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A Novel Method to Assay Proteins in Blood Plasma after Intravenous Injection of Plasmid DNA

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HANAWA, H., WATANABE, R., HAYASHI, M., YOSHIDA, T., ABE, S., KOMURA, S., LIU, H., ELNAGGAR, R., CHANG, H., OKURA, Y., KATO, K., KODAMA, M., MARUYAMA, H., MIYAZAKI, J. and AIZAWA, Y. *A Novel Method to Assay Proteins in Blood Plasma after Intravenous Injection of Plasmid DNA*. Tohoku J. Exp. Med., 2004, **202** (3), 155-161 —— Gene therapy is expected to lead to new and useful methods to treat diseases. The development of assays to quantitate gene-therapy-derived proteins circulating in blood will be essential to investigate the effects and side effects of the introduced proteins. The purpose of this study is to evaluate whether a protein circulating at trace concentrations in blood can be measured by tagging a peptide corresponding to glucagon residues 19-29 onto its C-terminal end. We constructed plasmids encoding chimeric proteins and transferred them into rats by hydrodynamics-based delivery. When plasmids encoding human IL8-glucagon 19-29 chimeric protein were injected into rats to evaluate the accuracy of this method, there was a high correlation between chimeric proteins measured by an enzyme-linked immunosorbent assay for human IL8 and one by a radioimmunoassay for glucagon. Furthermore, when plasmids coding rat IFN gamma receptor IgG-Fc glucagon 19-29 chimeric protein were injected to evaluate the time course of chimeric proteins in blood plasma, we could calculate the concentrations in blood from 10 μl plasma samples using glucagon 19-29 tag as follows: 2815±2318 ng/ml after 4 hours (mean±S.D.), 6061±2789 ng/ml after 8 hours, 5752±2270 ng/ml after 12 hours, 2870±1062 ng/ml after one day, 1440±334 ng/ml after three days, 1120±433 ng/ml after seven days, and 281±162 ng/ml after 16 days. Blood sugar levels which might have been increased by glucagon did not increase even at peak chime-

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ric protein concentrations. These results demonstrate a useful and convenient method to assay gene therapy products circulating in blood using a glucagon 19-29 tagging vector. ——— gene therapy; glucagon; pCAGGS; radioimmunoassay
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Recently, numerous attempts to accomplish gene therapy by using viral or plasmid vector-based methods have been published (Liu et al. 1999; Lawson et al. 2000; Quattrocchi et al. 2000; Maruyama et al. 2002; Matsui et al. 2002; Watanabe et al. 2001). It is expected that these new methods will be developed further and will prove useful in the treatment of various diseases. In many of these studies, it is essential to measure the concentration of the synthesized protein in blood. In general, proteins in blood are measured by an enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA). However, if antibodies for these assays are not available, the protein levels can be difficult to measure (Lawson et al. 2000). The proteins synthesized by these types of vectors are often tagged with peptides containing 6-20 amino acids to aid in their purification, for example poly-His-tags, the c-myc-tag, the glutathione *S*-transferase tag, or the FLAG-tag (Denbow et al. 1996; Lu et al. 1997; Bouchard et al. 1999; Hefti et al. 2003). We have previously attempted to measure recombinant proteins using poly-His-tags and Tag-100 and they were able to be detected in transfected Cos-7 cell culture medium, not in the blood of rat after intravenous injection of plasmid DNA. The levels of tagged, recombinant proteins derived from these plasmids cannot yet be assayed with sufficient accuracy. We decided to develop super-sensitive assays by exploiting the tags on the proteins themselves, as they are often unique markers for the recombinant products.

In this study, we investigated whether a plasmid vector that adds a peptide tag consisting of glucagon residues 19-29 to the C-terminal end of proteins could be exploited to measure the concentration of the tagged protein in blood following gene therapy. This type of plasmid was injected rapidly into rats via the tail vein using published protocol (Liu et al. 1999; Maruyama et al. 2002) and the synthesized protein in blood was measured by a commonly used glucagon assay kit (Imagawa et al. 1979; Nishino et al. 1981) over a 16-day period following the injection. Glucagon consists of 29 amino acids, and this peptide hormone plays a physiological role by increasing blood sugar and its amino acid sequence is the same in mouse, rat and human (Lefebvre 1995; Irwin 2001). Therefore, the expressed glucagon 19-29 peptides already exist in living bodies and are thought to have low antigenicity in all three species. The recombinant chimeric protein contained a glucagon-derived peptide tag (residues 19-29) corresponding to 38% of the entire peptide hormone. Assays of blood sugar in rats treated by gene therapy using this protein indicated that possible glucagon-like side effects in fact did not occur. The synthesized protein would be expected to have no glucagon-like side effects in the other species either, due to the sequence identity of the glucagon peptide in all three species.

