Establishment of new clonal pancreatic β-cell lines (MIN6-K) useful for study of incretin/cyclic adenosine monophosphate signaling

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ABSTRACT

Incretin/cyclic adenosine monophosphate (cAMP) signaling is critical for potentiation of insulin secretion. Although several cell lines of pancreatic β-cells are currently available, there are no cell lines suitable for investigation of incretin/cAMP signaling. In the present study, we have newly established pancreatic β-cell lines (named MIN6-K) from the IT6 mouse, which develops insulinoma. MIN6-K8 cells respond to both glucose and incretins, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), as is the case in pancreatic islets, whereas MIN6-K20 cells respond to glucose, but not to incretins. Despite the difference in incretin-potentiated insulin secretion between these two cell lines, the accumulation of cAMP after stimulation of GLP-1 is comparable in these cells. Interestingly, we also found that incretin responsiveness is drastically induced by the formation of pseudoislets from MIN6-K20 cells to a level comparable to that of pancreatic islets. Thus, these cell lines are useful for studying incretin/cAMP signaling in β-cells. **(J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00026.x, 2010)**

KEY WORDS: Incretin, cAMP, Pseudoislet

INTRODUCTION

Incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are released from enteroendocrine cells by ingestion of nutrients, and potentiate insulin secretion in a glucose-dependent manner by activation of cyclic adenosine monophosphate (cAMP) signaling through their specific receptors in the pancreatic β-cell membrane[1]. GLP-1 analogs and dipeptidyl peptidase IV (DPP-IV) inhibitors are currently being used as new hypoglycemic agents to treat patients with type 2 diabetes mellitus (T2DM)[2]. In contrast, it has been reported that GIP is ineffective for the treatment of T2DM[3,4], which shows that GIP receptor-mediated signaling is inactivated in T2DM[5]. Although cAMP is now known to potentiate insulin secretion mediated by both protein kinase A (PKA)-dependent and PKA-independent pathways[6–9], differences in the mechanisms between GLP-1 and GIP signaling in pancreatic β-cells are still unclear. In addition, the nature of incretin-mediated signaling in pancreatic β-cells of T2DM has not been characterized. This is mainly because there is no appropriate system for the study of the mechanisms of incretin/cAMP signaling.

Various clonal β-cells are useful models for the study of insulin secretion in pancreatic β-cells. Although several β-cell lines, such as RINm5F, HIT, βTC, INS1, and MIN6, have been established[10–14], these cells often show insulin secretory properties different from those of native pancreatic β-cells, and tend to lose glucose-stimulated insulin secretion (GSIS) during the course of passage[15,16]. We previously reported that MIN6-m9 cells subcloned from original MIN6 cells retain GSIS after repetitive passage[17]. However, because of their lack of incretin responsiveness, MIN6-m9 cells are not suitable for the investigation of incretin/cAMP signaling.

In the present study, we established two new pancreatic β-cell lines (designated MIN6-K8 and MIN6-K20) from the IT6 mouse, which develops insulinoma, and characterized their properties of insulin secretion. We found that these cells show distinct responses to incretins and that formation of pseudoislets drastically induces an incretin responsive state from the unresponsive state.

MATERIALS AND METHODS

Cloning of MIN6-K Cell Lines

An IT6 mouse was used to establish pancreatic β-cell lines[14]. Clonal β-cells were obtained by isolating β-cell colonies sprouted on culture dishes of mixed-cells prepared from the whole pancreas of an IT6 mouse, as previously described[18].

