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ENGINEERING VERO CELLS TO SECRETE HUMAN INSULIN

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SUMMARY

Cell therapy may have the potential for the treatment of Type I diabetes. To date, cells suitable for this purpose have not been developed. This study investigates the feasibility of modifying Vero, a cell line that may be considered safe to implant into humans, for this purpose. Stable Vero transfectants containing full-length human preproinsulin complementary deoxyribonucleic acid (cDNA) were generated using a liposomal transfection reagent. Reverse transcriptase–polymerase chain reaction, immunocytochemistry, Western blotting, and enzyme-linked immunosorbent assays were used to assess the resulting cells. Proinsulin was expressed but was not processed to insulin by these cells. Proinsulin cDNA was genetically modified, resulting in a form that is furin sensitive. The resulting stably transfected Vero clones constitutively release approximately 34%/h (32.68 ± 2.21 to 35.62 ± 3.14%) of the product formed, approximately 62% (59.99 ± 6.45 to 64.64 ± 4.57%) of which is mature insulin. These Vero transfectants did not exhibit glucose-stimulated insulin secretion. As GLUT2 and glucokinase (GCK) are not constitutively expressed by these cells, human GLUT2 cDNA and GCK cDNA were cotransfected with furin-sensitive preproinsulin cDNA into Vero cells. Insulin and GCK proteins were detected in the cytoplasmic region of the resulting cells, whereas GLUT2 was predominantly expressed in the nucleus. Coexpression of GLUT2 and GCK did not result in glucose-stimulated insulin secretion. The results from this study demonstrate the feasibility of engineering a relatively "safe" nonbeta cell line to produce human insulin. Coexpression of GLUT2 and GCK, at the levels achieved here, is not adequate enough to induce glucose-stimulated insulin secretion in such cells; the subcellular location of transfected components may be relevant.

Key words: diabetes; cell therapy; genetic engineering; furin-sensitive proinsulin; glucose sensory apparatus; glucose-stimulated insulin secretion.

INTRODUCTION

Type I diabetes is characterized by autoimmune destruction of the pancreatic beta cells. A suitable therapy for Type I (and possibly for some cases of Type II) diabetes will apparently require transplantation of cells to replace endogenous beta cells after the development of new and abundant sources of suitable replacement cells deemed safe to implant in humans.

One approach in developing replacement beta cells involves engineering non-beta cells to behave as beta cell substitutes. A potential advantage of using such "artificial" beta-like cells instead of genuine beta cells (from xenogenic sources or developed by differentiation from precursor cells) is that non-beta cells may not be recognized by the autoimmune response directed against beta cells in patients with Type I diabetes. Previous studies using this approach have involved the use of both neuroendocrine cells and non-neuroendocrine cells, including pituitary cells, fibroblasts, kidney (COS) cells, chinese hamster ovary (CHO) cells, and hepatocytes (Bailey et al., 1999). No cell lines have yet been developed that secrete human insulin in a glucose-regulated manner and that would be considered safe to implant into humans.

Hughes et al. (1992) introduced the human insulin gene into a mouse corticotrophic cell line, AtT20 because these cells contain the necessary machinery to secrete a protein (adenocorticotropic hormone [ACTH] being the naturally secreted protein) and the relevant endoproteases (prohormone convertases PC2 and PC3) required to convert proinsulin into biologically active insulin (proinsulin is only approximately 8 [Taniguchi et al., 1997] to 15% [Levine and Leibowitz, 1999] as active as insulin in maintaining blood glucose control). The resulting cells secreted insulin but not in a glucose-responsive manner. Further transfection of GLUT2 induced a glucose response, but maximal insulin secretion occurred at subphysiological glucose concentrations. Furthermore, anterior pituitary–derived cells may have limited therapeutic value because of the fact that they secrete ACTH, which would result in enhanced glucorticoid synthesis, opposing the action of insulin.

Fibroblasts have also been used in previous studies. Insulin-producing mouse fibroblast Ltk$^-$ cells have been engineered by transfecting the human insulin complementary deoxyribonucleic acid (cDNA) (Taniguchi et al., 1997). Because fibroblasts possess little endogenous ability to process proinsulin to insulin (Bailey et al., 1999), the resulting cells constitutively secrete mainly proinsulin. Despite this, encapsulation and implantation of these cells improved the hyperglycemic state of diabetic mice for at least 30 d, with some recipients being normoglycemic for over 50 d.

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A more recent study involving fibroblast engineering for this purpose has been described (Falqui et al., 1999). To overcome the problem associated with the lack of proinsulin processing, a genetically modified form of the human proinsulin cDNA was produced which had base mutations at both the C–A and the B–C junctions, rendering it sensitive to cleavage by a ubiquitously expressed enzyme furin. Approximately 58.5% processing of proinsulin to insulin was achieved after the transduction of this cDNA into primary fibroblasts. Injection of engineered insulin-producing (furin-sensitive) cells into the peritoneal cavity of diabetic mice reverted hyperglycemia when assessed up to 35 d later; proinsulin-producing cells were ineffective.