Materials and Methods

Plasmid DNA

To construct pCAGGS-IL8 glucagon 19-29, the first PCR products of glucagon 19-29 DNA were amplified using KOD Plus DNA polymerase (TOYOBO, Osaka) and the following primers: (5′-gaGAATTCATTTAAATgagaGCGGCCGCccc aggtaaagcccaagattttgtgcagtggttg-3′ with SwaI and NotI restriction sites and 5′-gagagagaGAATTCtcaggtattcatcaaccactgcacaaaatcttgggc-3′) (Heinrich et al. 1984). The amplified glucagon 19-29 DNA was inserted into the pCAGGS vector using the EcoRI sites. Escherichia coli JM109 competent cells were then transformed and recombinant plasmids, i.e., pCAGGS-glucagon 19-29, were

isolated using a Quantum Prep Plasmid Maxiprep kit (Bio-Rad Laboratories, Hercules, CA, USA). Next, human IL8 cDNAs were amplified from Cos-7 cells cDNA using the primers: (5′-gaGAATTCATTTAAATgacttccaagctggccgtggct-3′ and 5′-gcagcatcGCGGCCGCtgaattctcagccctcttcaaaaa-3′), as described above. These were inserted into the pCAGGS-glucagon 19-29 vector using the SwaI and NotI sites and the recombinant plasmids, i.e., pCAGGS-IL8 glucagon 19-29, were isolated as described above.

To construct a negative control plasmid, i.e., pCAGGS-IFN gamma receptor extracellular domain (ECD) IgG-Fc without glucagon 19-29, rat IgG1Fc cDNAs were amplified from rat spleen cDNA using the primers: (5′-gaGAATTCATTTAAATgagaGCGGCCGCcgtgcccagaaactgtg-3′ with SwaI and NotI restriction sites and 5′-gagagagaGAATTCactctggggtcatttacccggagagtgggag-3′) (Bruggemann et al. 1986) as described above. The amplified cDNA was inserted into the pCAGGS vector using the EcoRI sites. Escherichia coli JM109 competent cells were then transformed and recombinant plasmids, i.e., pCAGGS-rat IgG-Fc were isolated as described above. Next, rat IFN gamma receptor ECD cDNAs were amplified using the primers: (5′-gaGAATTCATTTAAATgattctgctggtggtcctgatg-3′ and 5′-gcagcatcGCGGCCGCttcttctctgtcatcatggagaaa-3′), as described above. These products were inserted into the pCAGGS-rat IgG-Fc vector using the SwaI and NotI sites and the recombinant plasmids, i.e., pCAGGS-IFN gamma receptor ECD IgG-Fc without glucagon 19-29, were isolated as described above.

To construct the plasmid pCAGGS-IFN gamma receptor ECD IgG-Fc glucagon 19-29, the first PCR products were amplified from the diluted negative control plasmid using the primers: (5′-gaGAATTCATTTAAATgagaGCGGCCGCcgtgcccagaaactgtg-3′, 5′-tcaaccactgcacaaaatcttgggcTTTACCCGGAGAGTGGGAGAGACT-3′) as described above. The final PCR product inserts were then amplified from the diluted products of the first PCR reaction with the primers: (5′-gaGAATTCATTTAAATgagaGCGGCCGCcgtgccccagaaactgtg-3′ and 5′-gagagagaGAATTCtcaggtattcatcaaccactgcacaaaatcttgggc-3′) (Heinrich et al. 1984). These products were inserted into the pCAGGS vector using EcoRI sites. The recombinant plasmids, i.e., pCAGGS-IFN gamma receptor ECD IgG-Fc glucagon 19-29, were then isolated as described above.

