Recent Advances in the Okamoto Model

The CD38-Cyclic ADP-Ribose Signal System and the Regenerating Gene Protein (Reg)-Reg Receptor System in β-Cells

Hiroshi Okamoto and Shin Takasawa

Twenty years ago, we first proposed our hypothesis on β-cell damage and its prevention (the Okamoto model), according to which poly(ADP-ribose) synthetase/polymerase (PARP) activation is critically involved in the consumption of NAD\(^+\), leading to energy depletion and cell death by necrosis. Recently, the model was reconfirmed by results using PARP knockout mice and has been recognized as providing the basis for necrotic death of various cells and tissues. Based on the model, we proposed two signal systems in β-cells: one is the CD38-cyclic ADP-ribose (cADPR) signal system for insulin secretion, and the other is the regenerating gene protein (Reg)-Reg receptor system for β-cell regeneration. The physiological and pathological significance of the two signal systems in a variety of cells and tissues as well as in pancreatic β-cells has recently been recognized. Here, we describe the Okamoto model and its descendents, the CD38-cADPR signal system and the Reg-Reg receptor system, focusing on recent advances and how their significance came to light. Because PARP is involved in Reg gene transcription to induce β-cell regeneration, and the PARP activation reduces the cellular NAD\(^+\) to decrease the formation of cADPR (a second messenger for insulin secretion) and further to cause necrotic β-cell death, PARP and its inhibitors have key roles in the induction of β-cell regeneration, the maintenance of insulin secretion, and the prevention of β-cell death. Diabetes 51 (Suppl. 3):S462–S473, 2002

The Okamoto model, a unifying model for β-cell damage and its prevention, was based on results obtained in streptozotocin- and alloxan-induced diabetes in rodents (1–8). After 18 years of controversy, irrefutable support for the model was provided by experiments using poly(ADP-ribose) polymerase (PARP) knockout mice (9–12). The model has since become accepted as valid for necrotic β-cell death. Recently, three major progresses were made on the Okamoto model. In this article, we first describe the significance of the Okamoto model as a model of necrotic death in a variety of cells and tissues as well as in β-cells. Next, we describe the physiological and pathological roles of the CD38-cyclic ADP-ribose (cADPR) signal system in insulin secretion and the regenerating gene protein (Reg)-Reg receptor system for β-cell regeneration, the elucidations for both of which were developed on the basis of the Okamoto model.

THE OKAMOTO MODEL

The Okamoto model for β-cell damage and its prevention. The removal of the insulin-producing pancreatic β-cells from an animal induces a syndrome that features many of the acute metabolic derangements of diabetes as they are known in humans. von Mering and Minkowski in 1890 were the first to produce this form of diabetes by surgically removing the pancreata of dogs. Alloxan in 1943 and streptozotocin in 1963 were found to be highly selective β-cytoxins in animals and to be extremely potent diabetogenic substances. Since then, alloxan and streptozotocin have been widely used to produce diabetes in experimental animals. Because alloxan and streptozotocin are structurally different, the two substances were commonly thought not to act in an identical way. In 1981, based on in vitro (13) and in vivo experiments (14), we hypothesized that the action of diabetogenic agents, streptozotocin and alloxan, on pancreatic β-cells would result from the induction of DNA strand breaks and PARP (1). These breaks in the nuclear DNA of β-cells would result from either the accumulation of free radicals or from the alklylation of DNA. PARP acts to repair the DNA breaks, consuming β-cell NAD\(^+\) as a substrate. Consequently, there would be a sharp drop in the intracellular levels of NAD\(^+\). The cellular NAD\(^+\) reduction would severely im-
pair such cellular functions as insulin synthesis and secretion and cause lethal injury to the β-cells. Therefore, in diabetes induction, pancreatic β-cells seem to be making a "suicide response" in their attempt to repair the damaged DNA. This NAD⁺ depletion and β-cell functional impairment were dose-dependently blocked by the radical scavengers superoxide dismutase and catalase and by PARP inhibitors such as nicotinamide (15,16). Recently, three independent groups in Germany, Japan, and the U.S. provided irrefutable support using PARP-deficient mice for the model shown in Fig. 1. The mice were extremely resistant to streptozotocin, and the β-cell death was prevented (9–12).

This model for the mechanism of action of alloxan and streptozotocin has received much attention because of its possible relevance to the effects of viruses and inflammation, especially those related to inflammatory damage of β-cells (1–8) because inflammation, virus infection, radiation, and chemical insult may independently or interactively produce β-cell DNA strand breaks. Therefore, although type 1 diabetes can result from immunological abnormalities, inflammatory tissue damage, and β-cytotoxic chemical substances, the final pathway is the same (Fig. 1A), involving DNA damage, PARP activation, and NAD⁺ depletion. Therefore, it may be possible to prevent type 1 diabetes by blocking immune reactions, scavenging free radicals, and inhibiting PARP. Concerning nitric oxide (see Fig. 1A), we found that in transgenic mice expressing nitric oxide synthase constitutively in pancreatic β-cells, the β-cell mass was markedly reduced, resulting in the development of severe diabetes (17). Nitric oxide synthase is usually induced by proinflammatory/anti-inflammatory cytokines such as interleukin (IL)-1β, γ-interferon, and tumor necrosis factor-α. Some experiments using cytokines as cytotoxic agents showed that nicotinamide did not effectively inhibit human β-cell death (18,19). However, there are two kinds of cell death: necrosis and apoptosis. PARP activation would result in necrotic cell death (3,4,7,8,20), whereas in apoptotic cell death, PARP is cleaved by caspases and inactivated. Therefore, PARP inhibitors can prevent necrosis but not apoptosis. The type of cell death, whether necrosis or apoptosis, would depend on the severity and duration of the insult, differences in signaling pathways, and the species of the cells. It has recently been suggested that dendritic cells/macrophages can distinguish between the two types of cell death and that necrosis may provide the critical control for the initiation of immunity (21,22). Upon recognition of necrotic cells, the proinflammatory responses of activated macrophages are increased. In contrast, apoptotic cells have a strong inhibitory effect on phlogistic macrophage responses. Thus, as shown in Fig. 1, the apoptotic β-cell

