Mechanisms Associated With Loss of Glucose Responsiveness in Beta Cells

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ABSTRACT

Background. Cell replacement therapies have been proposed as possible alternatives to the current treatments for controlling blood glucose in insulin-dependent diabetes. Beta cells, however, often lose their glucose-stimulated insulin secretion (GSIS) when maintained for prolonged periods in culture. For beta cell lines to be considered as a suitable source of transplantable tissue, it is essential that their GSIS is maintained. This study aimed to investigate cellular events involved in this loss of GSIS, to enable future optimization and enhancement of this response.

Methods. GSIS was investigated in low and high-passage murine insulinoma MIN-6 cells (using in vitro static procedures) and assessing levels of secreted (pro)insulin by enzyme-linked immunosorbent assays. Expression of relevant islet gene transcripts, including insulin, glucagon, somatostatin, and pancreatic polypeptide, was investigated by RT-PCR analysis.

Results. At low-passage, MIN-6 cells produced an approximately four- to fivefold increase in (pro)insulin secretion in response to 26.7 mmol/L glucose compared to 3.3 mmol/L glucose; at high passage, this response was lost. Expression of glucagon and somatostatin mRNAs were down-regulated with increased passage, while levels of insulin and pancreatic polypeptide mRNAs were apparently unchanged.

Conclusion. The maintenance of insulin mRNA levels in high-passage MIN-6 cells with down-regulation of glucagon (stimulates insulin secretion) and somatostatin (inhibits insulin secretion) gene transcript levels suggests that these cells have not lost their ability to maintain insulin production, but that the loss of glucose responsiveness may be due to a general effect on regulated secretion. Further studies investigating the regulated secretory pathway in these cells may further explain the mechanistic changes occurring with passing of beta cells.

MIN-6, A MURINE PANCREATIC beta cell line, was established from an insulinoma obtained by targeted expression of the simian virus 40T antigen gene in transgenic mice.¹ As a typical beta cell line, MIN-6 cells express insulin and islet amyloid polypeptide (IAPP).^{1,2} MIN-6 cells, however, have also been shown to express glucagon (generally produced by pancreatic alpha cells; stimulates insulin secretion) and somatostatin (generally produced by pancreatic delta cells; inhibits insulin secretion) proteins.³ The physiological relevance of expression of these genes by MIN-6 cells has not yet been established.

MIN-6 cells have been reported to secrete insulin in a glucose-regulated manner, as expected for a beta cell line; however, differing levels of glucose-stimulated insulin se-

cretion (GSIS), ranging from 1.2 to 30-fold, have been reported for these cells. Following the initial establishment of MIN-6 as a cell line, exposure to 25 mmol/L glucose (compared to 0.7 mmol/L) resulted in an approximately

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30-fold GSIS, with similar results reported to be achieved after 30 passages.¹ Results from studies of MIN-6 cells at passage 16 indicated a twofold GSIS that was reduced to approximately 1.2-fold by passage 35.⁴ Other reported glucose-stimulated insulin secretion results for these cells include a 7.3-fold GSIS⁵ and an approximately 4.8-fold GSIS from MIN-6 cells analyzed between passages 12 to 15.⁶

This study investigates GSIS in continuously cultured MIN-6 cells, comparing low (passage 17 to 22) and high (passage 40 to 49) cells and analyzing expression of insulin, glucagon, somatostatin, and pancreatic polypeptide (generally expressed by islet PP cells; proposed to be involved in energy metabolism and food intake⁷), to establish if expression of these genes is associated with changes in GSIS.

MATERIALS AND METHODS

MIN-6 Culture

MIN-6 cells (generously donated by Dr Per Bendix Jeppesen, Aarhus University Hospital, Denmark) were grown in DMEM containing 20% heat-inactivated FCS and were cultured at 37°C with 5% CO₂. Routine sterility checks, including screening for *Mycoplasma* sp, indicated that the cells were clear of contamination.