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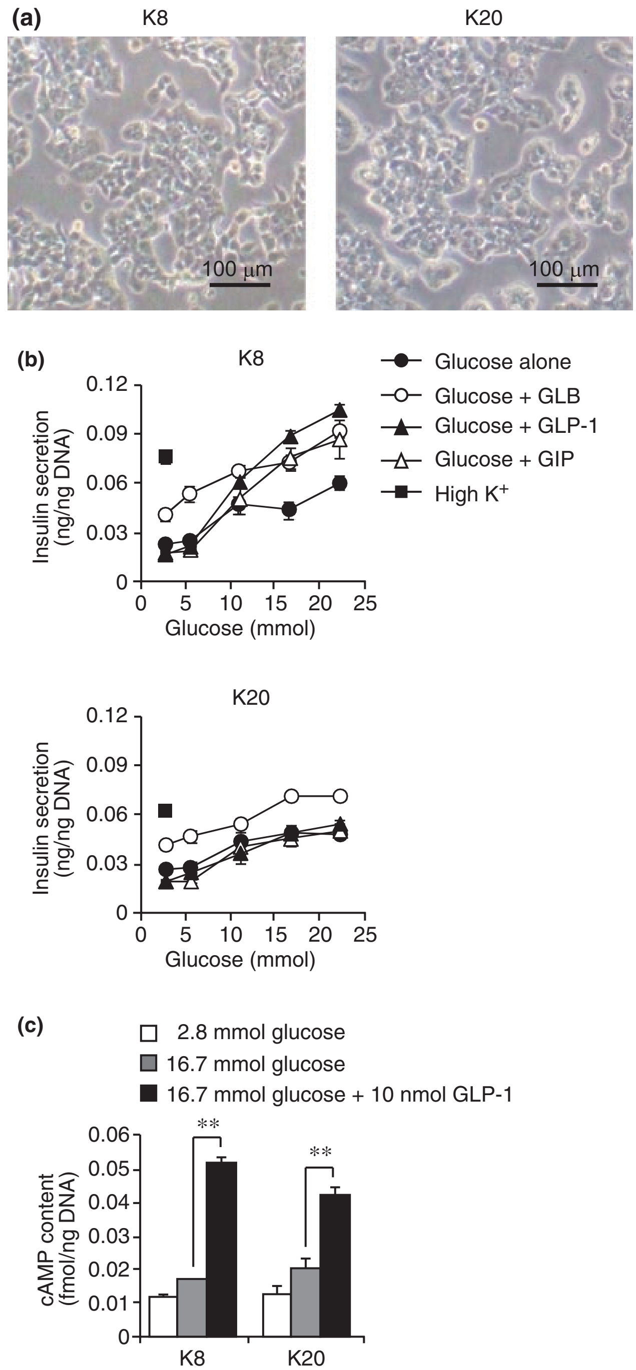

Figure 1 | Characterization of MIN6-K8 and MIN6-K20 cells. (a) Morphology of K8 and K20 cells. Cells were grown as monolayers on a tissue culture dish in DMEM containing 25 mmol/L glucose (scale bars, 100 μm). (b) Insulin secretory properties of K8 and K20 cells. Cells were precultured for 2 days in a 96-well plate and preincubated for 30 min in HEPES-Krebs buffer containing 0.1% BSA with 2.8 mmol/L glucose. Incubation was preformed in the presence of the indicated concentrations of glucose or glucose with 10 nmol/L glibenclamide (GLB), 10 nmol/L glucagon-like peptide-1 (GLP-1), or 10 nmol/L glucose-dependent insulinotropic polypeptide (GIP). Plasma membrane depolarization was induced by the addition of 60 mmol/L KCl in the presence of 2.8 mmol/L glucose. (c) Cyclic adenosine monophosphate (cAMP) levels in K8 and K20 cells were measured in the presence of 10 nmol/L GLP-1 with 16.7 mmol/L glucose. The amounts of insulin secretion and cAMP content were normalized by the cell DNA content. Data are means ± SEM (n = 4). Unpaired Student's t-test was used for the evaluation of statistical significance. $^{**}P < 0.01$.

Formation of Pseudoislets

Pseudoislets were formed as previously described[16], with slight modifications. Briefly, MIN6-K cells were seeded on dishes coated by 0.1% wt/vol gelatin, and cultured for 7 days in DMEM containing 25 mmol/L glucose.

