In diabetes, insulin secretion is either completely absent (insulin-dependent diabetes mellitus [IDDM]) or inappropriately regulated (non-insulin-dependent diabetes mellitus [NIDDM]). In recent years, new insights into the molecular and biochemical mechanism(s) of fuel-mediated insulin release coupled with advances in gene transfer technology have led to the investigation of molecular strategies for replacement of normal insulin delivery function. Such initiatives have included attempts to engineer glucose-stimulated insulin secretion in cell lines that might serve as surrogates for islets in IDDM. The development of DNA virus gene transfer systems of remarkable efficiency also has suggested ways in which the β-cell dysfunction of NIDDM might ultimately be repaired by gene therapy. The emerging work in these areas and implications for the future are summarized in this perspective. *Diabetes* 43:341–50, 1994

**Biochemical Mechanism of Glucose-Stimulated Insulin Secretion**

For the purposes of this perspective, a short summary of important biochemical events in the pathway of glucose-stimulated insulin secretion is provided and summarized in schematic form in Fig. 1. For more comprehensive discussion, the reader is referred to one of several excellent reviews of the subject (3–6). Glucose stimulates insulin secretion from the β-cells of the islets of Langerhans through its own metabolism (3,7). Thus, nonmetabolizable analogues of glucose, such as 3-O-methyl or 2-deoxyglucose, are not insulin secretagogues, and inhibitors of glucose phosphorylation, such as glucosamine or mannohexulose, suppress β-cell glycolysis and insulin secretion (3,7). Glucose metabolism appears to trigger a number of electrochemical events, including inhibition of ATP-sensitive K+ channels and activation of voltage-gated Ca2+ channels. Several studies have suggested that the link between enhanced glucose flux and ion channels resides in alterations in the cellular ATP:ADP ratio (8–10). Although this model is widely quoted and inherently attractive, it has yet to be...
fully reconciled with the finding that glucose administration to islets causes only very small changes in the levels of ATP or ADP (11). Mitochondrial metabolism appears to play an important role in the glucose response, because glucose stimulates islet cell respiration and agents that block mitochondrial electron transport or oxidative phosphorylation also block glucose-stimulated insulin secretion (3,12,13).

The precise role of mitochondrial metabolites, however, is as yet unclear. Interestingly, glyceraldehyde is known as a potent insulin secretagogue, whereas pyruvate, which can enter directly into mitochondrial metabolism, is ineffective. This has led to the suggestion that metabolic events occurring between the level of the trioses and pyruvate are instrumental in normal glucose signaling (3,6). Islets are known to possess unusually high levels of glycerol-3-phosphate dehydrogenase, the enzyme catalyzing the glycerol phosphate shuttle that transfers reducing equivalents from the cytosol to FAD$^+$ in the inner mitochondrial membrane (6). This shuttle may play an important role in maintenance of the $\beta$-cell redox state and provide a link between cytosolic and mitochondrial metabolism. Finally, previous studies have suggested a link between enhanced glucose flux and generation of malonyl CoA, a potent inhibitor of fatty acid oxidation (14). It has been suggested that generation of malonyl CoA, requiring both glycolytic and mitochondrial metabolism, may enhance cellular levels of long-chain acyl CoAs that could participate in activation of insulin secretion (14).

It is evident that defining the precise signaling pathway for the acute glucose-stimulated insulin secretion response will require further investigation. Although the identity of the glucose-related mediators remains an open question, the ultimate secretory response does appear to be tightly linked to increases in cytosolic free $Ca^{2+}$, much of which accumulates by influx through the voltage-gated $Ca^{2+}$ channel (4,5). Increases in $Ca^{2+}$ can have a wide range of effects in the $\beta$-cell, including activation of glyceraldehyde phosphate dehydrogenase and other mitochondrial enzymes, activation of protein kinases, and activation of phospholipase C to generate IP$_3$ and diacylglycerol. Previous studies have provided evidence in support of a role for protein kinase C in insulin secretion, because glucose clearly provokes translocation of the enzyme and phosphorylation of the myristoylated alanine-rich C-kinase substrate or MARCKS (15,16); this has traditionally been an area of some controversy (17,18). A role for other kinases, including members of the Ca$^{2+}$/calmodulin class in activation of insulin exocytosis also has been proposed (19), and this remains an important area for further investigation.