To passage (subculture), tissue culture flasks containing MIN-6 cells were emptied of conditioned medium and rinsed in prewarmed 1× Ca²⁺/Mg²⁺-free PBS (Gibco 14200-067). Trypsin/EDTA was then added (1 mL/25 cm² flask and 2 mL/75 cm² flask) and incubated at 37°C for 2 minutes. Flasks were tapped sharply to dislodge the cells and 5 mL of culture medium was added to inhibit the trypsin. The cell suspension was aspirated gently, with care to avoid separating the cells into a single-cell suspension. Following this, the cells were centrifuged at 900 rpm for 5 minutes, resuspended in prewarmed culture medium, counted, and reseeded to flasks or assay plates. MIN-6 cells were never reseeded back into culture vessels from which they had been trypsinized. Using this procedure, cells were subcultured to passage 50. Analysis, as described below, was performed using passage 17 to 22 (low) and passage 40 to 49 (high) cells.

GSIS Analysis of MIN-6 Cells

MIN-6 cells were seeded at a cell density of 2×10^5 cells/well in a 24-well plate. These cells were allowed to grow for 72 hours prior to the GSIS assay. Following this, 1× KRB was prepared from an aliquot of frozen 10× stock (36.525 g NaCl, 2.2 g KCl, 0.941 g CaCl₂ · 2H₂O, 1.22 g MgCl₂ · 6H₂O, 29.8 g HEPES dissolved in 500 mL H₂O), BSA was added to a final concentration of 0.1%, and the KRB-BSA was pH-adjusted to 7.36 exactly with 1 mol/L NaOH. This solution was warmed to 37°C and the pH was maintained in a 37°C incubator with a 5% CO₂ atmosphere (30 minutes). Glucose concentrations of 0, 3.3, 10, 16.7, and 26.7 mmol/L were prepared in the conditioned 1× KRB and were subsequently placed at 37°C and 5% CO₂ for 30 minutes. MIN-6 cells to be analyzed were rinsed (twice) in 1× KRB and were equilibrated at 3.3 mmol/L glucose for 30 minutes at 37°C. After equilibration, the glucose-containing stimulation media were added (1 mL/well), incubated at 37°C and 5% CO₂ for 60 minutes. The GSIS assay was then terminated by placing the plate on ice. Conditioned medium (500 µL) was removed from each well, placed in an ice-cold Eppendorf tube, centrifuged at 2500 rpm for 5 minutes and 200 µL of the

supernatant was removed for analysis by (pro)insulin ELISA (Mercodia, 10-1124-10; detects both proinsulin and insulin), following the manufacturer's instructions.

RT-PCR

Total RNA was isolated from cells by extracting with TriReagent (Sigma; Poole, England) according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 µg RNA using oligo dT primers (Oswel; Southampton, England). cDNA (5 µL) was then amplified in a 50-µL PCR reaction solution containing 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphates, 20 µmol/L oligonucleotide primers, and 2.5 U Taq polymerase enzyme (Sigma; D4545). Forward and reverse primers used for cDNA amplification were as follows: preproinsulin/insulin (PPI) (forward): 5' agcgtggtctctctacacacc 3' and (reverse) 5' ggtgcagcactgatccacaatg 3', amplifying a 158-bp product; glucagon (forward): 5' actcacagggcacattcacc 3' and (reverse) 5' ccagttgatgaagtcctgg 3', resulting in an amplified 353-bp product; somatostatin (forward): 5' ccgtcagtttctgcagaagt 3' and (reverse) 5' cagggtcaagttgacatcg 3', amplifying a 356-bp amplified product; pancreatic polypeptide (forward): 5' aggatggcgcgtgcatactg 3' and (reverse) 5' gagctgcactcagggaagtc 3', amplifying a 249-bp amplified product. β-actin was coamplified in all cases as endogenous control using the following primers: (forward): 5' gaaatcgtgcgtgacattaaggagaagct 3' and (reverse) 5' tcaggaggagcaatgatcttga 3', amplifying a 383-bp amplified product. The PCR cycle used was as follows: 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, relevant annealing temperature (54°C for PPI amplification and 60°C for glucagon, somatostatin, and pancreatic polypeptide) for 30 seconds 72°C for 30 seconds; completion step of 72°C for 5 minutes.

RESULTS

Static Incubation Glucose-Stimulated Insulin Secretion Study

Analysis of MIN-6 indicated that at low-passage numbers (p17 to 22) these cells secrete (pro)insulin in a regulated way, in response to changes in glucose concentrations in their environment, producing a four- to fivefold GSIS in response to 26.7 mmol/L glucose compared to 3.3 mmol/L glucose (Fig 1). At high-passage numbers (p40 to 49), the ability of these cells to regulate secretion of (pro)insulin was lost.