Measurements of Insulin Secretion

MIN6-K cells were preincubated for 30 min in HEPES-Krebs buffer[17] with 2.8 mmol/L glucose, and then stimulated for 30 min with various concentrations of glucose in the absence or presence of the incretins for 30 min. Released insulin was measured by insulin assay kit (CIS Bio International, Gif sur Yvette, France).

Measurement of cAMP Content

MIN6-K cells were incubated for 30 min in the presence or absence of GLP-1 with 16.7 mmol/L glucose. Cellular cAMP levels were determined by using a commercial kit (CIS Bio International).

Quantification of mRNA Expression

mRNA expressions were quantified by real-time RT–PCR using TaqMan probes (Applied Biosystems, Foster City, CA, USA).

RESULTS

Establishment and Characterization of MIN6-K Cells

We obtained more than 30 clonal pancreatic β-cell lines from the pancreas of an IT6 mouse. Among these, we selected two cell lines based on their insulin secretory response to glucose and GLP-1. We designated one line MIN6-K8 and the other line MIN6-K20, which were indistinguishable by their morphology (Figure 1a). MIN6-K8 cells secreted insulin in response to a physiological concentration of glucose and the sulfonylurea, glibenclamide. Potentiation of GSIS by incretins (both GLP-1 and GIP) was also evident (Figure 1b), showing that the cells resemble native pancreatic islets in terms of the property of insulin secretion. In contrast, while MIN6-K20 cells did respond to glucose and glibenclamide, the cells did not respond to either GLP-1 or GIP (Figure 1b). Because incretins potentiate insulin secretion through an increase in the intracellular cAMP concentration, we reasoned that the difference in incretin response between MIN6-K8 and -K20 cells might result from a difference in cAMP production. However, this is not the case, because the cAMP levels after the addition of GLP-1 in the two cell lines were found to be similar (Figure 1c). Therefore, signaling distal to cAMP production is likely to be responsible for the different