Rats

Eight-week-old male Lewis rats were purchased from Charles-River Japan, Inc. (Tokyo). One week later, we injected plasmid DNA into nine-week-old rats. Throughout the studies, all the animals were treated in accordance with the guidelines for animal experiments of our institute.

Gene transfer and assay of synthesized protein in blood

To compare IL8 glucagon 19-29 concentrations in blood calculated using glucagon tag and using IL8, eight rats were injected with 400 μg of pCAGGS-IL8 glucagon 19-29 which were added to 20 ml volumes of Ringer solution via the tail vein within 15 seconds (receiving approximately 80 ml/kg body weight) (Maruyama et al. 2002). Blood samples were taken 24 hours following injection. Glucagon concentrations were measured using a glucagon RIA Kit (DAIICHI RADIOISOTOPE LABS, Tokyo) (Imagawa et al. 1979; Nishino et al. 1981) and IL8 concentrations using IL-8 EASIA (BIOSOURCE, Nivelles, Belgium).

To measure rat IFN gamma receptor ECD IgG-Fc glucagon protein and blood sugar concentrations throughout the acute phase, ten rats were divided into two groups, a Glucagon 19-29 positive group (treated with plasmid pCAGGS-IFN gamma receptor ECD IgG-Fc glucagon 19-29, n=5), and a Glucagon 19-29 negative group (treated with plasmid pCAGGS-IFN gamma receptor ECD IgG-Fc, n=5). The plasmids (800 μg each) were added to 20 ml volume of Ringer solution and rats were injected via the tail vein within

15 seconds (receiving approximately 80 ml/kg body weight) (Maruyama et al. 2002). Blood samples were taken 4 hours, 8 hours, and 12 hours following injection. Glucagon concentrations were measured from 10 μl plasma samples diluted with 50-100 PBS using a glucagon RIA Kit as described above. Blood sugar was measured using a glutestsensor (SANWA KAGAKU KENKYUSHO, Nagoya).

To measure synthesized protein concentrations at subsequent time points, seven rats were injected with the plasmid pCAGGS-IFN gamma receptor ECD IgG-Fc glucagon 19-29 and three rats were injected with the control plasmid pCAGGS-IFN gamma receptor ECD IgG-Fc as described above. Blood samples were taken 1, 3, 7 and 16 days after injection, and glucagon concentrations were measured as described above.

Results

Synthesized protein concentration in blood and blood sugar levels

Human IL8 glucagon 19-29 protein concentrations 24 hours after injection of pCAGGS-IL8 glucagon 19-29 calculated using glucagon were similar to those using human IL8 (Fig. 1).

During the acute phase, IFN gamma receptor ECD IgG-Fc glucagon 19-29 concentrations were 2815±2318 ng/ml (mean±S.D.) 4 hours after injection of pCAGGS-IFN gamma receptor ECD IgG-Fc glucagon 19-29, 6061±2789 ng/ml 8 hours after injection, and 5752±2270 ng/ml 12 hours after injection. The protein concentration peaked at 8 hours and the protein concentrations in the Glucagon 19-29 negative group were not detected at any point (Fig. 2). The differences between blood sugar in the Glucagon 19-29 negative group and the Glucagon 19-29 positive group after 4