It is now commonly accepted that the magnitude of the insulin secretory response is proportional to the rate of glucose metabolism in the $\beta$-cell. Thus, proteins or enzymes that control glucose flux in $\beta$-cells can be thought of as glucose sensors controlling insulin release in response to changes in the external glucose concentration. Two proteins that have received considerable attention as candidates for a glucose-sensing role in the $\beta$-cell are the GLUT2-facilitated glucose transporter and the glucose-phosphorylating enzyme glucokinase (3,20–22). Of these, glucokinase is the more likely to play a rate-limiting role in glucose flux, at least in the normal islet. Glucokinase, also known as hexokinase IV, is a member of the family of hexokinases and is distinct from other members of the family in terms of its smaller size, its lack of allosteric regulation by glucose-6-phosphate, and its significantly higher $K_m$ for glucose (8 mM as opposed to 10–50 $\mu$M for hexokinase I–III [3,23,24]). Glucokinase is restricted in its tissue distribution, with its main sites of expression being the liver and the islet $\beta$-cell (25,26), although transcriptional activity also has been reported in discrete cell populations, such as anterior pituitary corticotrophs (27,28). Its $K_m$ for glucose of ~8 mM allows large changes in enzyme activity in response to small increments in glucose concentration over the physiological range (4–9 mM), an ideal property for a glucose-sensing protein. The maximal velocity of glucokinase in islet cell extracts is much less than the activities of other potential glycolytic regulatory enzymes, such as phosphofructokinase or pyruvate kinase (29), and also is well below the capacity for islet glucose uptake, consistent with a rate-limiting role for the glucose phosphorylation step. Glucokinase exerts its full effect on regulation of glucose metabolism even in the face of coexpression of low $K_m$ hexokinase activity in the $\beta$-cell, presumably because most of the hexokinase activity is inhibited by glucose-6-phosphate or other effectors within the intact cell (29,30).

The importance of glucokinase in regulation of insulin release was underscored recently by the finding that mutations in the glucokinase gene are tightly linked to a form of NIDDM known as maturity-onset diabetes of the young (MODY) (31). In such patients, glucose-stimulated insulin secretion is impaired, apparently contributing to the relatively mild hyperglycemia that is characteristic of the syndrome (31,32). All MODY patients with glucoki-
nase mutations are heterozygous, with one normal and one mutant allele. A large number of mutations have been described that predict glucokinase enzymes with a range of activities from completely inactive to near normal (33). Whether the diabetic phenotype of patients with the various mutant alleles is fully explained by a gene dosage model (i.e., a reduction in the total glucose phosphorylation capacity) or whether some alleles may interfere with effective glucose signaling in a more direct manner remains to be determined.

Like glucokinase, the GLUT2 glucose transporter is distinct from other members of its gene family in that it has a higher \( K_m \) (lower affinity) for glucose (34,35). GLUT2 is the major glucose transporter isoform expressed in \( \beta \)-cells (34,35), and its expression is found to be sharply decreased in islets from a wide variety of rodents that lack glucose-stimulated insulin secretion and that manifest syndromes resembling human NIDDM (22). Whether GLUT2 is a passive, permissive participant in glucose-stimulated insulin release that simply ensures that changes in external glucose concentrations are transmitted to the intracellular environment for reading by glucokinase or whether, as discussed in more detail below, GLUT2 is an active participant in the signaling process remains to be established.

INSULIN-SECRETING CELL LINES

The difficulty and expense associated with islet isolation has stimulated a long-standing effort to identify more accessible model systems for the study of mechanisms of glucose-induced insulin release. Cell lines derived from \( \beta \)-cells represent an apparently logical alternative to islets for such work, and many lines have in fact been prepared from rodent insulinomas over the past fifteen years. Whereas early lines were derived from radiation or virus-induced tumors (37,38), more recent work has centered on the application of transgenic technology (39,40). A general approach taken with the latter technique is to express an oncogene, most often SV40 T-antigen, under control of the insulin promoter in transgenic animals, thereby generating \( \beta \)-cell tumors that can be used for propagating insulinoma cell lines (39,40). Whereas insulinoma lines provide an advantage in that they can be grown in essentially unlimited quantity at relatively low cost, most exhibit differences in their glucose-stimulated insulin secretory response relative to normal islets. These differences can be quite profound, such as in the case of RINm5F cells, which were derived from a radiation-induced insulinoma and which in their current form are completely lacking in any acute glucose-stimulated insulin secretion response (41,42). RIN1046–38 cells also are derived from a radiation-induced insulinoma but can be shown to be glucose responsive when studied at low passage numbers (43). This response is maximal at subphysiological glucose concentrations and is lost entirely when these cells are cultured for more than 40 passages (43).