**FIG. 1.** The Okamoto model for necrotic cell death. The Okamoto model, originally proposed as a unifying model for β-cell damage and its prevention, well explains both how autoimmunity for β-cell necrosis is initiated and how necrotic cell death is involved in various diseases in many tissues other than β-cells. Under physiological conditions, apoptotic cell death constitutively occurs for renewal and maintenance in animal bodies, whereas necrotic cells initiate and enhance (auto)immune reactions under pathological conditions. On the other hand, apoptotic cells recognized by macrophages/dendritic cells inhibit phlogistic (auto)immune responses. For the initiation of massive and pathological apoptotic cell death, necrotic cell death, which triggers autoimmune responses in macrophages/dendritic cells, is required (A). The Okamoto model as the mechanism of necrotic cell death in many tissues and cells in various diseases (B).

### A. The Okamoto model for β-cell damage

- **Radiation**
- **Inflammation**
- **Immune Reactions**
- **NAD⁺ Depletion**
- **Neurodegenerative**

### B. Diseases and References

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<td>Diabetic endotheial dysfunction</td>
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death that is often observed in type 1 diabetes may be triggered by the immune reactions caused by the preceding necrotic cell death.

**The Okamoto model in various diseases.** PARP is one of the best-known proteins with DNA damage scanning activity, and poly(ADP-ribosyl)ation by PARP has been proposed to function in DNA repair by modifying architectural proteins proximal to DNA breaks, thus facilitating the opening of the condensed chromatin structure required for the recruitment of the repairing complex (23). Paradoxically, despite this beneficial effect, PARP can induce necrotic cell death through NAD$^+$ depletion, as described above. Despite the apparent Mr. Hyde–like character of this enzyme, it may represent an evolutionary strategy adopted by multicellular organisms to prevent the survival of cells that would otherwise transmit potentially dangerous genetic material. Recently, many other tissues and cells (such as those involved in cerebral ischemia, myocardial ischemia, laryngeal injury, intestinal mucosal injury, organ injury by hemorrhagic shock, ischemic renal injury, and diabetic myocardial and endothelial injury) have been reported to die by the same mechanism as that in pancreatic β-cell death (Fig. 1B). Accordingly, the inhibition of PARP activity may be a possible therapeutic approach in a wide number of disorders other than diabetes.

**THE CD38-cADPR SIGNAL SYSTEM**

The CD38-cADPR signal system for insulin secretion by glucose in β-cells. Glucose increases the intracellular Ca$^{2+}$ concentration in pancreatic β-cells to cause the secretion of insulin. Ashcroft et al. (24) proposed in 1984 that this increase in the Ca$^{2+}$ concentration is provided extracellularly. That is, in the process of glucose metabolism, the millimolar concentrations of ATP produced intracellularly. Thus, in 1993, we proposed that insulin secretion by glucose occurs via cADPR-mediated Ca$^{2+}$ mobilization from an intracellular Ca$^{2+}$ pool, the endoplasmic reticulum (26), as shown in Fig. 2A. That is, because ATP inhibits the cADPR hydrolase of CD38, there is an accumulation of cADPR, which acts as a second messenger for Ca$^{2+}$ mobilization from the endoplasmic reticulum for insulin secretion. Because it was first necessary to determine whether the accumulation of cADPR is actually caused by glucose stimulation in pancreatic islets, we incubated normal rat (Wistar) and mouse (C57BL/6J) islets with low (2.8 mmol/l) and high (20 mmol/l) glucose and assayed the cADPR content in the islets by radioimmunoassay using an anti-cADPR antibody (Fig. 2B). Incubation with high glucose caused the cADPR content of islets to increase within 5 min, whereas the cADPR content of islets incubated with low glucose did not increase (27).