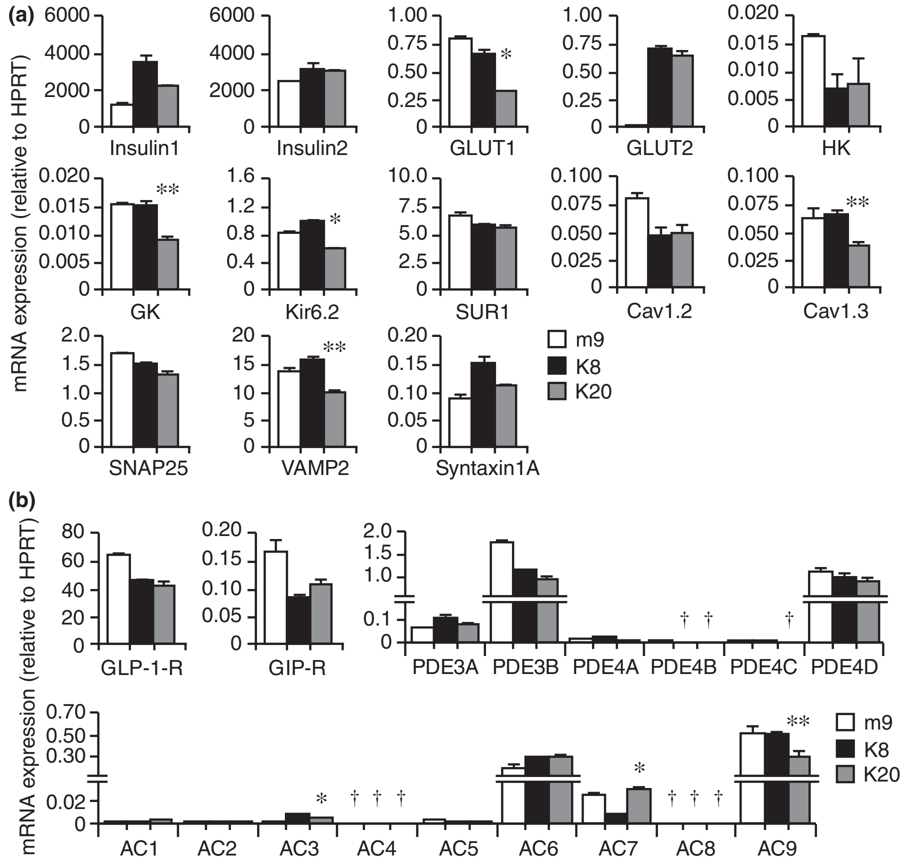

Figure 2 | Comparison of gene expressions among MIN6-K8, MIN6-K20 and MIN6-m9 cells. (a) Expressions of the genes involved in glucose-stimulated insulin secretion. (b) Expressions of the genes encoding incretin receptors, adenylyl cyclases and phosphodiesterases. The expressions are shown as relative to the level of hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression. Data are means ± SEM (n = 3). Unpaired Student's t-test was used for the evaluation of statistical significance between MIN6-K8 and MIN6-K20 cells. *P < 0.05, **P < 0.01, †, undetectable. AC, adenylyl cyclase; GIP-R, glucose-dependent insulinotropic polypeptide receptor; GK, glucokinase; GLP-1-R, glucagon-like peptide-1 receptor; HK, hexokinase; PDE, phosphodiesterase.

responses to the incretins between MIN6-K8 and MIN6-K20 cells.

Gene Expression in MIN6-K Cells

We then examined gene expression in MIN6-K8 and -K20 cells by quantitative RT–PCR. Although GSIS in the two cell lines was similar (Figure 1b), several genes, including GLUT1 and glucokinase, were downregulated in the incretin non-responsive MIN6-K20 cells (Figure 2a). The expressions of the receptors for GLP-1 and GIP were not different between MIN6-K8 and -K20 cells. In addition, no significant difference in expressions of adenylyl cyclases, which catalyze adenosine triphosphate to cAMP, and phosphodiesterases, which degrade cAMP, was detected (Figure 2b).

Normalization of Insulin Content, Basal Insulin Secretion and Incretin Responsiveness by Formation of Pseudoislets

Although experiments using cell lines are usually carried out in monolayer culture conditions, native pancreatic β-cells form three-dimensional structures (islets). We therefore examined insulin secretion in pseudoislets constituted from MIN6-K cells. Pseudoislets, which morphologically resemble native mouse pancreatic islets, were formed on gelatin-coated dishes (Figure 3a,b and Supporting Information Movie S1). Insulin contents in the pseudoislets were drastically increased to levels similar to those of native islets (Figure 3c), whereas the expressions of insulin genes were not changed or only slightly increased in pseudoislets (Figure 3d).

We then investigated insulin secretion in pseudoislets in comparison with that in monolayer-cultured cells (Figure 4). Insulin secretion from pseudoislets was significantly lower than that from monolayer-cultured cells at a low concentration of glucose. Interestingly, potentiation of GSIS by both GLP-1 and GIP was significantly enhanced in MIN6-K8 cells by the formation of pseudoislets. Most strikingly, even incretin non-responsive MIN6-K20 cells, as well as MIN6-m9 cells, clearly responded to both GLP-1 and GIP when pseudoislets were formed. These data show that these cell lines acquired insulin secretory