GLUT2 and glucokinase are expressed in low passage RIN 1046–38 cells but are gradually diminished with time in culture in synchrony with the loss of glucose-stimulated insulin release (S. Ferber, H. BeltrandelRio, J.H. Johnson, R. Noel, T. Becker, L.E. Cassidy, S. Clark, S.D. Hughes, C.B. Newgard, unpublished observations). Asfari et al. (45) have isolated a new insulinoma cell line called INS-1 that retains many of the characteristics of the differentiated \( \beta \)-cell, most notably a relatively high insulin content and a glucose-stimulated insulin secretion response that occurs over the physiological range. Consistent with the finding of physiological glucose responsiveness, a recent study indicates that INS-1 cells express GLUT2 and glucokinase as their predominant glucose transporter and glucose-phosphorylating enzyme, respectively (46). INS-1 cells grow very slowly, and whether glucose responsiveness and expression of GLUT2 and glucokinase are retained with prolonged culturing of these cells remains to be determined.

Cell lines derived by transgenic expression of T-antigen in \( \beta \)-cells also exhibit variable phenotypes (39,40,47,48). Some lines have little glucose-stimulated insulin release or exhibit maximal responses at subphysiological glucose concentrations (39,40,47), whereas others respond to glucose concentrations over the physiological range (40,48). It appears that the near-normal responsiveness of the latter cell lines is not permanent, because further time in culture results in a shift in glucose dose response such that the cells secrete insulin at subphysiological glucose concentrations (48). In some cases, these changes have been correlated with expression of glucose transporters and glucose-phosphorylating enzymes. Miyazaki et al. (40) isolated two classes of clones from transgenic animals expressing an insulin-promoter/T-antigen construct. Glucose-unresponsive lines, such as MIN-7, were found to express GLUT1 rather than GLUT2 as their major glucose transporter isoform, whereas MIN-6 cells were found to express GLUT2 predominantly and to exhibit normal glucose-stimulated insulin secretion (40). Recently, Efrat et al. (48) demonstrated that their cell line \( \beta \)TC-7, which exhibits a glucose-stimulated insulin secretion response that resembles that of the islet in magnitude and concentration dependence, expressed GLUT2 and contained a glucokinase-hexokinase activity ratio similar to that of the normal islet. With time in culture, glucose-stimulated insulin release became maximal at low, subphysiological glucose concentrations. GLUT2 expression did not change with time in culture, and glucokinase activity actually increased slightly, but the major change was a large (approximately sixfold) increase in hexokinase expression (48). This enhancement in hexokinase-glucokinase ratio may increase glucose flux at low glucose concentrations, thereby explaining the enhanced sensitivity of the glucose-stimulated insulin secretion response.

A fascinating alternative to insulinoma cell lines for studies on mechanisms of insulin secretion are non-islet cell lines of neuroendocrine origin that are engineered for insulin expression. The foremost example of this is the AIT-20 cell, which is derived from ACTH-secreting cells of the anterior pituitary. A decade ago, Moore et al. (49) demonstrated that stable transfection of AIT-20 cells with a construct in which a viral promoter is used to direct
expression of the human proinsulin cDNA resulted in cell lines that secreted the correctly processed and mature insulin polypeptide. Insulin secretion from such lines (generally termed AtT-20ins) can be stimulated by agents such as forskolin or dibutyryl cAMP, with the major secreted product in the form of mature insulin, suggesting that these cells contain a regulated secretory pathway that is similar to that operative in the islet β-cell (49,50). More recently, it has become clear that the peptidases that process proinsulin to insulin in the islet β-cell, termed PC2 and PC3, also are expressed in AtT-20ins cells (51,52). AtT-20ins cells do not respond to glucose as a secretagogue (27). Interestingly, AtT-20 cells express the glucokinase gene (27,28) and at least in some lines, low levels of glucokinase activity (27,53,54), but are completely lacking in GLUT2 expression (27,54).