Some reports did not fully support the view that the insulinotropic action of glucose involves major changes in the islet cell content of cADPR (28,29). As described previously (5,27), differences in experimental conditions, such as fasting of experimental animals, differences in glucose concentrations in islet isolation procedures, and the normalization of cADPR recovery using labeled cADPR as an internal standard, may be responsible for the different results. The increase in the cADPR concentration in response to high glucose was recently confirmed using mouse (BALB/c) islets (30). Next, in pancreatic islet microsomes used as a cell-free system to study Ca$^{2+}$ release, we found that cADPR released Ca$^{2+}$ from islet microsomes, as indicated by the prompt increase in fluo-3 fluorescence (26,27) (see Fig. 3A). The Ca$^{2+}$ release by cADPR was completely inhibited by 8-amino-cADPR, an antagonist of cADPR. However, inositol 1,4,5-trisphosphate (IP$_3$) did not cause the release of Ca$^{2+}$ from islet microsomes, although the islet microsomes remained responsive to cADPR. In rat cerebellum microsomes, both IP$_3$ and cADPR caused the release of Ca$^{2+}$. Although heparin, an inhibitor of IP$_3$ binding to its receptor, blocked the IP$_3$-induced Ca$^{2+}$ release from cerebellum microsomes, it did not block the cADPR-induced Ca$^{2+}$ release, indicating that islet microsomes respond to cADPR but not to IP$_3$. Although cerebellum microsomes respond to both cADPR and IP$_3$, cADPR induces Ca$^{2+}$ release via a different mechanism than that utilized by IP$_3$. When we examined the effect of cADPR on insulin secretion using digitonin-permeabilized pancreatic islets (Fig. 2C), cADPR as well as Ca$^{2+}$ induced insulin secretion, but IP$_3$ did not. The combination of cADPR and Ca$^{2+}$ did not induce significantly more insulin secretion than the addition of cADPR or Ca$^{2+}$ alone, and the cADPR-induced insulin secretion was inhibited by the addition of EGTA. The cADPR-induced insulin secretion therefore appears to be mediated by Ca$^{2+}$ mobilization from islet microsomes (26). Dose-response relationships between cADPR and insulin secretion from permeabilized islets were well fitted with those between cADPR and Ca$^{2+}$ release from islet microsomes. Thus, we concluded that glucose stimuli induce cADPR formation from NAD$^+$ and that cADPR then mobilizes Ca$^{2+}$ from the endoplasmic reticulum, serving as a second messenger for insulin secretion.

The next issue concerns the mechanism by which the glucose stimulus induces the formation of cADPR. We found that CD38, a 300–amino acid protein first recognized as a leukocyte antigen, is expressed in a variety of tissues, including pancreatic β-cells (31,32). We and others have found that CD38 has both ADP-ribosyl cyclase (synthesizing cADPR from NAD$^+$) and cADPR hydrolase to produce ADP (31–33). We expressed human CD38 cDNA in Escherichia coli, purified the human CD38 protein, and found that millimolar concentrations of ATP, produced in the process of glucose metabolism, inhibit the cADPR hydrolase activity of CD38, competing with the substrate cADPR (31,34). Based on the competitive inhibition of the cADPR hydrolisis by ATP, cADPR and ATP appear to bind to the same site of CD38. By labeling the purified CD38 with an ATP analog, 5'-fluorouridylbenzoyladenosine (FSBA), we identified the binding site for ATP and/or cADPR as the lysine-129 of CD38 (34). We introduced
cADPR in the extraction and concentration procedures and expressed as femtomoles per microgram of islet protein. The cADPR content was calculated by accounting for the recovery of cADPR by Cam kinase II.

RyR by Cam kinase II.

FKBP12.6 from RyR. Cam kinase II phosphorylates RyR to sensitize and activate the Ca\(^{2+}\) channel. Ca\(^{2+}\), released from intracellular stores and/or supplied from extracellular sources, further activates Cam kinase II and amplifies the process. In this way, Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) can be explained. The conventional insulin secretion mechanism by Ca\(^{2+}\) influx from extracellular sources is shown in black. Pi, phosphorylation of RyR by Cam kinase II.

The statistical analysis was performed using Student’s t test. *Significant difference from the value with 2.8 mmol/l glucose at P < 0.05. C: Effect of cADPR on insulin secretion from digitonin-permeabilized islets (adapted from 26). Values represent the means ± SE of n = 5–14 measurements. D: Glucose-induced intracellular concentration of Ca\(^{2+}\)\([\text{Ca}\text{\textsubscript{2+}}]\) changes in CD38\(−/−\) and CD38\(+/+\) islets (adapted from 41). A representative digital imaging of [Ca\textsuperscript{2+}] in the islets (D) and changes of the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in response to glucose stimulation (E and F) are shown. HG, high glucose; LG, low glucose. Islets were loaded with fura-2 by a 30-min incubation at 37°C in Krebs-Ringer buffer containing 0.2% BSA, 2.8 mmol/l glucose, and 25 mmol/l acetoxy methyl ester of fura-2. Islets were perfused with Krebs-Ringer buffer containing 2.8 mmol/l (low glucose) or 20 mmol/l (high glucose) at 37°C at a rate of 2.5 ml/min. Fura-2 dual excitation (340 and 380 nm) and fluorescence detection (510 nm) were accomplished, and the 340- to 380-nm fluorescence ratio was converted to [Ca\textsuperscript{2+}] using QuantiCell 900. The interval between successive images of recordings was 4 s. G: Insulin secretion from isolated islets under various glucose concentrations. n = 5–12 for each point.