RT-PCR Analysis of Islet Gene Transcripts

Expression of preproinsulin (PPI), glucagon, somatostatin, and PP gene transcripts was detected, by RT-PCR, in low-passage MIN-6 cells. However, with increased passage number, expression of glucagon was reduced and expression of somatostatin was no longer detectable, whereas expression of insulin and PP mRNAs were apparently unaffected by the passage number (Fig 2).

DISCUSSION

The regulated transcription, translation, processing, storage, and secretion of insulin by beta cells is very complex, involving control by many contributing factors, including the main secretagogue, glucose; other islet hormones (ie, glucagon and somatostatin); as well as the parasympathetic

Fig 1. (Pro)insulin secretion in response to increasing glucose concentrations in low (p17 to 22; white bars) and high (p40 to 49; black bars) passage MIN-6 cells. Data presents means $\pm$ SD for at least three experiments.

and sympathetic nervous system. This tightly regulated control is critical to maintain normoglycemia, avoiding episodes of hypo- and hyperglycemia.⁸ Culture of beta cells offers great potential as a possible alternative to the current treatments available for insulin-dependent diabetes. However, for such cells to be considered as a suitable source of transplantable tissue, it is essential that their secretion of

insulin is in a regulated manner, in response to physiological changes in blood glucose levels.

Conflicting results have been reported on the GSIS response of the murine beta cell line, MIN-6, with increasing passage numbers and time in culture, with some studies reporting maintenance of GSIS and others documenting loss of this response.^{1,4-6} Similarly to Kinoshita et al⁶ in this

Fig 2. RT-PCR analysis of preproinsulin (PPI), glucagon, somatostatin, and pancreatic polypeptide (PP) gene transcripts in low-(L) and high-(H) passage MIN-6 cells. β -actin was amplified as an endogenous control and water was included as a negative control. Results are representative of three experiments.

study we found low-passage MIN-6 cells to respond to changes in glucose concentrations, producing an approximately four- to five-fold GSIS in response to 26.7 mmol/L, compared to 3.3 mmol/L, glucose. After continuous culture to passage 40 to 49, this GSIS was no longer present.

Further analysis of these cells indicated that the low-passage MIN-6 cells transcribe the insulin, glucagon, somatostatin, and pancreatic polypeptide genes. Although the levels of transcripts encoding insulin and pancreatic polypeptide were apparently unchanged between high- and low-passage cells, levels of glucagon mRNA were reduced and somatostatin transcripts were no longer detectable at high passages. It has been established that glucagon (secreted by alpha cells and acting in a paracrine manner) stimulation of insulin secretion is mediated by activation of adenylate cyclase through a stimulatory GTP-binding protein, resulting in cAMP generation that, in turn, activates PKA, sensitizing the beta cell secretory machinery to calcium ions through the phosphorylation of intracellular proteins. The inhibitory effects of somatostatin, secreted by delta cells, on insulin (and glucagon) secretion is associated with decreased formation of cAMP, coupled with G-protein-mediated actions on ion channels, resulting in membrane hyperpolarization and a decrease in cytosolic calcium concentrations. Somatostatin has also been proposed to interfere with exocytosis.⁸⁻⁹ The reduced glucagon mRNA levels and loss of somatostatin mRNA expression in high-passage cells observed in this study therefore may cause many effects that are relevant to regulated secretion. These preliminary finds, however, must be supported by further research to determine if this proposed explanation is valid. For this to be so implies that the effects observed at the

mRNA level are translated to the protein level, and that glucagon and somatostatin thus produced by MIN-6 cells may act on these cells in an autocrine manner. It must also be considered that the changes in glucagon and somatostatin transcript levels found with loss of GSIS may not necessarily be involved in this process.

The maintenance of insulin mRNA levels with increasing passage numbers suggests that the cells are not losing their ability to produce insulin. Further studies investigating the significance of changes in glucagon and somatostatin expression, and the regulated secretory pathway as a whole, may potentially further explain the mechanistic changes occurring with passaging of beta cells, possibly enabling future restoration and maintenance of the glucose-stimulated insulin secretory response by these cells.

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