MOLECULAR ENGINEERING OF GLUCOSE RESPONSIVENESS IN INSULIN-SECRETING CELL LINES

The studies summarized above have provided some evidence for important roles for GLUT2 and the glucokinase:hexokinase ratio in conferring a glucose-stimulated insulin secretion response in cell lines. In recent years, our laboratory and others have attempted to move beyond correlative studies in favor of more direct manipulation of expression of the relevant gene products through techniques of gene transfer (55). Our initial work has focused on the effects of overexpression of glucose transporters in glucose unresponsive cell lines (54,56). We found that stable transfection of AtT-20ins cells with a plasmid containing the GLUT2 cDNA linked to the cytomegalovirus (CMV) promoter resulted in high levels of expression of the GLUT2 protein. Dramatic changes also resulted in the kinetics of 3-O-methyl glucose uptake, involving increases in both $K_m$ (from 2 mM in untransfected cells to 17–20 mM in GLUT2-expressing cells) and $V_{max}$ (from 0.5 mmol/liter cell space/h in untransfected cells to 18–25 mmol/liter cell space/h in GLUT2-expressing cells). AtT-20ins cells engineered for GLUT2 expression gained an acute glucose-stimulated insulin secretion response, showed an increased insulin content, and exhibited a potentiated response to the combination of glucose plus forskolin relative to untransfected cells (54,56). In contrast to the effects of GLUT2, overexpression of GLUT1 in AtT-20ins cells failed to enhance insulin release or biosynthesis, despite a 10-fold increase in the maximal velocity of 3-O-methyl glucose uptake relative to untransfected cells or cells transfected with the expression vector lacking a glucose transporter cDNA (56). The difference in secretory function between GLUT2 and GLUT1 transfected cells is highlighted in Fig. 2, in which several independent cell lines of each type were analyzed by perfusion.

These studies show that only GLUT2-transfected cells respond by secreting insulin within minutes of a change in the external glucose concentration, and just as importantly, that they rapidly reduce their insulin output when the glucose stimulus is removed. Surprisingly, overexpression of either GLUT1 or GLUT2 had little effect on the rate of glucose metabolism relative to untransfected cells or cells transfected with an empty vector, as measured by incubation of intact cells with 5-3H glucose (56). Nevertheless, glucose metabolism does appear to be critical for signaling in AtT-20ins cells, because 3-O-methyl glucose and 2-deoxyglucose fail to stimulate insulin secretion or potentiate forskolin-induced release in GLUT2-expressing lines (56). In light of all these data, our favored hypothesis for explaining the divergent effects of GLUT2 and GLUT1 on insulin release in AtT-20ins cells is that GLUT2 is uniquely capable of interacting with other cellular components to generate important secretory signals that require glucose metabolism for transmission. Potential partners for GLUT2 interaction might include GTP-binding proteins, either of the heterotrimeric or small-molecular weight classes, or metabolic enzymes complexed in a manner analogous to the metabolons that have been described for enzymes of the citric acid cycle (57). The latter construct might allow generation of metabolic signals in close proximity to activators of the insulin secretion machinery, such as K+ or Ca2+ channels. In an effort to gather experimental support for models in which GLUT2 participates in glucose sensing through physical coupling, we have prepared a number of GLUT2/GLUT1 chimeric glucose transporters that are
being tested in AtT-20ins cells for their ability to confer glucose-stimulated insulin release (L. Cassidy, C.B.N., unpublished observations). Other approaches to this issue involve overexpression of glucokinase or GTP-binding proteins in GLUT2 or GLUT1-expressing cell lines or in normal islets.

Glucose usage achieved half its maximal rate at glucose concentrations of 1–2 mM in AtT-20ins cells, regardless of which glucose transporter was expressed, suggesting that a low $K_m$ activity distal to glucose uptake is rate-limiting in AtT-20ins cells (56). A potential candidate for this low $K_m$ activity is hexokinase, because direct measurement of glucose phosphorylation in AtT-20ins cell extracts revealed that glucokinase comprises <10% of the glucose-phosphorylating activity (54). These data also may help to explain the fact that insulin secretion from AtT-20ins cells engineered for GLUT2 expression is maximal at 10–50 $\mu$M glucose, well below the threshold for responsiveness of the normal $\beta$-cell (5 mM glucose), but consistent with the $K_m$ of hexokinase (~50 $\mu$M).

More recently we have performed a series of studies on the effects of GLUT2 expression in glucose-unresponsive RIN cells. RIN 1046–38 cells of intermediate passage number (passages 30–40), in which GLUT2 expression was low or absent and glucokinase activity markedly reduced compared with low passage cells, were used for these studies. RIN cells of intermediate or high (>80 passages) passage numbers do not increase their insulin secretion in response to any concentration of glucose tested over the range from 10 $\mu$M to 20 mM. Stable transfection of intermediate but not high passage number RIN cells with the GLUT2 expression plasmid conferred a maximal 3.7-fold increase in insulin secretion in response to glucose, with the maximum achieved at 50 $\mu$M glucose. Remarkably, GLUT2 expression also induced a fourfold increase in glucokinase enzymatic activity in intermediate-passage RIN cells, but no such change in high passage cells relative to untransfected controls (S. Ferber, H. BeltrandelRio, J.H. Johnson, R. Noel, T. Becker, L.E. Cassidy, S. Clark, S.D. Hughes, C.B. Newgard, unpublished observations). These observations were consistent among four independent cell lines for each experimental group.

