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ARTICLE

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Implantation of primary cultured adipocytes that secrete insulin modifies blood glucose levels in diabetic mice

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Abstract *Aim/hypothesis:* In type 1 diabetic patients, basal insulin supplementation plays a central role in tight glycaemic control. Therefore, safe and steady supplementation of basal insulin is strongly desirable, despite the need for multiple injections. The aim of this study was to investigate a procedure for supplementation using genetically engineered, primary-cultured adipocytes in diabetic mice. *Methods:* Furin-cleavable human proinsulin cDNA was transferred into murine primary-cultured adipocytes using a retroviral vector. The cells were implanted subcutaneously into streptozotocin-induced diabetic mice. *Results:* The transfected cells secreted substantial amounts of mature insulin, as well as C-peptide, into conditioned medium. Syngeneic implantation of the cells significantly improved hyperglycaemia and blood HbA_1c concentrations in a manner that was dependent on cell number, without causing hypoglycaemia. The plasma insulin concentration was dependent on the implanted cell number, and the systemic effect of the circulating insulin was confirmed by marked improvement of body weight reduction and liver glycogen content. Additionally, surgical resection of the implants, in which the insulin secretion was immunologically confirmed after transplantation, diminished the glucose-lowering effect, suggesting that in vivo expression could be eliminated if necessary. *Conclusions/interpretation:* These results indicate that the autotransplantation of functionalised adipocytes may lead to a clinical application in the treatment of diabetes.

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Abbreviations FBG: fasting blood glucose · GFP: green fluorescent protein · PA: primary adipocyte · s1s2B10-Ins/PA: human proinsulin gene-transfected murine PAs · STZ: streptozotocin

Introduction

Protein injection therapy is frequently used to treat genetic and acquired protein hormone deficiencies; however, multiple injections remain a major limitation to the lifestyle of these patients. Thus, it would be better for the patients' quality of life to treat with a continuous supplementation system.

Type 1 diabetic mellitus is one of the typical disorders that require multiple injections to prevent various short- or long-term complications [1, 2]. Supplementation of basal insulin, the steady and low level of insulin that is constantly present in the circulation, is central to intensive glucose control; however, an adequate supply is still not easily achieved even with daily multiple injections, due to the occurrence of hypoglycaemia [3]. To overcome this problem, steady-state supplementation of basal insulin is highly desirable [4]. Implantation therapy with ex vivo gene-transferred cells is considered an attractive potential method for continuous insulin supplementation [5]. Moreover, an autotransplantation procedure using self-originated primary cells is highly desirable in order to avoid the need for immunosuppression.

Recently, it has been reported that adipocytes express and secrete various biologically active peptides called adipocytokines [6]. Leptin secreted from adipocytes regulates food intake and energy expenditure [7]. Adiponectin has

been reported to ameliorate insulin resistance in obese mice by enhancement of both fatty acid combustion and energy dissipation in muscle [8]. We have recently reported that 3T3-L1 adipocytes transplanted into the mesenteric area of mice expressed TNF-α, and the cytokine modulated the metabolic status of the recipient mice [9]. These results indicate that adipocytes can secrete bioactive proteins into the blood circulation, and thereby affect the systemic metabolic status. Thus, we hypothesised that adipocytes could be used as a source of protein production in vivo for the modulation of metabolic disorders. Based on the hypothesis, we generated insulin-expressing primary adipocytes (PAs) and evaluated the effect of implantation of these cells in diabetic mice.

Materials and methods

Mice

All mice in this study were obtained from Charles River Japan (Yokohama, Japan). They were allowed free access to regular chow and water unless otherwise specified. All work was carried out according to the guidelines of the Animal Care Committees of Chiba University Graduate School of Medicine and Eisai.

