rat apo-E due to the co-expression of the exogenous apoprotein, the same samples of plasma were analyzed for the expression of the endogenous apo-E at 15 and 45 days after injection (Fig.5). 1 μl of plasma was fractionated on a 12% SDS-polyacrilamide gel electrophoresis, transferred onto nitrocellulose and immunostained using polyclonal anti-rat apo-E antibodies. No differences in signal levels were demonstrated by densitometric scanning among both injected and non-injected Yoshida rats, at 15 or at 45 days after injection. Scanning between the signal of 1 μg rat apo-E standard and the signals from 1 μl specimens of injected and non-injected-rat plasmas allowed the calculation of endogenous apo-E plasma concentration to 5 mg/dl. No reaction to 1 μg human apo-E standard control was observed with anti-rat apo-E antibodies (Fig.5).

Lipoprotein analysis demonstrated no differences in lipoprotein distribution and amount between treated and untreated Yoshida rats (data not shown). Plasma cholesterol and triglyceride plasma levels were similar in treated and untreated Yoshida rats confirming mild hypercholesterolemia (115 ± 20 mg/dl) and hypertriglyceridemia (81 ± 20 mg/dl) (11) .

DISCUSSION

We have analyzed the possibility to use direct intramuscular injection of naked DNA as a tool for delivering recombinant protein to the circulation. An expression vector containing the CMV promoter, the complete cDNA coding for the signal peptide and the human receptor-binding defective apolipoprotein E2, plus the hGH terminator sequence, was constructed (pCMV-E2). We demonstrated that the injection of pCMV-E2 plasmid into the quadriceps of Yoshida rats, affected by a complex heritable alteration of lipoprotein metabolism and decreased expression of LDL-receptor in the liver (11), caused local expression of the recombinant gene and plasma accumulation of the defective protein. Plasma concentration of human recombinant apo-E2 was about 0.5 mg/dl at 15 days but lowered and varied significantly among different animals at 45 days after plasmid injection. The endogenous rat apo-E expression was not altered by the concomitant expression of human recombinant apo-E and was constantly found, at 15 and 45 days, both in injected and control animals, at the concentration of 5 mg/dl.

The low concentration of the human receptor-binding defective apo-E was not able to overcome the function of the endogenous normal apo-E. For this reason, both lipoprotein pattern and cholesterol levels were not influenced by the presence of the abnormal human apolipoprotein. This is in agreement with findings in transgenic mice, where the expression of a receptor-binding defective variant of human apo-E at very low levels (~ 0.5 mg/dl) does not cause alterations in plasma cholesterol and triglyceride levels (20).

These results imply that the previous inability to identify circulating recombinant proteins, produced in the muscle by direct injection of naked DNA, was likely due to the low efficiency of gene expression and low number of transduced cells, and not to any obstacle to the secretion from muscle to blood stream. By designing an experimental strategy that allowed a double block to the plasma clearance and liver uptake of the exogenous apo-E we induced a temporary plasma accumulation of the recombinant protein. In fact, when we injected a normal human apo-E3 cDNA into the muscle of normal rats, we were able to identify the local expression of human apo-E in the muscle but we did not find any trace of the human recombinant protein in the circulation. This is the first direct demonstration that muscle tissue injected with naked plasmid DNA can express and secrete the encoded protein in the blood stream, inducing plasma accumulation of the recombinant protein.

In addition to treatment of primary myopathies or to elicit cellular and humoral immune responses against specific DNA-encoded antigens, intramuscular direct gene transfer may have a potential therapeutic role in the chronic systemic delivery of functional recombinant protein, for the treatment of inherited or acquired diseases. Several methods have been developed for *in vivo* expression of DNA-encoded proteins and long-time persistence of the recombinant protein

in the circulation. *Ex vivo* transduced primary autologous myoblasts transplanted in muscles of recipient animals were demonstrated to express significant levels of the recombinant protein for several months (14). Analogous results were obtained by implants of transduced primary fibroblasts (15, 16). Direct *in vivo* uptake of naked DNA by muscle cells may offer several advantages over these methods, such as the economy, simplicity, and safety of the procedure.

The principal problems associated with this method have been insufficient levels of expression and variable results (1, 2, 5), even if expression of the exogenous gene has been shown to persist up to 19 months (17). Numerous recent observations have given abundant technical suggestions to enhance gene expression, stability and transducing efficiency of intramuscularly injected genes. Different improvements have been very recently demonstrated in the technics of intramuscular injection (18) or in the preparation of the muscle tissue (19). Moreover, no specific vectors for direct intramuscular gene transfer have been so far designed. Many specific characteristics of the technic procedure, target tissue and fate of injected DNA may be exploited to construct specific and useful DNA expression vectors. According to these observations the efficiency of this technique can be greatly improved in the near future.

Apolipoprotein E is one of several apoprotein components associated with plasma lipoproteins (12). It plays a central role in the redistribution of cholesterol and triglycerides in the plasma and various cells. Defective apo-E has been associated with Familial Dysbetalipoproteinemia, a genetic disorder of lipoprotein metabolism predisposing to premature coronary and/or peripheral vascular disease (10, 12). Moreover, it has been demonstrated that intravenous infusion of human functional apo-E can protect animals from the consequence of increased cholesterol feed (13) and that the ectopic expression does not alter the partecipation of apo-E in the constitution of lipoproteins and cholesterol/triglyceride transport (13, 20). On the basis of these considerations, apolipoprotein-E seems to be an appropriate candidate for gene therapy of both single gene or multifactorial diseases characterized by hyperlipidemia. However, the present study reports successful results only for the receptor-binding defective apo-E2, and not for the normally active apo-E3, which evidently represents the ideal tool for correction of hyperlipidemias. Work in this direction is currently under way in our laboratory.

Setting an efficient method for chronic systemic delivery of recombinant protein may offer a new important therapeutic tool for the treatment not only of hereditary single gene diseases but also of multi-gene or acquired diseases. We believe that many characteristics of intramuscular direct gene transfer technique, such as safety, technical facility, economy and possible technical improvements, will make this approach more advantageous than others.

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