Even in light of these consistent results, we were concerned about the possibility that the enhanced glucokinase activity and glucose responsiveness might have been achieved by clonal selection as opposed to being a direct effect of GLUT2 expression. To address this issue, we repeated the GLUT2 gene transfer studies with recombinant adenovirus, which allows high efficiency gene transfer to cells and cell lines without the need for selection of clones with antibiotics (58,59). The general strategy used for preparation of such viruses is shown in Fig. 3. The recombinant virus is rendered replication-defective by deletion of its E1A gene, but can be propagated in human kidney 293 cells because such cells contain the E1A gene in their genome. The resultant virus is broadly infectious, but because it can only replicate in permissive host cells such as 293, it has no harmful effects on targeted cells. Incubation of RIN 1046–38 cells with recombinant adenovirus containing the $\beta$-galactosidase gene (termed AdCMV-$\beta$Gal [60]) and evaluation of expression of the gene with the X-gal chromophore 72 h later demonstrated transfer of $\beta$-galactosidase with an efficiency of nearly 100% (44). This led us to construct a recombinant adenovirus containing the GLUT2 cDNA expressed from the CMV promoter (AdCMV-GLUT2). Incubation of intermediate passage RIN cells with this virus resulted in expression of GLUT2 in all cells and an increase in the $K_m$ and $V_{max}$ for 3-O-methyl glucose uptake to values similar to those achieved in stably transfected clones. As was the case for the stable transfection experiments, adenovirus-mediated transfer of the GLUT2 gene increased glucokinase activity in intermediate, but not high passage insulinoma cells (S. Ferber, H. BeltrandelRio, J.H. Johnson, R. Noel, T. Becker, L.E. Cassidy, S. Clark, S.D. Hughes, C.B. Newgard, unpublished observations).

These studies establish that expression of GLUT2 in glucose-unresponsive RIN cells enhances glucokinase enzymatic activity, regardless of whether the GLUT2 gene is introduced stably into the cell genome or whether it is delivered via recombinant adenovirus that integrates into genomic DNA with very low frequency. The mechanism by which GLUT2 enhances glucokinase activity in these experiments is not yet resolved. Note that there is
a growing precedent for regulation of glucokinase activity in islets by mechanisms that do not involve changes in gene expression. For example, prolonged fasting of rodents is known to reduce their islet glucokinase activity by ≈50% (61,62), yet no changes in glucokinase mRNA or immunodetectable protein is observed in islets from fasted rats relative to those from ad-lib fed or refed animals (25). Furthermore, exposure of isolated islets to high concentrations of glucose (63) or glucose infusion into whole animals (64) causes increases in glucokinase enzymatic activity that are only partially accounted for by increases in glucokinase mRNA or protein. The mechanism of the GLUT2 or glucose-mediated induction of glucokinase activity remains to be elucidated.

One possible explanation is that enhanced glucose influx, induced either by elevation of the extracellular glucose concentration or by expression of GLUT2 results in altered activity of the glucokinase regulatory protein described by Van Schaftingen et al. (65). This protein is thought to inhibit glucokinase by binding directly to the enzyme and is expressed in liver and the islets of Langerhans. The regulatory protein is itself regulated by hexose phosphates, becoming a more efficient inhibitor in the presence of fructose-6-phosphate and being antagonized by fructose-1-phosphate. Interestingly, the enhancement of glucokinase expression observed in RIN cells that express GLUT2 is not observed in AT-20ins cells (54). These observations could be explained by expression of the regulatory protein in RIN but not AT-20ins cells, but this has not yet been studied. Recent cloning of the regulatory protein cDNA should facilitate this analysis (66). Determining whether GLUT2-expressing cells contain altered levels of the putative regulatory hexose phosphates also will be of interest. Possibly, GLUT2 expression and/or elevated glucose levels alter the cellular localization of glucokinase in a manner analogous to hexokinases I and II, which alternate between cytoplasmic and mitochondria-associated fractions. Hexokinases bound to mitochondria have a higher intrinsic activity, attributable to enhanced affinity for substrates and reduced allosteric inhibition by glucose-6-phosphate (24).