Adipocytes primary culture

PAs were obtained by means of the ceiling culture method reported by Sugihara et al. [10]. Briefly, 0.5–1 g of inguinal s.c. fat was isolated from male 4-week-old C57BL/6 mice. The tissues were minced and digested with collagenases-1 (Nitta Gelatin, Osaka, Japan). The floating layer after centrifugation was seeded into T-25 flasks filled with DMEM (4.5 g/l D-glucose; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (as normal medium), the flasks placed upside down, and cultured for 2 weeks. The cells attached on the ceiling surface were harvested with trypsin, and grown according to standard procedures.

cDNA isolation

Green fluorescent protein (GFP) cDNA was purchased from BD Biosciences Clontech (Tokyo, Japan). Human preproinsulin gene was amplified with Expand Hi-Fi (Roche Diagnostics KK, Tokyo, Japan) polymerase mixture from a human pancreas cDNA library (BD Biosciences Clontech). The primers were designed as follows: 5′-CATAAGCTT ACCATGGCCCTGTGGATGCGC-3′ (forward, containing a start codon) and 5′-CATTCTAGACTAGTTGCAG TAGTTCTCCAG-3′ (reverse, containing a stop codon). The furin-cleavable modified proinsulin gene [11] (named s1s2B10-Ins in this study) was generated using a Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The furin-cleavable sites at the B–C junction (as site 1) and the C–A junction (as site 2) were created using the oligomers 5′-CTTCTACACACCCAGGACCA AGCGGGAGGCAGAGGAC-3′ (site 1) and 5′-CCCTGG AGGGATCCCGGCAGAAGCGTGG-3′ (site 2). The resulting substitution of amino acids are as follows: lysine to arginine at amino acid number 29 and arginine to lysine at number 31 in site 1, and leucine to arginine at number 62 in site 2. A mutation of the tenth amino acid in the B-chain (from histidine to aspartic acid) was created using the primer 5′-CACCTGTGCGGATCCGACCT GGTGGAAGC-3′. These modifications were confirmed by sequencing.

Construction of retroviral vectors and gene transduction

In this study, we used the vesicular stomatitis virus G-protein-pseudotyped retroviral vector [12] for stable gene transfer to PAs. The vector structures are shown in Fig. 1a.

Preparation of the pseudotyped vector was largely based on a ViraPort Retroviral Gene Expression System (Stratagene). The retroviral vector was harvested by transfection of the plasmid mixture composed of the ViraPort system and the insulin-expression vector into 293-EBNA cells (Invitrogen, Tokyo, Japan; 2×10^6 cells/10 ml in 100 mm dish) using TransIT (Mirus, Madison, WI, USA). Two days later, the conditioned medium containing vector particles (200 ml) was collected and concentrated by ultracentrifugation (50,000×*g*, 100 min at 4°C). The concentrated vec-

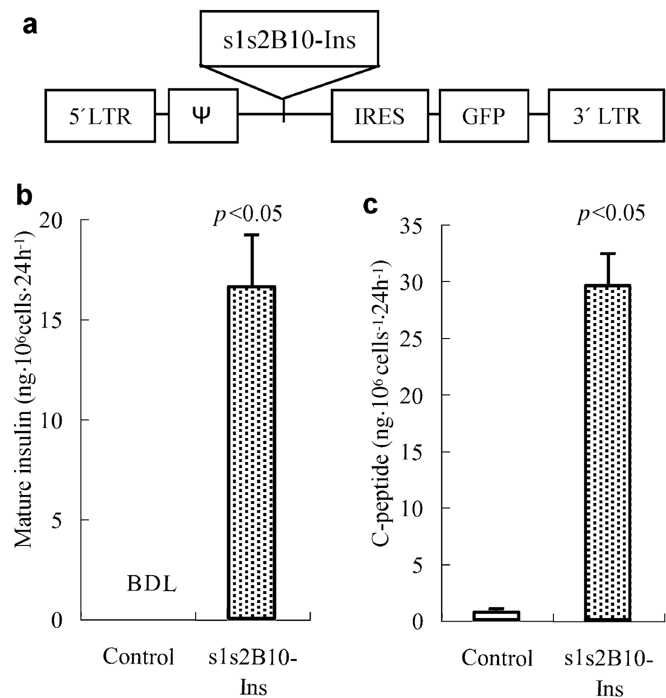

Fig. 1 Transduction to PAs of a retrovirus vector expressing modified human insulin. Gene structure of the retroviral vector (**a**). ψ packaging signal sequence. The concentrations of mature insulin (**b**) and C-peptide (**c**) were measured in 24-h-conditioned medium. *IRES* Internal ribosome entry site *LTR* long terminal repeat; *BDL* below the detection limit (0.08 ng/ml in our study)

tor solution was transduced into subconfluent PAs (six-well plates) with 2 μg/ml Polybrene (Sigma). The transduction of retroviral vector containing GFP showed that more than 95% of PAs were transduced with the vector. The reserve cell stocks were cryopreserved with Cellbanker solution (Wako Pure Chemical Industries, Osaka, Japan).