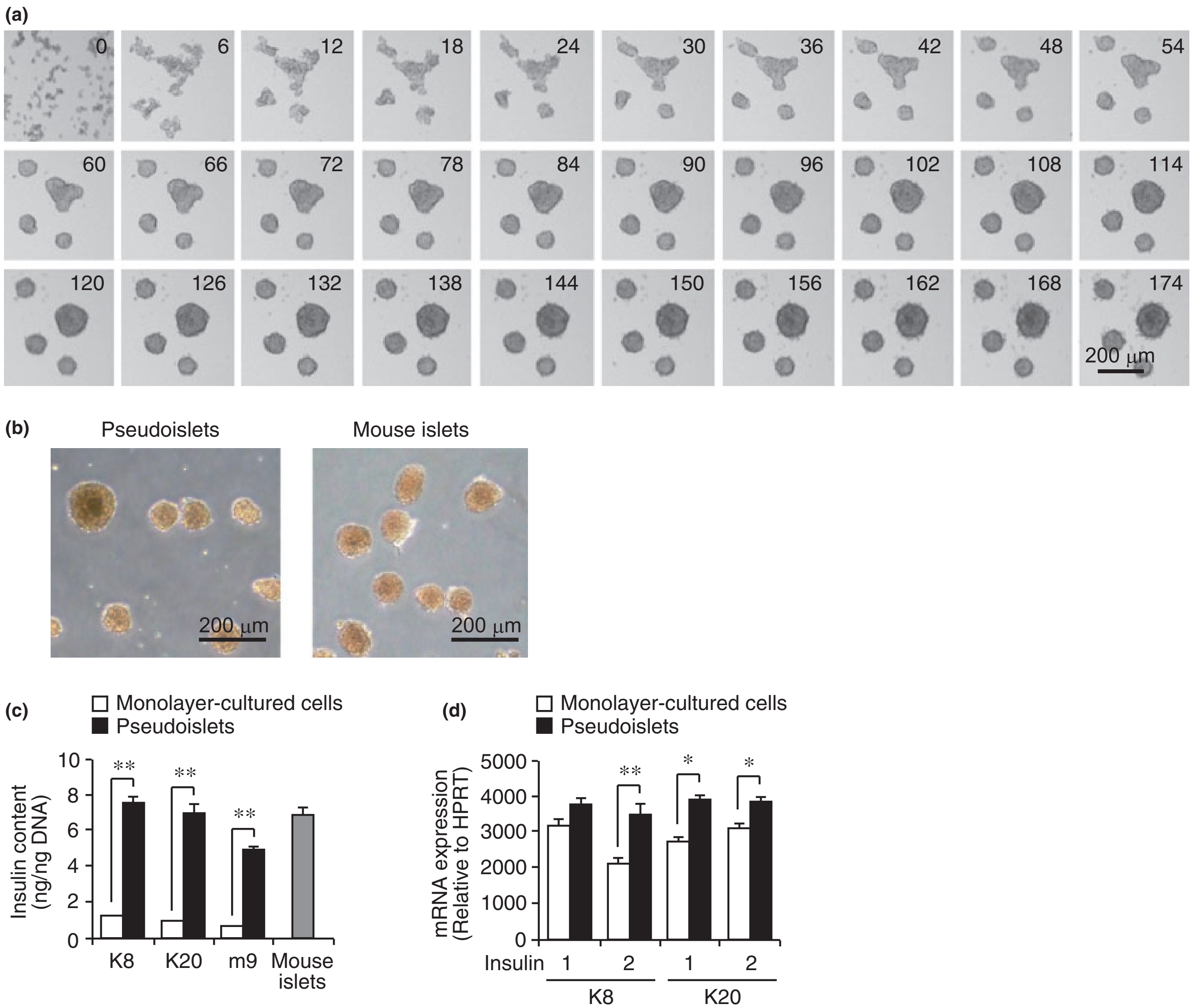

Figure 3 | Formation of pseudoislets. (a) The course of formation of pseudoislets. Sequential images acquired every 6 h for 7 days are shown (scale bar, 200 μm). (b) Morphology of pseudoislets and mouse pancreatic islets (scale bars, 200 μm). (c) Comparison of insulin contents between pseudoislets and monolayer-cultured cells. Data are means ± SEM (n = 4–8). The amount of insulin content was normalized by the cell DNA content. (d) mRNA expression levels of insulin1 and insulin2 in pseudoislets and monolayer-cultured cells. Expressions of these genes are shown as relative to the level of hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression. Data are means ± SEM (n = 3). Unpaired Student's t-test was used for the evaluation of statistical significance. *$P < 0.05$, **$P < 0.01$.

properties similar to those of native pancreatic islets by the formation of pseudoislets.