Site-directed mutations to change the Lys-129 to Ala and Arg. Neither mutant was labeled with FSBA or catalyzed the hydrolysis of cADPR to ADPR. Furthermore, the mutants did not bind cADPR, whereas they still used NAD\(^{+}\) as a substrate to form some cADPR (34). These results indicate that Lys-129 of CD38 participates in cADPR binding, and ATP, produced in the process of glucose metabolism, competes with cADPR for the binding site, resulting in both the inhibition of the cADPR hydrolase activity of CD38 and the accumulation of cADPR in \(\beta\)-cells.

cADPR is generally believed to activate a ryanodine receptor to release Ca\(^{2+}\) from the intracellular stores, the endoplasmic reticulum (26,35). We have reported that the type 2 ryanodine receptor (RyR-2) is expressed in rat pancreatic islets (27). Our experiments indicated that cADPR did not bind directly to the ryanodine receptor but acted on the receptor through a mediator such as FK506 binding protein 12.6 (FKBP12.6) to release Ca\(^{2+}\). The cellular target for FK506, one of the most widely used immunosuppressive agents, is thought to be FKBP12 and FKBP12.6. Rat FKBP12 is composed of 108 amino acids and is highly conserved among human, mouse, bovine, and rabbit FKBP12. Rat FKBP12 is also a 108–amino acid protein, as are human and bovine FKBP12.6. We isolated microsomes from rat islets, carried out immunoblot analyses, and found that rat islet microsomes contained FKBP12.6 but did not contain FKBP12. Interestingly, cADPR was found to bind to FKBP12.6 at a dissociation constant \(K_d\) value of 35 mmol/l. The cADPR-binding was inhibited by FK506, and neither structurally nor functionally related analogs of cADPR inhibited the cADPR binding to FKBP12.6 (36). These results not only indicate that FKBP12.6 acts as a cADPR binding protein, but they also strongly suggest that cADPR is the actual ligand for FKBP12.6 because FK506 does not normally exist in mammalian cells. As mentioned above, FKBP12.6 occurs in rat islet microsomes. When rat islet microsomes were treated with cADPR, FKBP12.6 dissociated from the microsomes and moved to the supernatant, releasing Ca\(^{2+}\).
from the intracellular stores. The microsomes that were treated with FK506 or cADPR and were then devoid of FKBP12.6 did not show Ca\[^{2+}\]/H\[^{11001}\] release by cADPR. These results and those from other experiments strongly suggest that when cADPR binds to FKBP12.6 in the ryanodine receptor and causes the dissociation of FKBP12.6 from the ryanodine receptor to form the FKBP12.6-cADPR complex, the ryanodine receptor channel activity is thereby increased to release Ca\[^{2+}\]/H\[^{11001}\] from the endoplasmic reticulum (36). When FK506 is present, cADPR cannot act on the ryanodine receptor to release Ca\[^{2+}\], and the glucose-induced insulin secreting machinery ceases to function.

Recently, the role of FKBP12.6 in cADPR-induced activation of the ryanodine receptor described above was confirmed by the study of Tang et al. (37) using bovine arterial smooth muscle. In humans, when FK506 was used as an immunosuppressant in kidney transplantation, hypoglycemia was observed in 20–35% of the recipients (38). The diabetogenic side effect of FK506 may be explained by the mechanism shown in Fig. 2A. It should also be noted that in the presence of calmodulin (CaM), islet microsomes became sensitized to cADPR at much lower concentrations for Ca\[^{2+}\] release, and the Ca\[^{2+}\] release was greatly increased (8,39). Inhibitors for CaM-dependent protein kinase II (CaM kinase II) completely abolished the glucose-induced insulin secretion as well as the cADPR-mediated and CaM-activated Ca\[^{2+}\] mobilization. Western blot analysis revealed that rat microsomes contain CaM kinase II but do not contain CaM. When the active 30-kDa chymotryptic fragment of CaM kinase II was added to the microsomes, fully activated cADPR-mediated Ca\[^{2+}\] release was observed in the absence of CaM (8,39). These results strongly suggest that CaM kinase II is required to phosphorylate and activate the ryanodine receptor for the cADPR-mediated Ca\[^{2+}\] release. We also found that cyclic AMP-dependent protein kinase (A-kinase) activates the cADPR-mediated Ca\[^{2+}\] release from islet microsomes. In the absence of A-kinase, only a small amount of Ca\[^{2+}\] was released from the microsomes by low concentrations of cADPR. On the other hand, when the catalytic subunit of A-kinase was added to the islet microsomes, the Ca\[^{2+}\] release was sensitized at lower concentrations of cADPR (8). Because CaM kinase II was observed to be activated by glucose stimulation and several incretin peptide hormones (such as VIP, PACAP, GLP-1, and GIP) increase the intracellular concentrations of cyclic AMP to activate A-kinase, it is quite possible that the cADPR-mediated Ca\[^{2+}\] mobilization for insulin secretion is activated by CaM kinase II and A-kinase. Possibly, the activated kinases phosphorylate the ryanodine receptor to sensitize the Ca\[^{2+}\] channel for the cADPR signal.