On the basis of the progress reported by previous studies, which involved modifying fibroblast cells, and with the aim of engineering a cell line (to secrete human insulin in a regulated manner) which may potentially be considered safe to implant in humans, we selected Vero cells for our study. Vero (African green monkey kidney cells) cells have been standardized for the production of the polio virus vaccine for human use, and so, like the fibroblast cell line BHK21, which has previously been genetically engineered and used in clinical trials for amyotrophic lateral sclerosis and Huntington's disease, they may be considered as a potentially safe cell type for future implantation in humans. Some of the other advantages of Vero cells for such an application are that they can be grown as anchorage-dependent cells or as cell aggregates in suspension (Litwin, 1992a), they grow in defined serum-free media (avoiding contaminants, including viruses, being introduced to the cells) without the addition of insulin or bovine serum albumin (BSA), the size and density of the cell aggregates formed may be controlled, to some extent, based on the medium used to support their growth (Litwin, 1992b), and like other cell lines, large banks of these cells can be cryopreserved after all sterility and safety tests have been performed.

Materials and Methods

Cell line culture. Vero cells (ATCC CCL 81) were grown in modified Eagle's medium with 5.6 mmol/L glucose, 1% L-glutamine, 1% nonessential amino acids, and 5% fetal calf serum and were cultured at 37° C in an atmosphere of 95% air and 5% CO_2. Routine sterility checks, including screening for *Mycoplasma*, indicated that the cells were clear of contamination.

Plasmid constructs. The human preproinsulin (PPI)-coding region (333 bp) was cloned from a pancreatic cDNA library and inserted into the pTarget mammalian expression vector (Promega, Southampton, U.K.), conferring geneticin resistance, resulting in the PPI construct (pT-PPI plasmid). After sequencing, to ensure no mismatches were included, a specific mutation was introduced at base position 257 (distance from ATG) to alter the gene product's susceptibility to furin cleavage, resulting in furin–PPI in pTarget (pT-F-PPI plasmid). Human GLUT2 cDNA (P-7-GLUT2), a gift from Prof. M. A. Permutt, Washington University School of Medicine (St. Louis, MO), as a full-length coding sequence of the human islet glucose transporter in puC19 vector, was subcloned from the KnpI-XbnI site and inserted into pcDNA3.1/Zeocin (Invitrogen, Groningen, The Netherlands), resulting in the ZeoGLUT2 plasmid; human glucokinase (GCK) cDNA (phIGLK-2), a gift from Prof. M. A. Permutt, as a full-length coding sequence of the human islet GCK cDNA in pBluescript SK+ vector, was subcloned into pcDNA3.1/Hygromycin (Invitrogen), resulting in the HygGCK plasmid.

Stable transfection of cell lines. Vero cells were seeded at 2×10^5 cells/25-cm² flask 24 h before transfection. LipofectAMINE PLUS® reagent (GIBCO BRL, Life Technologies, Paisley, Scotland) was used for all transfections. Human PPI (pT-PPI plasmid) or furin-sensitive PPI (pT-F-PPI plasmid) was transfected alone into Vero cells. Clonal populations (resistant to 1 mg/ml of geneticin) resulting from pT-PPI plasmid transfections were further cotransfected with GLUT2 and GCK. The Vero parental population was also cotransfected with furin–PPI, GLUT2, and GCK.

Reverse transcriptase–polymerase chain reaction analysis. For ribonucleic acid (RNA) analyses, cells were grown in 75-cm² tissue culture flasks until approximately 80% confluency was reached. Total RNA was isolated from pelleted cells by extracting with TriReagent (Sigma, Poole, U.K.) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 μg RNA using oligo dT primers (Oswel, Southampton, U.K.). Five microliters of the cDNA was then amplified in a 50-μl polymerase chain reaction (PCR) solution, containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, 20 μmol/L oligonucleotide primers, and 2.5 U *Taq* polymerase enzyme (Sigma). Forward and reverse primers used for cDNA amplification were as follows: PPI, 5′ AGCCTGGCTTCTTCTACACACC 3′ (forward) and 5′ GGTGCAGCACTGATCCACAATG 3′ (reverse) amplifying a 158-bp product; GLUT2, 5′ TGGCAGCTGCTCAACTAATCAC 3′ (forward) and 5′ AAACAGGTTTGCTGATACCAGC 3′ (reverse) resulting in an amplified 759-bp product; GCK, 5′ GATGCTGGATGACAGAGCCACGATG 3′ (forward) and 5′ AGATGCACTCAGAGATGTAGTCGA 3′ (reverse) amplifying a 392-bp amplified product; β-actin, 5′ TCGACATCCGCAAAGACCTGTAC 3′ (forward) and 5′ TCACGACGAGCAATGATCTTGA 3′ (reverse) primers were used, together with GLUT2 and GCK primers, to amplify a 142-bp β-actin product. Forward, 5′ GAAATCGTGCGTGACATTAAGGAGAAGCT 3′, and reverse, 5′ TCAGGAGGAGCAATGATCTTGA 3′, primers were included with PPI primers, resulting in the amplification of a 383-bp β-actin product.