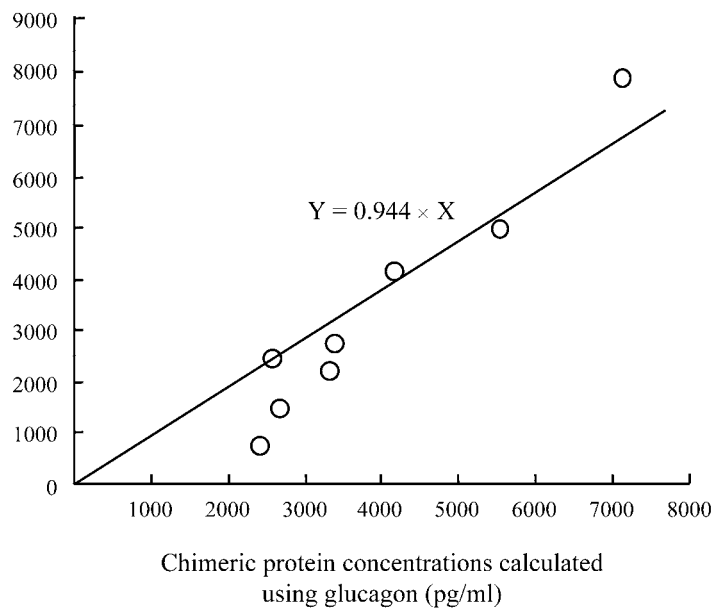

Fig. 1. The chimeric protein (human IL8 glucagon 19-29) concentrations calculated using glucagon or human IL8 in blood 24 hours after injection of plasmids.
Chimera protein (human IL8 glucagon 19-29) concentrations were calculated using the following formula: (human IL8 glucagon 19-29 protein concentration)=(actually measured glucagon concentration)×(human IL8 glucagon 19-29 protein molecular weight)/(whole glucagon molecular weight), or (human IL8 glucagon 19-29 protein concentration)=(actually measured human IL-8 concentration)×(human IL8 glucagon 19-29 protein molecular weight)/(whole human IL-8 molecular weight) Regression line: Y=0.944×X.

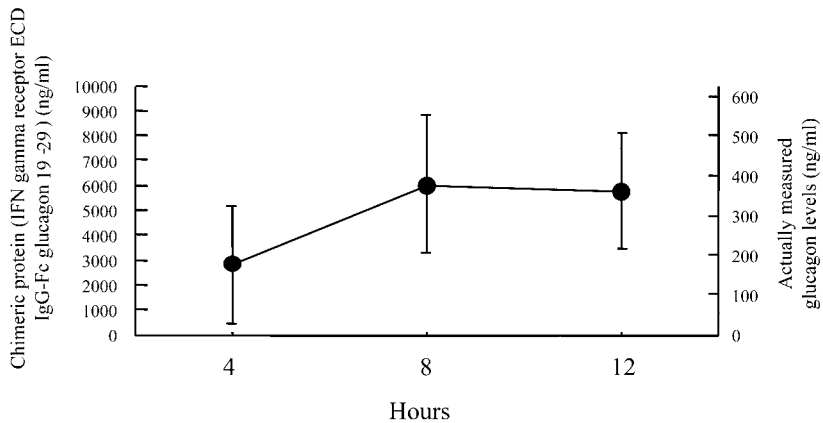

Fig. 2. The chimeric protein (IFN gamma receptor ECD IgG-Fc glucagon 19-29) concentrations in blood during the acute phase following injection of plasmids. Chimeric proteins were not indicated by increased glucagon antigen levels in the blood of any rat in the control (Glucagon 19-29 negative) group (data not shown). Chimera protein (IFN gamma receptor ECD IgG-Fc glucagon 19-29) concentrations were calculated using the following formula: (IFN gamma receptor ECD IgG-Fc glucagon 19-29 protein concentration)=(actually measured glucagon concentration) ×(IFN gamma receptor ECD IgG-Fc glucagon 19-29 protein molecular weight)/(whole glucagon molecular weight).

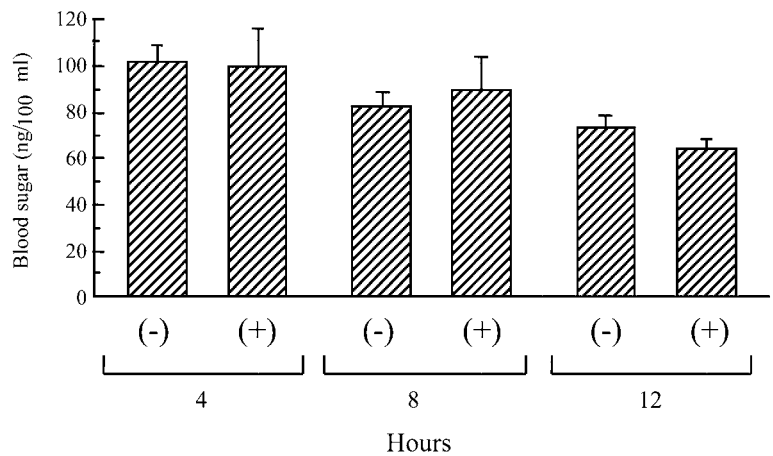

Fig. 3. Blood sugar levels following gene therapy. Differences between the Glucagon 19-29 positive group and the Glucagon 19-29 negative group were not significant at any time. The symbol "(+)" denotes the Glucagon 19-29 positive group and the symbol "(–)" denotes the Glucagon 19-29 negative group.