Toward verifying our model of insulin secretion, the CD38-cADPR signal system, we created CD38 transgenic and knockout mice (40,41). We produced transgenic mice overexpressing human CD38 in pancreatic \(\beta\)-cells using a rat insulin promoter (40). In immunohistochemistry, pancreatic islets of the two transgenic lines were densely stained for human CD38. We isolated islets from the transgenic mouse pancreas. The insulin secretion in transgenic islets was significantly higher than that of the control
insulin secretion by glucose. When islets were exposed to ketoisocaproate (KIC), which, like glucose, is metabolized to form ATP, the transgenic insulin secretion was significantly higher than that of the controls. However, with tolbutamide and KCl, the transgenic insulin secretion was not altered as compared with the control secretion. Tolbutamide blocks the ATP-sensitive $K^+$ channel and facilitates $Ca^{2+}$ influx from extracellular sources through voltage-dependent $Ca^{2+}$ channels, and KCl directly induces cell membrane depolarization, resulting in $Ca^{2+}$ influx. In glucose tolerance tests, the serum insulin level of CD38 transgenic mice was higher than that of control mice.

In addition, we created CD38 gene–disrupted mice (41). The pancreatic islets of CD38 knockout mice showed almost no ADP-ribosyl cyclase activity. The glucose-induced increase in the intracellular $Ca^{2+}$ concentration was severely impaired in the knockout mouse islets, and the glucose-induced insulin secretion was severely decreased (Fig. 2D–G). CD38–/– mice showed impaired glucose tolerance, and the serum insulin level was lower than in controls. We further tested whether the observed phenotype could be rescued by the pancreatic β-cell–specific expression of CD38 cDNA. Transgenic mice carrying a human CD38 CDNA under the rat insulin promoter described above were crossed with CD38–/– mice. By intercrossing their offspring, CD38+/- mice carrying the human CD38 transgene were generated. The human CD38 transgene ameliorated the glucose intolerance and the decreased insulin secretion. CD38–/– mice did not show insulin resistance, suggesting that the observed phenotype is indeed caused by the absence of CD38 in pancreatic β-cells (35). The knockout islets, however, responded normally to the extracellular $Ca^{2+}$ influx stimulants tolbutamide and KCl to secrete insulin (41). Thus, as shown in Fig. 2A, the CD38-cADPR signal system as well as the ATP-sensitive $K^+$ channel system appear to function in insulin secretion by glucose.

This paradigm of insulin secretion based on the CD38-cADPR signal system is supported by a wide body of evidence obtained in rat and mouse, and recent results indicate that the CD38-cADPR signal system also functions in insulin secretion in humans. As a result of a missense mutation (Arg140Trp) found in the CD38 gene in Japanese diabetic patients, the CD38 protein showed altered catalytic activities and a decreased production of cADPR (42). The decreased function of the CD38 mutant may have contributed to the impairment of glucose-stimulated insulin secretion in type 2 diabetic patients. It is also significant that circulating anti-CD38 autoantibodies were detected in 10–14% of Japanese (43) as well as Caucasian diabetic patients (44–46). The autoantibody altered the in vitro enzymatic activity of islet CD38 and insulin secretion. These findings provide further support for the concept that the CD38-cADPR signal system functions in insulin secretion in humans.

**Intracellular $Ca^{2+}$ mobilization for insulin secretion.** The CD38-cADPR signal system for insulin secretion is different from the hypothesis of Ashcroft et al. (24), in which $Ca^{2+}$ influx from extracellular sources is involved in insulin secretion by glucose. In the signal system proposed by Berridge and Irvine (47), $IP_3$ induces $Ca^{2+}$ release from the intracellular pool, the endoplasmic reticulum. Accordingly, the CD38-cADPR signal system we proposed was the focus of intense debate (5,48–51). Discrepant results were reported in diabetic β-cells such as ob/ob mouse islets and RINm5F cells, which are often used for studying insulin secretion. However, the $Ca^{2+}$ release responses of these diabetic β-cell microsomes differed greatly from those of normal islet microsomes (27). As shown in Fig. 3A, microsomes from normal C57BL mouse islets released $Ca^{2+}$ in response to cADPR but scarcely in response to $IP_3$. This response to cADPR was completely attenuated by the prior addition of 8-amino-cADPR (Fig. 3A, inset). In ob/ob mouse islet microsomes, however, only a small amount of $Ca^{2+}$ was released by cADPR, but much $Ca^{2+}$ was released by $IP_3$. RINm5F cell microsomes also responded well to $IP_3$ to release $Ca^{2+}$ but did not respond to cADPR (Fig. 3A). However, RINm5F cells are rat insulinoma–derived immortal cells that show almost no glucose-induced insulin-secreting ability.