Detection of gene products

In vitro secretion of mature insulin and C-peptide was assayed using ELISA kits from Linco Research (St Charles, MO, USA) and IBL (Gunma, Japan), respectively. All assay procedures were according to the manufacturers. The insulin detection kit is known to detect site-1-undigested proinsulin as well as mature insulin, and not proinsulin or site-2-undigested proinsulin. Taking into account the cross-reactivity of antibodies with the proinsulin in the kit, conversion to mature insulin was expected to about 5% of total secreted insulin.

Implantation of insulin-expressing PAs into streptozotocin-induced diabetic mice

Prior to the experiment using PAs expressing insulin, we examined the implantation of PAs expressing placental alkaline phosphatase as a secretion marker (data not shown). For this purpose, we implanted transplants between individual mice of this strain (syngeneic), because it does not require immunosuppression after transplantation. Based on the results, we confirmed the long-term and stable expression of exogenous genes in PAs, and secretion into the circulation, by implantation of gene-transferred PAs in mice.

Streptozotocin (STZ) (Sigma) was dissolved in 1 mmol/l cold sodium citrate (pH 4.5) just prior to i.v. injection at a dose of 170 mg/kg into the tail vein of 9-week-old male C57BL/6 mice (groups of four to six). Blood samples were collected from the tail vein in a 16-h-fasted state (10.00 hours). The blood glucose levels were determined with a Glucose CII (Wako). Two to three weeks later, mice in which the fasting blood glucose (FBG) levels rose to over 19.4 mmol/l were selected for experiment.

In order to accelerate the differentiation of implanted cells, gene-transferred PAs were treated, prior to implantation, with medium supplemented with 0.5 mmol/l 3-isobutyl-1-methylxanthine, 0.25 μmol/l dexamethasone and 10 μg/ml recombinant human insulin (all from Sigma) for 3 days. The harvested cells were suspended at 2×10^6 cells/ml in Matrigel (BD Biosciences) supplemented with 1 μg/ml recombinant human basic fibroblast growth factor (Genzyme Techne, Minneapolis, MN, USA) and injected s.c. in the back of the mice. FBG and body weight were monitored at 1, 3, 5, 7, 9 and 10 weeks after implantation. At 9 weeks, plasma samples were collected from the retro-orbital venous plexus of mice and plasma insulin concentrations were assayed by an ultrasensitive ELISA (Morinaga, Yokohama, Japan). HbA_1c values at 10 weeks were evaluated with a DCA2000 HbA_1c analyser (Bayer Medical, Tokyo, Japan).

Resection of implanted cells from mice

Cells from cryopreserved stock were used in the study. Implantation (8×10^6 cells/mouse of control PAs or s1s2B10-Ins/PA) and FBG evaluation were performed as described above. Three weeks after implantation, mice were anaesthetised by i.p. administration of sodium pentobarbital (50 mg/kg). Then the implanted area was shaved, the skin incised and the implants removed, followed by suturing and disinfection. The FBG levels were determined 2 weeks after operation; however, direct statistical comparison with control mice could not be performed because they had already died. Since two of three resected mice died at 3 weeks after operation, the plasma concentration of insulin was determined using the remaining resected mouse at 4 weeks after operation.

Histological staining

At 5 weeks after implantation, livers were isolated and sections were embedded in paraffin for a periodic acid–Schiff reaction. The resected implants were collected, fixed in 10% buffered formalin and embedded in paraffin for haematoxylin/eosin staining.

Immunohistochemical staining of insulin in fixed implants was done using guinea-pig anti-human insulin antibody (Linco Research) with EnVision plus reagent (Dako Cytomation, Kyoto, Japan), as recommended by the manufacturers.