Immunocytochemical analysis. For immunocytochemical analysis, cells were grown on 6-well plates (Costar), rinsed three times with phosphate-buffered saline (PBS), and fixed at −20° C for 5 min in methanol, which had been prechilled to −20° C. Primary antibodies used were as follows—insulin (Clone 5B6/6: insulin–proinsulin rat antibody raised in mouse; 1:300 dilution in Tris-buffered saline [TBS, 0.05 mol/L Tris–HCl, 0.15 mmol/L NaCl, pH 7.4]) (Biogenesis, Poole, U.K.), GLUT2 (AB1342: raised in rabbit; 1:300 dilution in TBS) (Chemicon, Harrow, U.K.), and GCK (GCK N-19, SC-1980: raised in goat; 1:30 dilution in TBS) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunocytochemical analysis was performed using the avidin biotin–horse radish peroxidase (AB-HRP) technique (ABC visualization kit) (DAKO Labs., Cambridgeshire, U.K.), according to the manufacturer's instructions. Briefly, endogenous peroxidase activity was quenched using 3% H_2O_2. Nonspecific binding was then blocked using dilute (1:5 in TBS) rabbit (for insulin and GCK analyses) or swine serum (for GLUT2 analysis), as appropriate. Primary antibodies were added and incubated for 2 h at 37° C or overnight at 4° C. Secondary antibodies were added for 30 min at room temperature. This was followed by a further 30 min of incubation at room temperature with streptABCcomplex/HRP. After counterstaining with 0.01% methyl green, HRP activity was visualized using a 3,3-diaminobenzidine tetrahydrochloride chromogen, which resulted in a brown color precipitation.

Western blot analysis. For Western blots, cells were harvested by trypsinization and washed three times with PBS. Cells were then lysed in a buffer containing 62.5 mmol/L Tris–HCl pH 6.8, 12.5% glycerol, 2% Nonidet P40 (Sigma), 2.5 mmol/L phenylmethylsulphonyl fluoride (Sigma), 1.25 mmol/L ethylenediaminetetraacetic acid, 12.5 μg/ml leupeptin (Sigma), 116 μg/ml aprotinin (Sigma) for 40 min on ice and sonicated, and the protein was quantified by the BioRad assay. Aliquots (50 μg) of samples were separated by running them at 250 V and 45 mA on a 12% polyacrylamide gel; proteins were transferred onto nitrocellulose membranes and, in the case of GLUT2, were blocked for 2 h at room temperature with 5% semiskimmed dried milk in TBS. For GCK analysis, membranes were blocked for 2 h at room temperature with 2.5% semiskimmed dried milk in TBS. Membranes were then probed overnight at 4° C, with the primary antibodies already discussed in the "Immunocytochemical analysis" section, using appropriate dilutions (GLUT2, 1:2500; GCK, 1:100; β-actin, 1:10,000). After a series of washes, the membranes were probed with an appropriate peroxidase-labeled secondary antibody for 1 h and were visualized by chemiluminescence using electrochemiluminescence (ECL) reagents (Amersham Pharmacia Biotechnology, Buckinghamshire, U.K.).

Proinsulin and insulin content and secretion. Before insulin content and secretion analyses, 2×10^5 cells were seeded per well of six-well plates and allowed to attach overnight. After this, the cells were rinsed three times with PBS (preheated to 37° C) to remove proinsulin and insulin secreted overnight and were subsequently incubated in a glucose-free medium twice for 15 min. To assess proinsulin and insulin cellular content and constitutive secretion, cells were then exposed to a serum-free medium containing 0.1% BSA for 0, 1, 2, 4, 8, and 24 h of incubation. Glucose-stimulated proinsulin and

insulin secretions were assessed after exposure to 0, 5, 10, 15, and 20 mmol/L glucose in serum-free medium for 2 h. After all the incubations, the medium was collected for analysis. For measurements of the intracellular proinsulin and insulin contents, cells were lysed in a lysis buffer (20 mmol/L Tris pH 8.0, 2 mmol/L ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1% Triton X-100, 10% glycerol, 1.5 mmol/L $MgCl_2$, 137 mmol/L NaCl, 1 mmol/L Na_3VO_4, 1× protease inhibitor cocktail [Boehringer Mannheim, Germany], 5% BSA). Proinsulin and insulin levels were measured by enzyme-linked immunosorbent assays (ELISAs). Proinsulin and insulin were codetected using a rat insulin kit, which is 120, 71, and <0.05% cross-reactive with human insulin, proinsulin, and c-peptide, respectively (Mercodia AB, Uppsala, Sweden; 10-11-24-10). Mature insulin was assessed using a specific human insulin ELISA (Mercodia AB; 10-11-13-10), which is <0.01% cross-reactive with proinsulin and c-peptide, following procedures provided by the manufacturers.

Results

Cell lines. Transfection of Vero cells with pT-PPI plasmid or pT-F-PPI plasmid resulted in several geneticin-resistant (1 mg/ml geneticin) clones (i.e., seven clones transfected with pT-PPI plasmid and five clones transfected with pT-F-PPI plasmid). Clonal populations resulting from the transfection of the pT-PPI plasmid were cotransfected with ZeoGLUT2 plasmid and HygGCK plasmid, resulting in PPI + GLUT2 + GCK cell populations, which were selected in increasing concentrations of antibiotics, in combination, up to 1 mg/ml geneticin, 80 μg/ml zeocin, and 120 μg/ml hygromycin. Nine clonal populations were isolated, i.e., PPI-GLUT2 + GCK Clones 1–9. Similarly, Vero cells were cotransfected with pT-F-PPI, ZeoGLUT2, and HygGCK (F-PPI + GLUT2 + GCK) and were finally grown in 1 mg/ml geneticin, 80 μg/ml zeocin, and 120 μg/ml hygromycin, resulting in the isolation of F-PPI + GLUT2 + GCK Clones 1–11.