Concerning intracellular $Ca^{2+}$ release channels, the mRNA expression of RyR-2, which is postulated to be a $Ca^{2+}$ release channel for cADPR (27,36), was clearly detected in normal islets but not in ob/ob islets (Fig. 3B). In contrast, $IP_3$ receptor (IP$_3$R)-1, -2, -4, and -5 mRNAs were not detected in normal islets but were clearly detected in ob/ob islets, and although IP$_3$R-3 mRNA was slightly detected in normal islets, the mRNA was significantly increased in ob/ob islets, fitting well with the observation that $IP_3$-induced $Ca^{2+}$ mobilization preferentially worked in ob/ob islet microsomes. More importantly, as shown in Fig. 3C, the CD38 mRNA level was significantly decreased in ob/ob islets (27), and CD38 mRNA was not expressed in RINm5F cells (32). The decrease of CD38 mRNA in ob/ob islets may explain the low response in the cADPR content of β-cells by glucose stimulation (27) because CD38 has both the ADP-ribosyl cyclase and the cADPR hydrolase activity, and the increase of cADPR by glucose stimulation is achieved by inhibition of the cADPR hydrolase activity of CD38. Decreased CD38 mRNA was also reported in islets of Goto-Kakizaki (GK) diabetic rats (52), which show impaired glucose-induced insulin secretion. These results show that the CD38-cADPR signal system for insulin secretion acts under normal physiological conditions, but the $IP_3$ system becomes dominant in diabetic β-cells such as ob/ob mouse islets and RINm5F cells. In fact, it was more recently confirmed by Rutter and colleagues (35,53) using aequorin chimera that MIN6 cells show a dramatic $Ca^{2+}$ mobilization in response to cADPR via the ryside receptor, even though no response to $IP_3$ was observed (35). Moreover, An et al. (30) demonstrated that BALB/c mouse islets exhibited distinct increases in intracellular cADPR, $Ca^{2+}$, and insulin secretion by glucose stimulation.

**The CD38-cADPR signal system in other tissues and cells.** Although $IP_3$ had been thought to be a second messenger for $Ca^{2+}$ mobilization from intracellular stores, cADPR induces $Ca^{2+}$ release from pancreatic islet microsomes, but $IP_3$ does not, as described above. In cerebellum microsomes, $Ca^{2+}$ release is induced by both cADPR and $IP_3$. It is therefore apparent that cells can utilize two second messengers, $IP_3$ and cADPR, for $Ca^{2+}$ mobilization, depending on the species of cells as well as differences in...
cellular conditions, physiological or pathological, performing a variety of cellular functions (Fig. 3D). Recently, various physiological phenomena in both animal and plant cells have been found to utilize this novel signal system (7,8). In pancreatic acinar cells of CD38-knockout mice, the acetylcholine-induced Ca\(^{2+}\) oscillation was greatly reduced or completely disappeared under a physiological concentration of acetylcholine (54). Furthermore, acetylcholine induced cADPR formation in normal acinar cells but not in CD38 knockout acinar cells. The IP\(_3\) formation was very small in the presence of a physiological concentration of acetylcholine and showed no difference between normal and CD38 knockout cells. It appears likely that acetylcholine induces the cADPR formation via the G-protein-coupled CD38 system (55). In pancreatic \(\beta\)-cells, glucose is metabolized and induces the CD38-cADPR signal system to secrete insulin. In other cells, hormones and neurotransmitters may regulate the CD38-cADPR signal system in a receptor-coupled manner, such as in a G-protein-coupled manner, for various other types of physiological responses (8).

Recently, the possible involvement of the CD38-cADPR signal system in cardiovascular abnormalities was reported: myocardial hypertrophy was observed in male mice with null mutations of CD38, and FKBP12.6 (a cADPR binding protein) (56,57) and the altered stoichiometry of FKBP12.6 versus RyR-2 was reported as a cause of abnormal Ca\(^{2+}\) leaks through the ryanodine receptor in heart failure in humans (58). As diabetic complications in the cardiovascular system are frequently observed, screening of the abnormalities in the CD38-cADPR signal system may provide us a clue of the underlying molecular mechanism.

THE REG-REG RECEPTOR SYSTEM

\(\beta\)-Cell regeneration by PARP inhibitors. According to the Okamoto model, streptozotocin- and alloxan-induced diabetes can be prevented by PARP inhibitors. At the end of the 19th century, von Mering and Minkowski found that a dog became glycosuric and hyperglycemic after pancreaticectomy. From this observation there followed attempts to isolate the active pancreatic principle toward finding a possible treatment for diabetes. In 1984, we demonstrated that surgical diabetes could be ameliorated by using PARP inhibitors to induce the regeneration of pancreatic \(\beta\)-cells (59). Male Wistar rats were 90% depancreatized, and nicotinamide or 3-aminobenzamide was injected intraperitoneally every day. The administration of PARP inhibitors ameliorated the surgical diabetes, the islets in the remaining pancrea of rats that had received PARP inhibitors for 3 months were extremely large, and the areas of the enlarged islets were almost entirely stained for insulin.

Isolation of Reg (regenerating gene) and its function for \(\beta\)-cell regeneration. We isolated the regenerating islets and constructed a cDNA library. By differential screening of the regenerating islet-derived cDNA library, we found a novel gene expressed in regenerating islets. The cDNA had one large open reading frame encoding a 165-amino acid protein, and the deduced protein has a signal sequence. We named the novel gene Reg, that is, regenerating gene, with the implication that the gene may be involved in islet regeneration (60). We then isolated the human REG gene (60,61). Rat Reg protein increased \[^{3}H\]thymidine incorporation in rat islets, and mitosis was often observed (62). Reg protein (1 mg \cdot kg\(^{-1}\) \cdot day\(^{-1}\)) was injected intraperitoneally in 90% depancreatized rats. On the 30th and 60th postoperative day, the fasting plasma glucose level of the rats that had received Reg protein was significantly lower than that of the 90% depancreatized control rats. After 2 months, almost all of the islets of the 90% depancreatized control rats were destroyed, whereas the islets of the remaining pancrea in the Reg protein-treated rats were enlarged and almost entirely stained for insulin (62) (Fig. 4A–C). Human REG protein administration also ameliorated diabetes in NOD mice, with an increase in the \(\beta\)-cell mass (63). These results indicate that Reg protein stimulates the regeneration and/or growth of pancreatic \(\beta\)-cells, thereby ameliorating animal diabetes. This was confirmed by our recent observations using Reg transgenic and knockout mice (see 64 in the same issue).