DISCUSSION

It is well known that incretins, such as GIP and GLP-1, activate adenylyl cyclase to increase cAMP in pancreatic β-cells[2]. Elevation of cAMP levels activates both PKA-dependent and PKA-independent pathways, the latter involving Epac2/Rap1 signaling[6–9]. While PKA modulates insulin secretion through phosphorylation of various proteins associated with the secretory process of insulin[8,19], Epac2/Rap1 signaling is also important for the potentiation of GSIS by cAMP, probably by increasing the size of a readily releasable pool near the plasma membrane[9,18,20]. However, the link between these pathways and insulin secretion is largely unknown. Although native pancreaticβ-cells are an ideal source for investigation of these mechanisms, the limited numbers of pancreatic β-cells that can be isolated from the native pancreas hampers the study of incretin/cAMP signaling at the cell level.

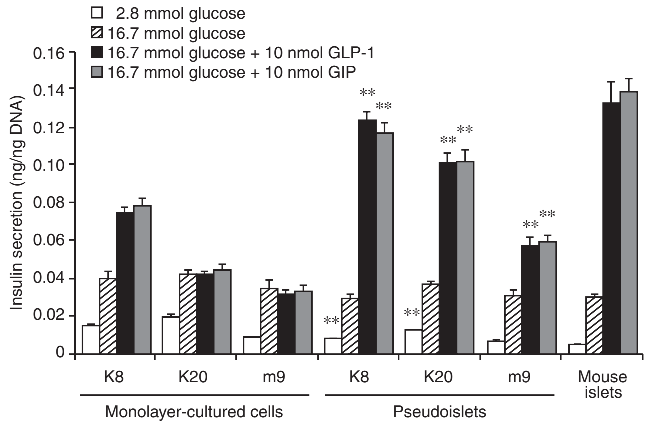

Figure 4 | Normalization of insulin secretion in pseudoislets. Pseudoislets and monolayer-cultured cells were preincubated for 30 min with HEPES-Krebs buffer containing 0.1% BSA with 2.8 mmol/L glucose, and incubated for 30 min with 2.8 mmol/L or 16.7 mmol/L glucose with or without 10 nmol/L glucagon-like peptide-1 receptor or 10 nmol/L glucose-dependent insulinotropic polypeptide. The amount of insulin secretion was normalized by the cell DNA content. Data are means ± SEM (n = 8). Unpaired Student's t-test was used for evaluation of statistical significance between monolayer-cultured cells and pseudoislets. *$P < 0.05$, **$P < 0.01$.

In the present study, we show that our newly established mouse pancreatic β-cell lines are useful for such study. GLP-1 increased the intracellular cAMP concentration similarly in both incretin responsive MIN6-K8 and incretin non-responsive MIN6-K20 cells, showing that signals distal to cAMP production differ between the two cell lines. Comparative analysis of MIN6-K8 and -K20 cells should therefore advance understanding the link between cAMP and insulin secretion.

In the present study, we show for the first time that the formation of pseudoislets induces incretin responsiveness in pancreatic β-cell lines. Cell–cell adhesion is known to have important roles in cell function and differentiation[21,22]. Cell–cell interactions mediated by gap junctions and/or EphA-ephrin-A[23–25] might participate, at least in part, in the improved insulin secretion in pseudoislets. Pseudoislets constituted from these newly established MIN6-K lines are a useful system for elucidation of incretin/cAMP signaling in β-cells.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Movie S1 | Time-lapse images of pseudoislet formation of MIN6-K20 cells were acquired every 1 h for 7 days by using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan). Timestamp (h : min : sec) and calibration bar (200 μm) are overlaid on the images.

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