A total of 32 clones resulted: seven PPI-only transfectants; nine PPI + GLUT2 + GCK transfectants; five F-PPI–only transfectants; and 11 F-PPI + GLUT2 + GCK transfectants. After the preliminary analysis of (pro)insulin formation, F-PPI + GLUT2 + GCK Clone 3 (C3) and F-PPI + GLUT2 + GCK Clone 9 (C9) were selected for extensive analysis. The results of all of the analyses reported here were similar when studies were performed immediately after transfection and selection, when compared with those obtained approximately 1 yr after the removal of the selection agents from the culture medium. This indicates that stable transfectants have been produced.

Reverse transcriptase–polymerase chain reaction analysis. Transcription of PPI, GLUT2, and GCK messenger RNAs (mRNAs) were assessed in Vero, C3, and C9 cells, as well as in two F-PPI–only transfected clones that were not transfected with GLUT2 and GCK cDNAs, i.e., Clone 7 (C7) and Clone 8 (C8). All newly constructed Vero cell lines (C3, C7, C8, and C9) expressed human insulin mRNA. Vero (nontransfected parent) did not produce insulin transcripts (Fig. 1*A*). Human GLUT2 mRNA (Fig. 1*B*) and GCK mRNA (Fig. 1*C*) were transcribed in cell lines derived from cotransfection

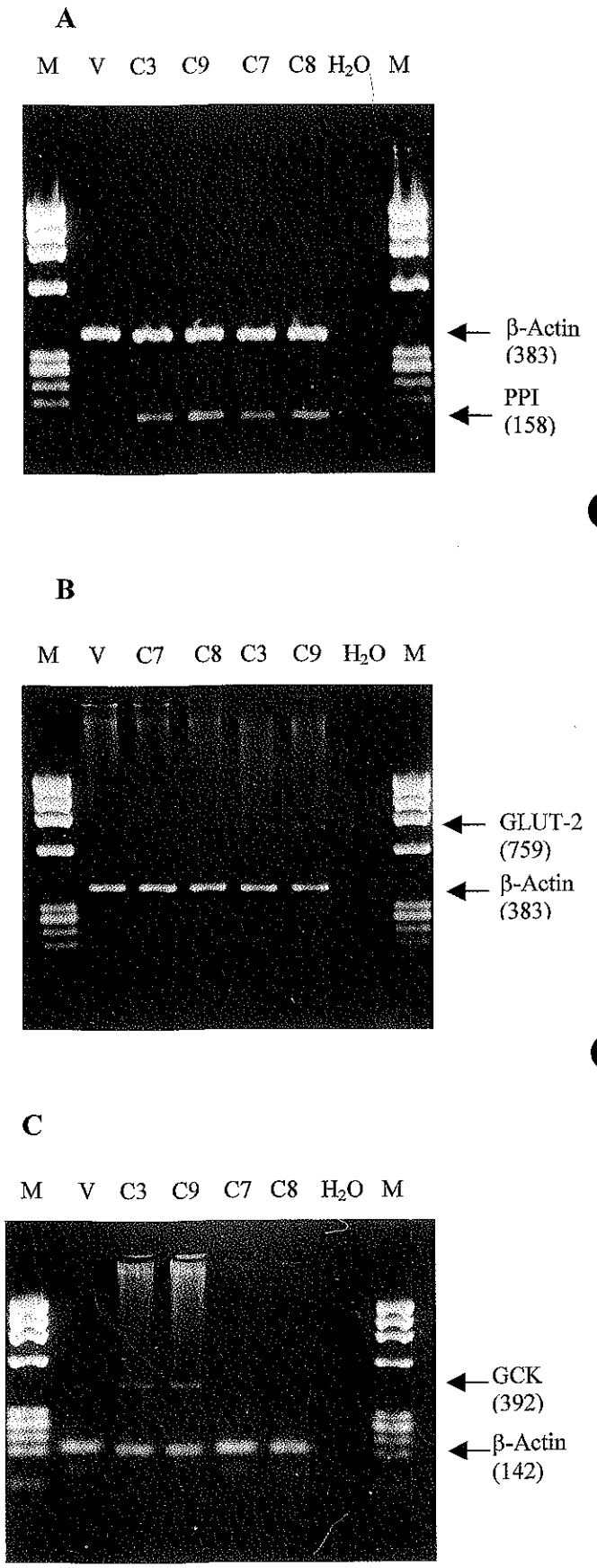

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FIG. 1. Reverse transcriptase–polymerase chain reaction analysis of (*A*) insulin, (*B*) GLUT2, and (*C*) glucokinase (GCK) messenger ribonucleic acid transcripts in nontransfected Vero parent cell line (V); F-PPI + GLUT2 + GCK Clones 3 (C3) and 9 (C9); and two clonal populations (C7 and C8) transfected with F-PPI but not with GLUT2 and GCK. β-Actin was included as an endogenous control and H_2O as the negative control. *M* indicates molecular weight markers.