We produced transgenic mice expressing Reg in \(\beta\)-cells, and the islets from the transgenic mice showed increased \[^{3}H\]thymidine incorporation. By intercrossing, we produced NOD mice carrying the Reg transgene and found that the development of diabetes in the resultant Reg transgenic NOD mice was significantly retarded. We then produced Reg knockout mice by homologous recombination and found that the \[^{3}H\]thymidine incorporation in the islets was decreased. When hyperplastic islets were induced by the injection of goldthioglucone, the islet sizes of Reg knockout mice were significantly smaller than those from control Reg\(^{+/–}\) mice.

Reg receptor. We isolated the cDNA of a Reg protein receptor from a rat islet cDNA expression library (65). The cDNA encoded a 919-amino acid protein, and based on the amino acid sequence, the protein appears to be a type II transmembrane protein with a long extracellular domain. We also isolated a human cDNA that shows \(>97\%\) amino acid identity to the rat homologue. Surprisingly, a homology search against DNA/protein databases revealed that the rat cDNA and its deduced amino acid sequences show significant homologies to those of multiple exostoses (EXT) family genes, especially to human EXT-like gene 3 (EXTL3)/EXT-related gene 1 (EXTR1). The EXTL3/EXTR1 gene was isolated as a member of EXT family genes by homology screening. The human cDNA sequence is identical to that of EXTL3/EXTR1, indicating that EXTL3/EXTR1 is human REG receptor (65). The rat Reg receptor–expressing CHO cells bound rat Reg protein with high affinity (\(K_d = 4.4\) nmol/l). The binding of \(^{125}\)I-labeled rat Reg protein was displaced by increasing the concentration of unlabeled rat Reg protein. Human REG protein also bound to the CHO cells (\(K_d = 14.0\) nmol/l), but higher concentrations of human REG protein were required for the displacement of the rat Reg protein. We then established several cell lines of RINm5F cells overexpressing the Reg receptor. The cell lines showed significant increases in 5-bromo-2′-deoxyuridine (BrdU) incorporation in the presence of 0.3–100 nmol/l rat Reg protein, and the cell numbers were increased in response to Reg protein. The receptor mRNA was expressed in normal pancreatic islets, regenerating islets, and a pancreatic ductal cell line (ARIP cells, which proliferate in a Reg protein–dependent manner). These results indicate that the receptor mediates...
a growth signal of Reg protein for β-cell regeneration and that Reg protein acts as a growth factor via the receptor in an autocrine/paracrine fashion (Fig. 4D).

**Transcriptional activation of Reg gene by PARP inhibitors.** The Reg gene is only expressed during islet regeneration (60,66,67), whereas there is no difference in the receptor mRNA expression between normal and regenerating islets (65). The regeneration and proliferation of pancreatic β-cells would therefore appear to be primarily regulated by the Reg gene expression. Accordingly, transcriptional activation would have an important role in β-cell regeneration. In this context, we found that Reg gene is activated by IL-6, dexamethasone, and PARP inhibitors (68). The combined addition of IL-6 and dexamethasone increased the Reg mRNA level, and furthermore, addition of nicotinamide or 3-aminobenzamide increased the mRNA even more. Progressive deletion of the 5'-flanking region of rat Reg gene revealed that the region between nucleotides −81 and −70 “TGCCCTC-CCAT” is essential for the Reg gene promoter activity. In gel mobility shift assays with the Reg promoter, the intensity of the band, which was detected in the nuclear extracts of RINm5F cells treated with IL-6, dexamethasone, and/or nicotinamide, was correlated with the luciferase activity, and the protein binding to the sequence was revealed to be PARP (68). We have also shown that the inhibition of PARP activity facilitates Reg transcription by preventing excessive PARP self-poly(ADP-ribose)ylation and consequent dissociation of PARP from the active transcriptional DNA/protein complex. Transcriptional regulation by PARP activity and the transcription-regulating PARP-binding sequences were followed in other genes such as chemokine CXCL1 (69), nuclear factor-κB–regulated genes (70), and PARP (71), and a novel view that PARP acts as a transcriptional regulator is established.

Thus, as shown in Fig. 4E, inflammatory mediators, IL-6, and glucocorticoids induce the formation of an active transcriptional complex involving PARP for the Reg gene, and the Reg gene transcription therefore proceeds. However, during inflammation, superoxide (O₂⁻) and nitric oxide (NO⁻) are produced, causing DNA damage. In this situation, PARP is activated by DNA nicks to repair the DNA. Then, PARP poly(ADP-ribose)ylates PARP itself, the poly(ADPR) chains on the PARP protein inhibit the formation of the active transcriptional complex, and the Reg gene transcription is stopped. In the presence of PARP inhibitors such as nicotinamide, the PARP is not poly-(ADP-ribose)ylated, the transcriptional complex is stabilized, and the Reg gene transcription proceeds. Therefore, PARP inhibitors maintain the function of PARP as a transcription factor for β-cell regeneration. This can account for the islet regeneration found in 90% depancre-
atized rats treated with PARP inhibitors (59) and also supports the notion that the restriction of H9252-cell replication is relieved by PARP inhibitors (2). When DNA is massively damaged, PARP is rapidly activated to repair the DNA, as described above, and the complex for Reg gene transcription is not formed at all.