Statistical analysis

Data are expressed as means±SEM. Statistical analysis was conducted using the software package SAS 8.1 (SAS Institute Japan, Tokyo, Japan). In animal studies, the evaluation was performed by one-way ANOVA, followed by a Dunnett-type multiple comparison test with the control. The results of ELISA were evaluated with the Mann–Whitney *U*-test. A value of $p<0.05$ was considered significant.

Results

In vitro expression of modified insulin from PAs

A retroviral vector that expressed modified insulin was constructed as shown in Fig. 1a. The mature insulin production in conditioned medium of s1s2B10-Ins/PA was 16.6 ± 2.59 ng·10^6 cells^{-1}·24 h^{-1} (Fig. 1b). The marked increase of C-peptide suggested successful processing by furin in the adipocytes (Fig. 1c). The biological activity of

engineered insulin was confirmed by autophosphorylation activity against endogenous insulin receptor in HepG2 cell (data not shown).

Implantation of insulin-expressing PAs ameliorated hyperglycaemia

Diabetic mice were created by administration of STZ. Implantation of s1s2B10-Ins/PA significantly lowered the blood glucose levels in the fasting state, this reduction being dependent on cell number (Fig. 2). In mice implanted with 8×10^6 cells of s1s2B10-Ins/PA (high-cell-number group), FBG levels were close to the normal range through the experimental period (10 weeks) without the occurrence of hypoglycaemia in the fasting state.

Plasma insulin concentrations in implanted mice were determined at 9 weeks (Fig. 3). In the high-cell-number group, in which the average FBG level was controlled to a normal level, plasma insulin was significantly increased (160±55 pg/ml, $p<0.05$) compared with the control group (below the detection limit). The value in the low-cell-number group (4×10^6 cells of s1s2B10-Ins/PA) was not significantly different from the control ($p=0.058$), but was nevertheless elevated to nearly half that of the high-cell-number group (92.0±38.1 pg/ml), indicating that the lowering effect on FBG is cell-number-dependent.

Subcutaneous implantation improved systemic metabolic status

To assess the systemic effects of insulin from implants, we analysed liver glycogenesis by the periodic acid–Schiff reaction (Fig. 4). In accord with previous studies [13, 14], STZ treatment caused depletion of liver glycogen. Subcutaneous implantation of s1s2B10-Ins/PA dramatically increased the glycogen content.

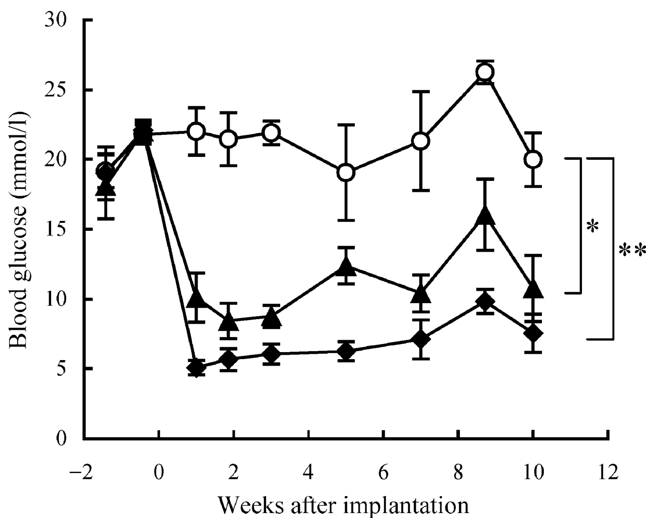

Fig. 2 FBG levels of s1s2B10-Ins/PA-implanted STZ mice. Implantation of control cells (*open circle*, $n=6$), or a low cell number (*solid triangle*, 4×10^6 cells/mouse, $n=5$) and high cell number (*solid diamond*, 8×10^6 cells/mouse, $n=4$) of s1s2B10-Ins/PA cells was performed at day 17 after STZ administration. *$p<0.05$; **$p<0.01$ vs control