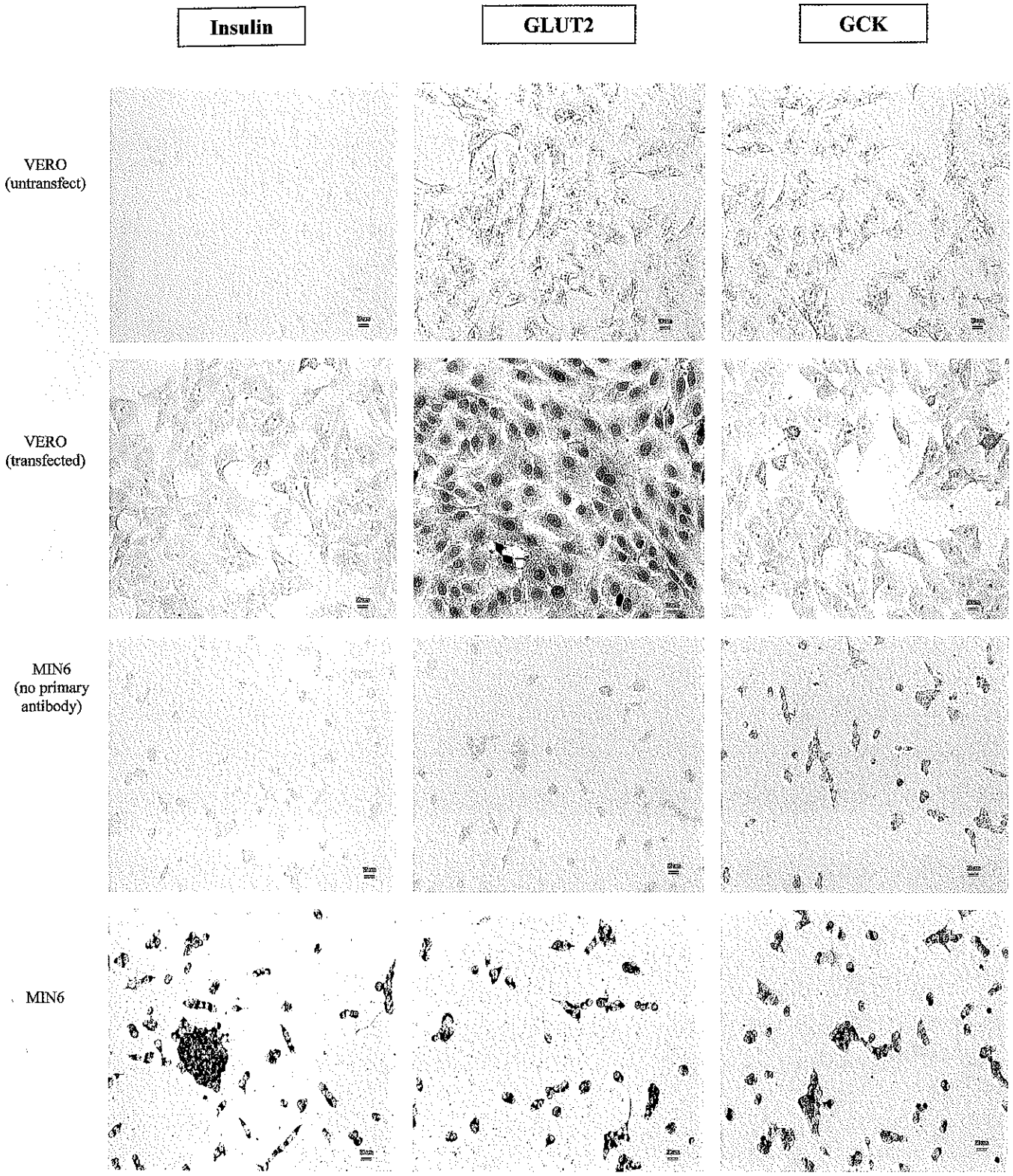

FIG. 2. Immunocytochemical detection of insulin, GLUT2, and glucokinase (GCK) in nontransfected Vero cells, transfected populations of Vero cells, and MIN6 cells. Indirect immunoperoxidase staining, using 3,3-diaminobenzidine tetrahydrochloride as the substrate, was performed followed by counterstaining with methyl green. Positive staining is shown in *brown*. MIN6 (no primary antibody) represent immunocytochemical studies on MIN6 cells, where the primary antibodies (for insulin, GLUT2, and GCK, respectively) were omitted, indicating that staining for each of the proteins under analysis is not a result of the nonspecific binding of the secondary antibody. Three replicate experiments gave comparable results. Magnification: ×40. *Bar*, 50 μm.