GLUT2-expressing intermediate-passage RIN cells exhibit a glucose-stimulated insulin secretion response that is maximal at 50–100 μM glucose. Although GLUT2 expression enhances glucokinase activity, most glucose phosphorylation in RIN cell extracts is still inhibitable by glucose-6-phosphate and is therefore likely caused by hexokinase I. We (S. Ferber, H. BeltrandelRio, J.H. John-son, R. Noel, T. Becker, L.E. Cassidy, S. Clark, S.D. Hughes, C.B. Newgard, unpublished observations, 54–56) and others (42,47,48) have postulated that an imbalance in the glucokinase:hexokinase ratio in favor of hexokinase will result in enhanced glycolytic flux at low glucose levels and consequently increased sensitivity of the glucose-stimulated insulin secretion response. This model would suggest that reducing hexokinase activity in glucose-responsive cell lines will recalibrate the glucose dose response toward that found in normal islets. We have recently gathered support for this idea, using both chemical and molecular approaches. For the former experiments, we used 2-deoxyglucose, an analogue that is transported and phosphorylated but not further metabolized. Accumulation of 2-deoxyglucose-6-phosphate in cells causes inhibition of hexokinase but has little effect on glucokinase. Incubation of GLUT2-expressing intermediate-passage RIN cells with 2-deoxyglucose before performing a secretion assay caused a shift in the glucose concentration required for maximal response from 50 μM in untreated cells to 5 mM in pretreated cells (44). More recently, we have prepared a recombinant adenovirus containing the hexokinase I cDNA in antisense orientation. Treatment of cells with this virus resulted in an ~75% reduction in the immunodetectable hexokinase I protein and a shift in the glucose dose-response curve similar to that observed with 2-deoxyglucose (H. BeltrandelRio, T. C. Becker, C.B.N., unpublished observations). Note that hexokinase inhibition by either method does not fully establish a physiological glucose dose response because normal islets only begin to respond to glucose at concentrations of ≥5 mM. Nevertheless, these early results are encouraging and provide motivation for continuing studies aimed at more complete and permanent downregulation of hexokinase in insulin secreting lines.

**POTENTIAL UTILITY OF ENGINEERED CELL LINES FOR CELL-BASED INSULIN DELIVERY IN IDDM**

With the spectacular success of the recently completed Diabetes Control and Complications Trial, the therapeutic benefits of tight control of blood glucose levels are firmly established. Current approaches to tight control involve either multiple daily insulin injections or graded infusion of the hormone via programmable pumps, both regimens requiring intensive interaction with a physician and considerable discipline on the part of the patient. Furthermore, neither approach simulates the function of normal islet β-cells, which provide insulin at the correct time and in precisely the right amount in response to metabolic and hormonal signals. Recognition of this fact has stimulated extensive work on islet and pancreas transplantation as an alternative means of insulin delivery (67–71). Years of investigation have resulted in significant improvements in the areas of islet isolation and storage, encapsulation devices for protection of implanted islets, and surgical techniques for whole pancreas transplantation. Despite these advances, whether transplanted islets can be protected from autoimmune destruction on a long-term basis is still unclear. Even if this becomes possible, the cost and difficulty associated with islet or pancreas procurement may serve to limit the applicability of the approach to a very few patients. For these reasons, engineered cells might be considered as a potential alternative to islets for cell-based insulin delivery in IDDM.

Engineered cells have potential advantages relative to isolated islets as a vehicle for insulin delivery. They can be grown at relatively low cost and in essentially unlimited numbers under pathogen-free conditions. Because they are clonal, engineered cell lines should theoretically retain highly reproducible functional features, as opposed to islets, which can be quite variable on a batch-
to-batch basis. These positive features must of course be balanced against the sizeable obstacles that remain before stable cell lines with fuel-mediated insulin secretion responses similar to those of the normal islet are available. Efforts at engineering cells with the correct threshold for glucose responsiveness have been summarized above, with emphasis on the key contributions of GLUT2 expression and proper balancing of the glucokinase:hexokinase ratio.