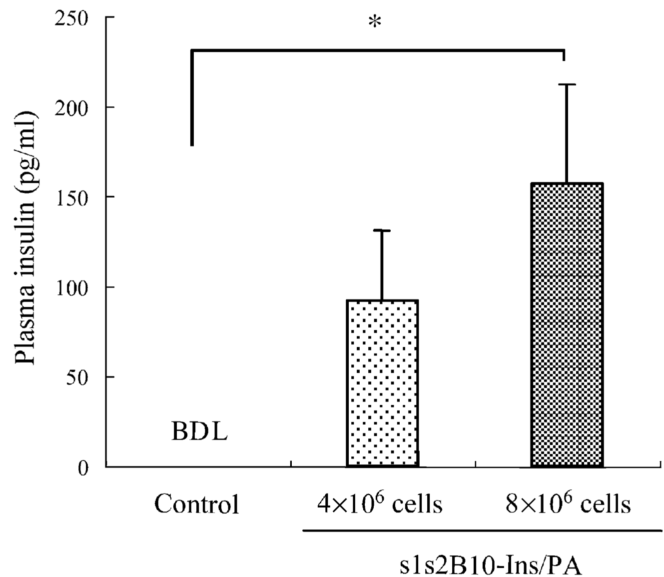

Fig. 3 Plasma insulin concentrations in implanted mice. Eight weeks after implantation, plasma samples were collected and the concentration of insulin determined. The values in plasma from all control mice were below the detection limit (BDL, 50 pg/ml in our assay). Data are means±SEM of four mice (control and high cell number of s1s2B10-Ins/PA) or five mice (low cell number of s1s2B10-Ins/PA). *$p<0.05$

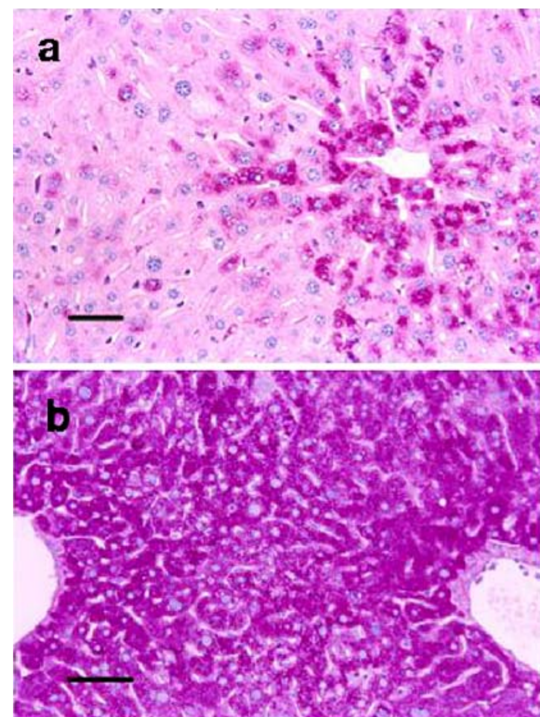

Fig. 4 Liver glycogen contents in implanted mice. The liver sections isolated from killed animals were stained for glycogen. **a** Control-cell-implanted mice. **b** s1s2B10-Ins/PA-implanted mice. *Scale bar*=100 μm

The systemic effect of the implantation was also confirmed by an improvement of body weight loss. The values were significantly different in s1s2B10-Ins/PA groups compared with control from 1 week, and the effects were sustained to the end of the study. The values at 10 weeks were 21.7±0.5 g in the high-cell-number group ($p<0.001$), 19.7±0.4 g in the low-cell-number group ($p<0.01$) and 16.5±0.9 g in the control group.

In order to examine the quality of glycaemic control by the implantation, HbA_1c concentration was determined at the end of the experimental period. In comparison with control mice (9.8±0.7%), the HbA_1c value was significantly lowered in the high-cell-number group (8.4±0.5%, $p<0.01$).