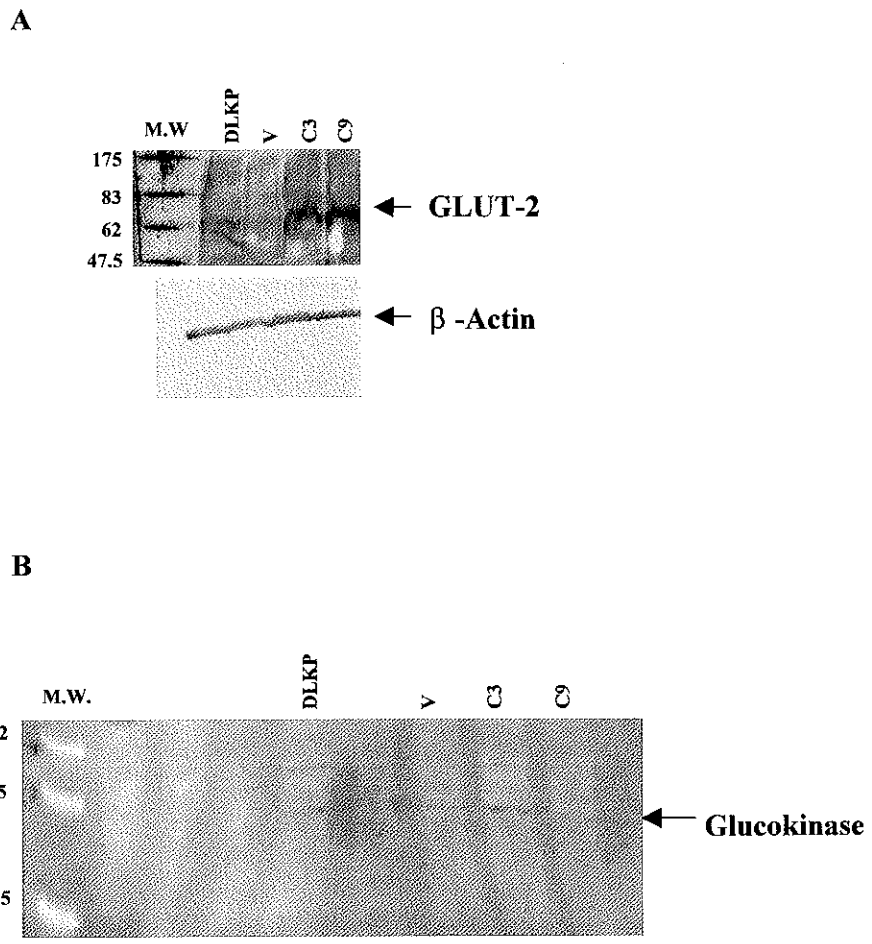

FIG. 3. Western blot analysis of GLUT2 and glucokinase (GCK) protein expression in cell lines. Cellular lysate (50 μg) from the nontransfected Vero parent cell line (V) and the clonal populations transfected with F-PPI + GLUT2 + GCK, i.e., C3 and C9, and DLKP ("in-house" control) were subjected to polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and hybridized with (*A*) rabbit anti-serum to GLUT2 (or β-actin [44 kDa] as endogenous control) and (*B*) goat anti-serum to GCK. Chemiluminescence was used as the detection system. GLUT2 was expressed in DLKP and, more significantly, in C3 and C9 but not in Vero cells. Similarly, GCK was apparently expressed by DLKP, and C3 and C9 cells, but not by Vero cells. M.W. indicates molecular weight markers.

of these plasmids with PPI cDNA, i.e., C3 and C9. GLUT2 and GCK transcripts were not detected in cells into which they had not been transfected, i.e., Vero parent cell line, C7 and C8. Amplification of β-actin (383 or 142 bp, depending on the primer pair used), as an endogenous control in all cases, indicated that the mRNA was of sufficient quality and that reverse transcription and PCR were conducted successfully.

Immunocytochemical analysis. Expression of insulin, GLUT2, and GCK were determined by immunocytochemical analysis. Transfection of PPI (wild type or furin sensitive), GLUT2, and GCK cDNAs into Vero cells resulted in the translation of the corresponding proteins by the cells. Indicative results are shown in Fig. 2, including C9 as an example of the result of insulin, GLUT2, and GCK expression. A murine beta cell line, MIN6, was included in

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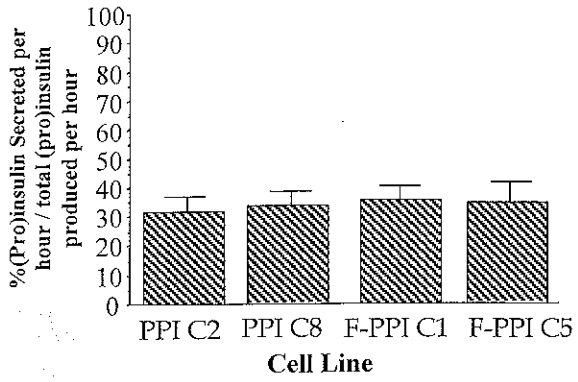
FIG. 4. (Pro)insulin (i.e., proinsulin + insulin [*bars* with *hashed design*]) and insulin (i.e., insulin only [*black bars* on the *graphs*]) production and secretion assessed by enzyme-linked immunosorbent assay. (*A*) Constitutive secretion: analysis of (pro)insulin indicates that Vero cells constitutively secrete approximately 34% (32.68 ± 2.21 to 35.62 ± 3.14%) of the total (pro)insulin formed per h. Similar levels of constitutive secretion were determined with cells expressing wild-type preproinsulin (PPI) (PPI C2 and PPI C8) and those expressing furin-sensitive PPI (F-PPI C1 and F-PPI C5). (*B*) Approximately 62% (59.99 ± 6.45 to 64.64 ± 4.57%) of the constitutively secreted (pro)insulin from cells expressing furin-sensitive PPI (C1 and C5) is mature insulin; no significant levels of mature insulin were detected in the secreted products from cells expressing wild-type PPI (C2 and C8). (*C*) and (*D*) To determine the effects of glucose concentration on insulin secretion, cells were incubated with a range of glucose concentrations (0–20 mmol/L) for 2 h. No glucose regulation of (pro)insulin or insulin was detected ([*C*] and [*D*] are F-PPI C1 and F-PPI C5, respectively, shown as examples of the results). (*E*) and (*F*) Introduction of GLUT2 and glucokinase into these cells did not induce glucose-regulated (pro)insulin or insulin secretion ([*E*] and [*F*] are C3 and C9, respectively, shown as examples of the results). All data represent means ± SD of at least three independent experiments.