Permanent increases in GLUT2 and glucokinase activity should be achieved with relative ease by stable transfection, but long-term downregulation of hexokinase will likely require more challenging maneuvers, such as stable expression of antisense hexokinase or hexokinase gene knock-out by homologous recombination. Even if efforts at engineering cell lines with a physiological glucose response are successful, the resultant cell lines could be different from normal β-cells with regard to their responses to both positive effectors, such as glucagon-like peptide-1, and suppressors of secretion, such as catecholamines. Should such differences exist, whether they would be translated into altered responses to perturbations in glucose homeostasis in patients is unclear. It also will be necessary to increase the amount of insulin produced by cell lines, because most insulinoma lines or AtT-20 lines secrete only a fraction of the insulin that is produced by β-cells.

We are encouraged in this area by our recent success in overexpressing human insulin in RIN1046–38 cells, resulting in cell lines that secrete ~20 times more than the parental cells, and about half as much as freshly isolated rat islets on a per cell basis (S. Ferber, S. Clark, H. Constandy, C.B.N., unpublished observations). These cells still produce rat insulin, however, which likely must be eliminated by molecular strategies similar to those suggested for hexokinase. Furthermore, numerous studies have demonstrated that insulinoma cell lines can thrive and produce insulin in syngeneic hosts (72) or in immunocompromised animals such as the nude rat (73,74), but whether these cells can be grown and protected in permissive hosts in the context of autoimmune diabetes remains to be determined. Finally, whether cellular proteins other than insulin will escape from encapsulation devices in sufficient quantity to cause adverse immunological or biological responses is as yet unclear. This issue pertains not only to transplantation of rodent cell lines but also to transplantation of isolated islets from non-human sources such as dogs or pigs.

LESSONS FROM GENE TRANSFER EXPERIMENTS IN NORMAL ISLETS

Until recently, gene transfer into the islets of Langerhans was mainly accomplished either by relatively inefficient physical techniques, such as electroporation (75,76), or by the creation of transgenic animals in which genes were targeted to the islet β-cells (77,78) or α-cells (79), using the insulin or glucagon promoters, respectively. Some of these experiments have provided important information about the relative importance of glucose phosphorylation in dictating the magnitude and dose dependence of glucose-mediated effects on insulin expression and release. For example, German (76) has used electroporation to transfer the hexokinase I gene into fetal islets with a transfection efficiency of ~10–20% of the cells. This maneuver caused a reduction in the glucose concentration threshold for activation of a cotransfected chimeric gene consisting of the rat insulin 1 promoter linked to chloramphenicol acetyl transferase from 4–5 mM glucose in untransfected fetal islets to 1 mM in hexokinase-transfected islets. Preincubation of hexokinase-transfected islets with 2-deoxyglucose resulted in a return of the threshold for glucose stimulation to the physiological range (76). These studies provide important information about the role of glucose phosphorylation in controlling insulin promoter activity, but the physical gene transfer techniques used are not efficient enough to allow evaluation of the effects of specific genes on metabolic rate or acute insulin secretion.

In another study of interest, yeast hexokinase was expressed in β-cells of transgenic mice under control of the insulin promoter (78). Unlike mammalian hexokinases, yeast hexokinase is not inhibited by glucose-6-phosphate. Perhaps as a consequence of this difference, modest overexpression of yeast hexokinase in mouse islets caused enhanced insulin release in response to glucose, with a threshold for response that was lower than observed in normal islets. Furthermore, a cross of the hexokinase transgenic strain with an independent transgenic line that exhibits diabetes because of overexpression of calmodulin in islets resulted in normoglycemic progeny, indicating that enhancing glucose phosphorylating capacity is sufficient to repair dysfunctional islets. It is unclear from these experiments whether calmodulin altered glucose phosphorylation in diabetic animals and whether overexpression of mammalian hexokinase would have the same effect as expression of yeast hexokinase, because the mammalian enzyme that is normally present in islets is apparently in an allosterically inhibited state (29,30).

Recently, recombinant adenovirus has emerged as a system that allows gene transfer into primary cells with efficiencies much higher than those achieved by the physical transfection methods (58). Adenovirus also is distinct from other viral gene transfer systems, such as retrovirus, in that integration of viral DNA is not required for expression of transferred genes, meaning that genes can be transferred efficiently into cells with low mitotic activity, such as hepatocytes and islets. Our first experiences with recombinant adenovirus involved transfer of the muscle glycogen phosphorylase cDNA into primary hepatocytes. These studies indicated that the muscle phosphorylase gene was expressed in 86% of the cells, resulting in altered regulation of their glycogen metabolism relative to cells infected with virions lacking the muscle phosphorylase cDNA (59).