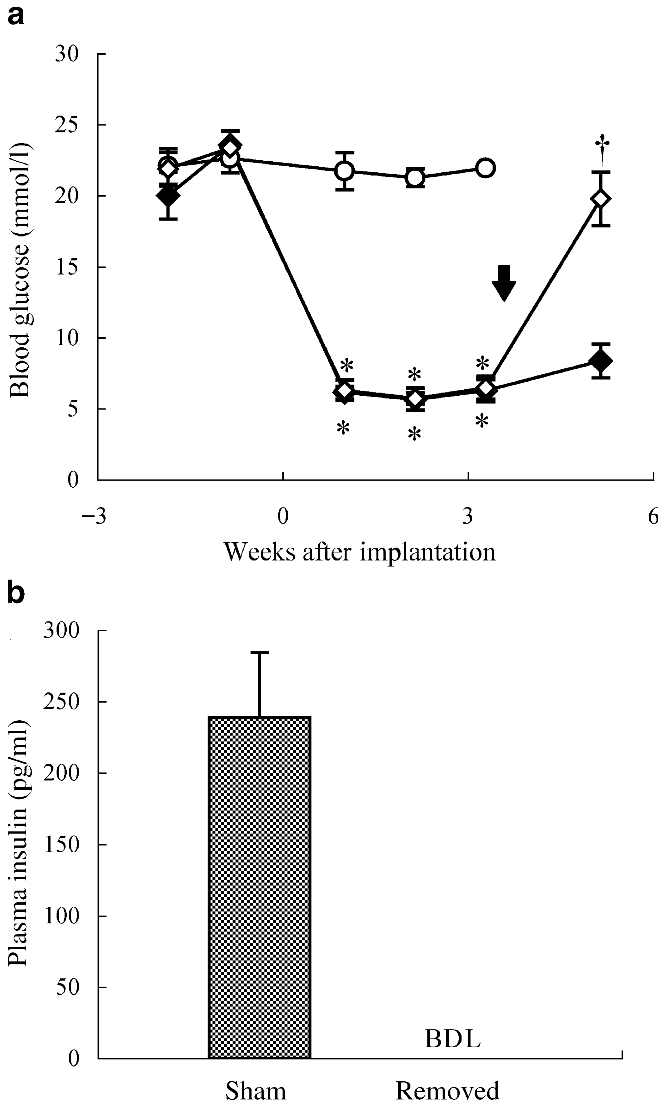

Fig. 5 Resection of the implants diminished the glucose-lowering effect and plasma insulin concentration in mice. **a** FBG levels. Implantation of control cells (*open circle*, $n=6$) or s1s2B10-Ins/PA was performed as shown in Fig. 3. In s1s2B10-Ins/PA-implanted mice, the gross implants were removed surgically (*open diamond*, $n=3$) at 3 weeks after implantation (*arrow*). A sham operation was performed in the non-resected group (*solid diamond*, $n=6$). **b** Plasma insulin concentration at 5 weeks after implantation. The value in control mice was below the detection limit (BDL, 50 pg/ml in our assay). $^{*}p<0.01$ vs control; $^{\dagger}p<0.01$ vs the sham-operated group

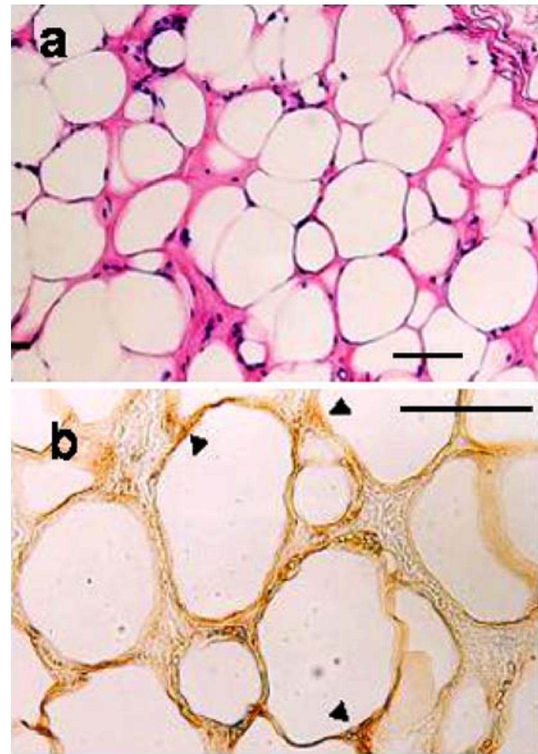

Fig. 6 Tissue staining of resected implants from mice. **a** Haematoxylin/eosin stain. **b** Immunohistochemistry with anti-human insulin IgG. *Arrowheads* indicate typical insulin-positive areas. *Scale bars*=100 µm

Reversal of the glucose-lowering effect by resection

One advantage of our procedure lies in the possibility of resection of the implants in the case of adverse effects and/or over-reaction, such as hypoglycaemia. We studied the effect of removal of the implants from treated STZ mice. FBG levels after surgical resection of the implants significantly increased from those of sham-operated mice, and reached almost the level seen previously in the control mice (Fig. 5a). Plasma insulin was dramatically reduced by the operation (Fig. 5b).