A


%(Pro)insulin Secreted per hour / total (pro)insulin produced per hour
100
90
80
70
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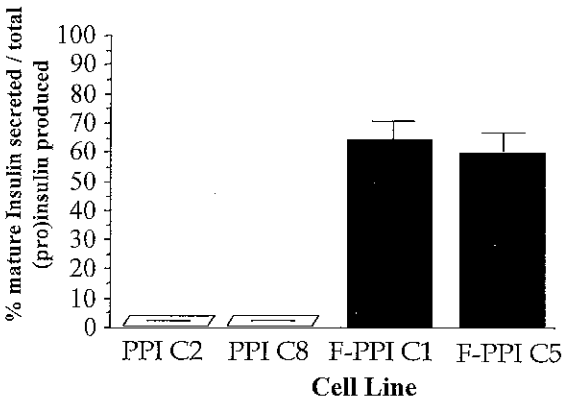
PPI C2
PPI C8
F-PPI C1
F-PPI C5
Cell Line

B


% mature Insulin secreted / total (pro)insulin produced
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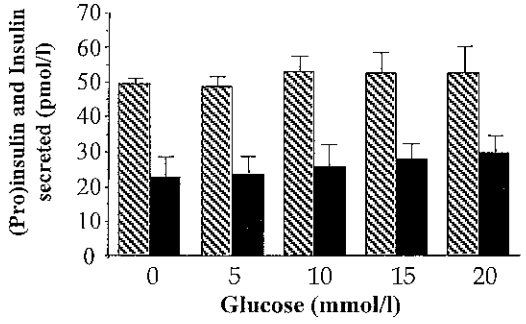
PPI C2
PPI C8
F-PPI C1
F-PPI C5
Cell Line

C


(Pro)insulin and Insulin secreted (pmol/l)
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Glucose (mmol/l)

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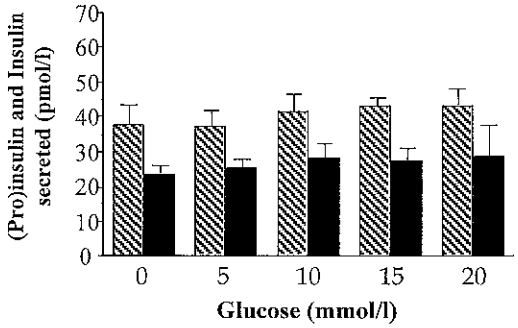
(Pro)insulin and Insulin secreted (pmol/l)
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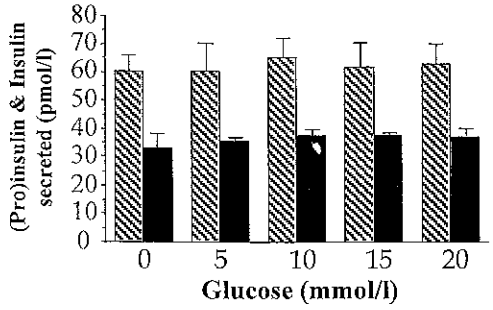
(Pro)insulin & Insulin secreted (pmol/l)
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Glucose (mmol/l)

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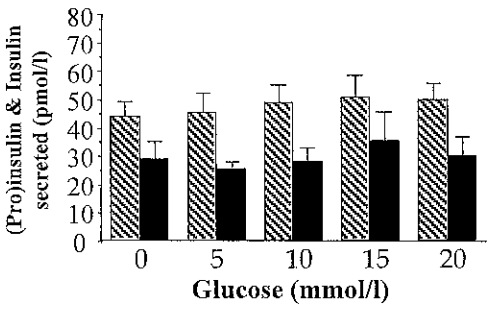
(Pro)insulin & Insulin secreted (pmol/l)
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Glucose (mmol/l)

these analyses as a positive control. It is interesting to note that whereas insulin and GCK are expressed in the cytoplasmic region, as expected, GLUT2 is apparently predominantly expressed in the nuclear region of the transfected Vero cells. GLUT2 expression in the MIN6 cells is, as expected, localized in the extranuclear region of the cells (β-actin, as endogenous control, indicated equal protein loading).

Western blot analysis. Single bands (approximately 62 kDa) corresponding to the GLUT2 protein were detected in C3 and C9 cells and, to a lesser extent, in the positive control cell line DLKP (Fig. 3*A*). In contrast, GLUT2 was not detected in nontransfected Vero cells.

Bands (approximately 50 kDa) corresponding to the GCK protein were detected in C3, C9, and DLKP cells (Fig. 3*B*) but not in the Vero parental population.

Enzyme-linked immunosorbent assays. Preliminary analysis for the constitutive secretion of a random selection of 20 of the 32 clonal populations isolated, i.e., PPI Clones 1–7, F-PPI Clones 1–5, PPI + GLUT2 + GCK Clones 1–9, and F-PPI + GLUT2 + GCK Clones 1–11, by (pro)insulin ELISA indicated that all the clones were forming and secreting human (pro)insulin to some extent. Analysis over a 24-h period indicated that all transfected cells constitutively secrete approximately 34% (32.68 ± 2.21 to 35.62 ± 3.14%) of their formed (pro)insulin per h (Fig. 4*A*, as an example of results).