hours (100.6±8.8 mg/100 ml vs 99.0±17.5 mg/100 ml), 8 hours (81.8±7.5 mg/100 ml vs 89.3±15.1 mg/100 ml), and 12 hours (71.4±6.9 mg/100 ml vs 63.5±5.7 mg/100 ml) were not significant (Fig. 3).

At later time points, the synthesized protein concentrations decreased gradually: 2870±1062 ng/ml after one day, 1440±334 ng/ml after three days, 1120±433 ng/ml after seven days, and 281±162 ng/ml after 16 days. Chimeric protein levels in diluted plasma samples from rats treated with the control plasmid (without glucagon residues 19-29) were below the detection levels of this assay (Fig. 4).

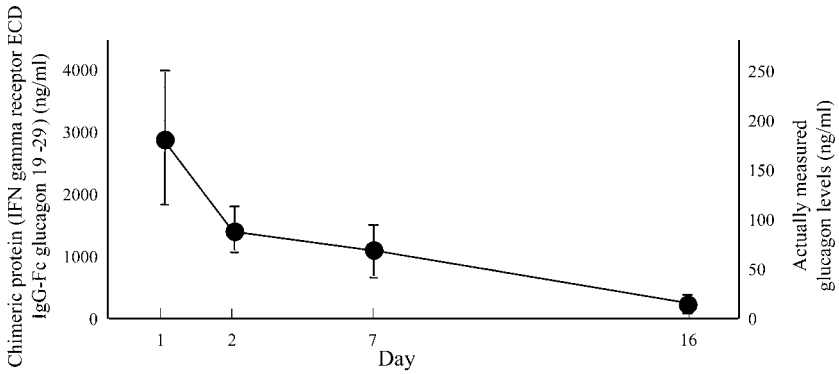

Fig. 4. Chimeric protein (IFN gamma receptor ECD IgG-Fc glucagon 19-29) levels in plasma of the Glucagon 19-29 positive group decreased following the acute phase. Chimeric proteins were not indicated by increased glucagon antigen levels in the blood of any rat in the control (Glucagon 19-29 negative) group (data not shown).

Discussion

The chimeric recombinant protein with a C-terminal tag consisting of glucagon residues 19-29 synthesized as a result of this gene therapy protocol could be measured using an RIA-based method for 16 days following injection of plasmid vectors into rats. This tag was detected by RIA, which also reacted with endogenous glucagon. The normal range of glucagon concentration in humans is 0.023-0.197 ng/ml. Glucagon antigen was detected at levels far above endogenous glucagon levels for 16 days following injection of the protein expression plasmid. Glucagon levels in blood were too low to be detected in any of the 50-100 diluted plasma samples taken from rats that received a control plasmid encoding the protein minus the glucagon tag. Therefore, we concluded that the glucagon levels measured for the Glucagon 19-29 positive rats accurately reflected the concentrations of the synthesized recombinant protein. Peak chimera protein synthesis in the Glucagon 19-29 positive group occurred eight hours following plasmid injection. Therefore, protein synthesis after intravenous injection of plasmid DNA into rats may begin earlier than in vitro transfection. Blood sugar levels in this group did not rise, even during the acute phase, indicating that the recombinant tag did not cause the type of physiological response that would be expected following a rise in normal glucagon levels. This implies that the Glucagon 19-29 tag will be useful and well tolerated in other gene therapy protocols. Injection of naked plasmid into the muscle or vein is an easy and cheap gene therapy method that is being explored in many laboratories. The synthesized protein levels following these protocols, however, can vary significantly between different animals. In gene therapy, the accurate measurement of synthesized protein in blood is essential to investigate the effects and side effects of the therapy. We present here a novel and convenient method to assay expression of gene therapy protein products, through use of a glucagon residue 19-29 C-terminal protein tag.

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