Finally, we identified the insulin-positive cells in the implants. Histological and immunohistochemical analysis showed that insulin-positive cells existed in implants as mature adipocytes, which seemed to store lipid droplets (Fig. 6). No positive signal was obtained with control IgG (data not shown).

Discussion

The majority of systemic protein deficiencies are currently treated by multiple injections of the lacking polypeptide. Safe administration procedures that would eliminate this unpleasant duty are highly desirable. In this report, we have

established the procedure for in vivo gene expression based on gene-transferred PAs using type 1 diabetes model mice. We confirmed that protein secretion from s.c. implanted cells could improve metabolic status systemically (e.g. in liver, shown in Fig. 4). Furthermore, as shown in Fig. 5, the glucose-lowering effects were retained when the cryopreserved cells were used, suggesting that sufficient cells for repeated implantations during clinical treatment might be obtainable by growth from stable reserve stocks. From these results, autotransplantation of gene-transferred PAs is thought to be an attractive candidate method for our aim.

A similar approach using self-originated adipocytes might be applicable for humans. Isolation of human fat tissue is being established as liposuction in plastic surgery and cosmetic surgery [15, 16]. Human PAs can also be obtained by ceiling culture [17]. Our investigations may thus provide a basis for the treatment of patients by long-term supplementation of therapeutic protein without systemic immunosuppression.

Supplementation of basal levels of insulin is known to act not only centrally for adequate control of hyperglycaemia, but also has a key role in preventing the development of ketoacidosis in humans [18]. In our study, sustained insulin supplementation from implants successfully lowered blood glucose in a manner that was dependent on cell number, suggesting that the procedure might make it possible to achieve long-term, steady-state insulin supplementation without the need for multiple injections. Therefore, it could be particularly helpful for elderly patients or patients with impaired vision, who find such injections hard to administer by themselves. Implantation of the s1s2B10-Ins/PA is also thought to supply C-peptides (Fig. 1c), which are not provided by the usual insulin injections. Since the peptides have been reported to have a protective effect on microvascular complications [19–21], the implantation might contribute to prevention of such complications. Moreover, removal of the implants reversed the glucose-lowering effect, suggesting that in vivo expression of insulin generated in this way could be surgically eliminated if necessary.

Recently, a long-acting human insulin analogue has been developed and is reported to improve hyperglycaemia and HbA_1c levels in type 1 [22] and type 2 diabetic patients [23]. These results suggest that a sustained supply of basal insulin is effective clinically in both forms of diabetes.

In our experiment, a single implantation retained its glucose-lowering effect for several months without causing hypoglycaemia. Previous trials showed hypoglycaemia at 3–4 weeks due to unregulated cell proliferation in vivo, resulting in increased insulin production [24, 25]. We speculate that the apparent advantage of PAs is due to their ability to differentiate, since the implanted PAs existed as mature adipocytes in the implants as assessed by histological analysis (Fig. 5).

This unproliferative property of implanted PAs may also contribute to safety if the procedure is applied to clinical treatment. Recently, it was reported that retrovirus-mediated gene transfer could cause uncontrolled T-cell proliferation in clinical trials for SCID-X1 [26]. This proliferation is thought to be triggered by retrovirus vector insertion in proximity to the LIM domain only-2 proto-oncogene promoter. However, it is expected that such aberrant proliferation could be minimised by our strategy of using PAs because of the containment and removability of the cells.

In summary, this is the first report that syngeneic implantation of gene-transferred PAs has successfully improved a systemic disorder in mice. This approach might be useful clinically for the treatment of patients with diabetes and other disorders caused by loss of circulating polypeptide(s).

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