The processing of proinsulin to insulin was investigated by ELISAs. The secreted product from the PPI clones and the PPI + GLUT2 + GCK clones was all proinsulin; no processed mature insulin was detected (Fig. 4*B*, PPI C2 and PPI C8). Approximately 62% (59.99 ± 6.45 to 64.64 ± 4.57%) of the secreted product from the clones transfected with the furin-sensitive form of the human PPI cDNA was mature insulin (Fig. 4*B*, F-PPI C1 and F-PPI C5). This was consistent after encapsulation and maintaining the cells for more than a mo in culture (data not shown). Based on these results, further extensive analysis was restricted to clones expressing furin-sensitive proinsulin.

In the next series of experiments investigating glucose-stimulated insulin secretion (GSIS), we analyzed the regulated secretion of (pro)insulin (i.e., proinsulin + insulin) and insulin alone in response to incubation for 2 h within a range of glucose concentrations. No stimulated release of proinsulin or insulin was detected above the levels constitutively secreted (Fig. 4*C* and *D*). Coexpression of GLUT2 and GCK in these cells did not induce glucose responsiveness (Fig. 4*E* and *F*) and did not significantly alter the processing of proinsulin to insulin.

Discussion

The data presented in this study demonstrate that the non-beta cell line, Vero, can be engineered to produce human proinsulin by transfection of the human PPI cDNA. Because of the lack of expression of the endopeptidase enzymes PC2 and PC3, Vero cells cannot process proinsulin to insulin. To address this issue, we generated a genetically-modified human proinsulin, containing furin-sensitive sites, cleavable to mature insulin, in non-beta cells. Previous studies have reported that after the introduction of furin-sensitive sites at both the C–A and the B–C peptide junctions of the human proinsulin cDNA, approximately 58.5% processing to mature insulin by human primary fibroblasts was achieved (Falqui et al., 1999). The single modification included in our studies resulted in similar levels (approximately 62%) of processing by Vero cells, without the need for introducing any changes in the mature insulin structure. In subsequent studies, it may be possible to optimize the conversion process by increasing furin levels in these cells by F-PPI cotransfection with a furin-encoding vector, as for therapeutic purposes, it may be useful to maximize the production of mature insulin per cell.

The results from this study indicate that Vero transfectants secrete mature human insulin but fail to show GSIS. Because mRNA (by reverse transcriptase–PCR) and protein (by immunocytochemical and Western blotting) analyses indicate that Vero cells do not constitutively express detectable levels of the high-capacity glucose transporter, GLUT2, and the glucose-phosphorylating enzyme, GCK, which are thought to be key components of the glucose sensory apparatus (GSA), regulating insulin release from beta cells in response to changes in the external glucose concentrations, we constructed cells that overexpress GLUT2 and GCK and studied their cellular characteristics. We found that the resulting cells cotranscribe and cotranslate GLUT2 and GCK with PPI. However, these cells did not secrete insulin in a glucose-regulated manner. Immunocytochemical analysis of the translated proteins indicated that although insulin and GCK were expressed in the cytoplasmic region of the Vero cells (as expected and as seen with the beta cell line, MIN6), GLUT2 expression was predominantly nuclear in Vero cells (this was not the case for MIN6 cells). The reason for nuclear expression of GLUT2 in Vero cells is unclear, but it may explain, at least in part, why a functional GSA was not formed by these cells.

In conclusion, Vero cells can be engineered to produce mature human insulin. This was consistent after encapsulation in alginate and maintaining in culture for more than a mo. Vero cells do not have a regulated secretory pathway, do not have secretory granules suitable for storage of high levels of insulin, and cannot be engineered to secrete insulin in a regulated manner by simply introducing GLUT2 and GCK expression. Insulin-producing Vero cells may, however, have potential application as a solution short of regulated insulin secretion "in which a constant trickle of insulin into the circulation occurs which may provide a basal control of hepatic glucose output, and may well improve glycaemia and the long-term control of diabetes" (Freeman et al., 1999).

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References

Bailey, C. J.; Davies, E. L.; Docherty, K. Prospects for insulin delivery by ex-vivo somatic cell gene therapy. J. Mol. Med. 77:244–249; 1999.

Falqui, L.; Martinenghi, S.; Severini, G. M., et al. Reversal of diabetes in mice by implantation of human fibroblasts genetically engineered to release mature human insulin. Hum. Gene Ther. 10:1753–1762; 1999.

Freeman, D. J.; Leclerc, I.; Rutter, G. A. Present and potential future use of gene therapy for the treatment of non-insulin dependent diabetes mellitus [review]. Int. J. Mol. Med. 4:585–592; 1999.

Hughes, S. D.; Johnson, J. H.; Quaade, C.; Newgard, C. B. Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells. Proc. Natl. Acad. Sci. USA 89:688–692; 1992.

Levine, F.; Leibowitz, G. Towards gene therapy of diabetes mellitus. Mol. Med. Today 5:165–171; 1999.

Litwin, J. The growth of Vero cells in suspension as cell-aggregates in serum-free media. Cytotechnology 10:169–174; 1992a.

Litwin, J. The growth of Vero cells as suspended aggregates in serum-free medium. In: Spier, H. E.; Griffiths, J. B.; MacDonald, C., ed. Animal cell technology: developments, processes and products. London: Butterworth–Heinemann; 1992b:414–417.

Taniguchi, H.; Fukao, K.; Nakauchi, H. Constant delivery of proinsulin by encapsulation of transfected cells. J. Surg. Res. 70:41–45; 1997.