The remarkable efficiency of gene transfer with the adenovirus system in hepatocytes led us to study the utility of this method for gene transfer into the islets of Langerhans. Using the AdCMV-βGal virus, we observed gene transfer to 70% of islet cells, with expression persisting for up to 1 month in cultured islets (80). Glucose-stimulated insulin secretion was studied by se-
that recombinant adenovirus has no discernable effect on glucose-stimulated insulin release in its own right, and virus to a similar extent relative to the baseline at low glucose concentration stimulated insulin release sequentially perifusing islets with buffer containing 3, 20, and 3 mM glucose, for 15 min under each condition. The high glucose concentration stimulated insulin release from uninfected islets or islets infected with AdCMV-βGal virus to a similar extent relative to the baseline at low glucose. This result is important in that it demonstrates that recombinant adenovirus has no discernable effect on glucose-stimulated insulin release in its own right, and is therefore a useful vehicle for gene transfer in these experiments. These results have encouraged us to construct a number of viruses containing the cDNAs encoding various glucose phosphorylating enzymes, including the islet and liver isoforms of glucokinase (27,53) and hexokinase I (81). Early results with these viruses indicate that they all cause sharp enhancements in glucose phosphorylation capacity, but that they induce divergent effects on glucose-stimulated insulin secretion (T.C. Becker, R.J. Noel, J.H. Johnson, C.B. Newgard, unpublished observations). Islets overexpressing the liver or islet isoforms of glucokinase exhibit enhanced glucose responsiveness relative to uninfected or AdCMV-βGal-infected islets, but the effect is clearly more pronounced for islet glucokinase. Hexokinase I overexpression, in contrast, has no effect on the magnitude of the response to high glucose relative to the control groups, but instead increases insulin output at basal glucose concentrations (3 mM). Basal hyperinsulinemia is a component of β-cell dysfunction in rodent models of obesity and NIDDM (82). The fact that hexokinase overexpression in islets can recapitulate this phenotype suggests a potential involvement of the enzyme in the β-cell abnormality of the animal models.

From a more practical perspective, these data provide support for the idea that enhancement of islet glucokinase activity may have therapeutic benefit if strategies for specific targeting of the gene to the islets in vivo can be developed. They also suggest that it may be possible to enhance the performance of islets destined for transplantation by ex vivo genetic manipulation. The GLUT2 gene would be another candidate for in vivo gene delivery experiments, given that its expression has been shown to be sharply downregulated in a large array of rodent models of NIDDM with compromised islet function (22). Previous studies involving systemic delivery of recombinant adenovirus have demonstrated highly efficient delivery of the cystic fibrosis (83) and LDL receptor (60) genes to lung and liver of intact animals, respectively. Preliminary experiments in our laboratory with the AdCMV-βGal virus have demonstrated that the islets of Langerhans are a preferential site for adenovirus-mediated gene delivery within the pancreas (W. Coats, T. Alam, R. Meidell, R. Gerard, C.B.N.; unpublished observations) probably because of their intense, fenestrated vasculature (84).

Gene transfer efficiencies of up to 20% have been observed in these experiments, but further work will be required to evaluate the consistency and reproducibility of this approach. Note that recombinant adenoviruses in their current form are unlikely to represent the ultimate vehicle for human gene therapy, given their poor efficiency of integration and short life span as episomal DNA within the cell (85). Other related or derivative systems that are currently under investigation include adenoviruses with genomic segments other than E1A deleted to reduce immunogenicity and enhance duration of expression, and adenovirus, a virus that appears to integrate stably and with reasonable efficiency at a specific site in human chromosome 19 (86). Other non-viral approaches may also emerge, an example being the recent demonstration of effective gene delivery to tissues via infusion of DNA/cationic liposome complexes (87). Although these approaches must all be rigorously tested before they can be applied to human subjects, they provide, for the moment, exciting new tools for experimentation in the area of islet function and fuel-mediated insulin release.

GENERAL CONCLUSIONS
Significant progress has been made in recent years in the area of cellular engineering for the purpose of studying the molecular basis of fuel-mediated insulin secretion. There is reason to hope that engineered cell lines that closely mimic the glucose-stimulated insulin secretion response of normal islets will eventually emerge. The therapeutic relevance of such lines will likely be dictated by a number of factors, including the stability of their phenotype over time in the in vivo environment, and whether the presence of the encapsulated cells causes deleterious immune responses. With the advent of new technologies such as the adenovirus system, one can envision the extrapolation of the approaches outlined for engineering of cell lines in culture to the level of gene therapy for syndromes of β-cell dysfunction. Many challenges remain, but the potential therapeutic implications of these approaches cannot be ignored.

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