The Reg gene family in tissue regeneration. The Reg and Reg-related genes were isolated and revealed to constitute a multigene family, the Reg gene family (66,67). Based on the primary structures of the encoded proteins, the members of the family are grouped into four subclasses: type I, II, III, and IV. Dots indicate amino acids identical to human REG Ia. Dashes indicate gaps for maximal alignment. Arrowheads indicate six conserved cysteines in the mature proteins.

FIG. 5. Alignment of amino acid sequences of the Reg gene family. Based on the primary structures of the encoded proteins (adapted from 67, and see also 74 and 75), the members of the Reg gene family are grouped into four subclasses: type I, II, III, and IV. Dots indicate amino acids identical to human REG Ia. Dashes indicate gaps for maximal alignment. Arrowheads indicate six conserved cysteines in the mature proteins.

atized rats treated with PARP inhibitors (59) and also supports the notion that the restriction of β-cell replication is relieved by PARP inhibitors (2). When DNA is massively damaged, PARP is rapidly activated to repair the DNA, as described above, and the complex for Reg gene transcription is not formed at all.

The Reg gene family in tissue regeneration. The Reg and Reg-related genes were isolated and revealed to constitute a multigene family, the Reg gene family (66,67). Based on the primary structures of the Reg proteins, the members of the family are grouped into four subclasses: type I, II, III, and IV (Fig. 5). In humans, four REG family genes, i.e., REG Iα (61), REG IB (72), REG-related sequence (RS) (61), and HIP/PAP, are tandemly ordered in the 95-Kbp region of chromosome 2p12 (73), whereas REG IV locates on chromosome 1 (74). In mouse genome, all of the Reg family genes, i.e., Reg I, Reg II, Reg IIIα, Reg IIIβ, and Reg IIIγ, were mapped to a contiguous 75-Kbp region of chromosome 6C (75). Type I (and type II) Reg proteins are expressed in regenerating islets (66). Type III Reg proteins have been suggested to be involved in cellular proliferation in intestinal cells, hepatic cells, and neuronal cells. Importantly, mouse Reg III was shown to be a Schwann cell mitogen that accompanies the regeneration of motor neurons (76), and Reg protein functions as a neurotrophic factor for motor neurons (77). Reg was also shown to mediate gastric mucosal proliferation in rats (78,79). The expression of Reg protein receptor mRNA has also been detected in liver, kidney, stomach, small intestine, colon, adrenal gland, pituitary gland, and brain (65), suggesting that the Reg-Reg receptor signal system is involved in a variety of cell types other than pancreatic β-cells. In fact, our preliminary histopathological analyses of Reg knockout mice showed that there are some structural abnormalities in tissue organization of alimentary tract and liver (unpublished data).

FUTURE PROSPECTS AND POSSIBLE CLINICAL APPLICATIONS
As described in the first part of this paper, PARP activation causes NAD⁺ depletion to form poly(ADPR), resulting in necrotic β-cell death. To prevent the necrotic β-cell death in type 1 diabetes, clinical trials of nicotinamide, a PARP inhibitor, have been performed in several groups in the world, and the results suggest that the nicotinamide treatment may be a promising one for the prevention and cure of type 1 diabetes (80–84). PARP inhibitors are also shown to induce Reg gene expression by stabilizing the transcriptional complex for the Reg gene, resulting in β-cell regeneration and neogenesis from progenitor cells such as pancreatic duct cells. It is therefore quite possible that the amelioration of diabetes observed in the clinical nicotinamide trials is caused not only by the prevention of necrotic β-cells but also by the induction of β-cell regeneration/neogenesis. Furthermore, because cADPR is produced from NAD⁺ by ADP-ribosyl cyclase of CD38 and PARP is the major enzyme for NAD⁺ degradation/con-
sumption, the inhibition of PARP by inhibitors such as nicotinamide may maintain the cADPR formation from NAD$^+$ for $\beta$-cells to achieve their functions, such as insulin secretion by glucose. Therefore, PARP inhibitors and their derivatives appear to be one of the most promising therapeutic approaches for diabetes treatment, although some toxic effects, such as reversible liver toxicity, teratogenicity, and growth-retardation, were reported in animal models at very high doses of the inhibitor. However, it has been revealed that there is no evidence in humans of most of the toxic effects of nicotinamide that have been reported in animal models (85). As shown in Fig. 6, PARP inhibitors induce at least three important events in $\beta$-cells: PARP inhibitors prevent the necrotic cell death, keep PARP functional as a transcription factor for cell regeneration, and maintain the formation of a second messenger, cADPR, to achieve the cell function.

Complications of diabetes are very serious problems for patients in their quality of life. The damages, dysfunction, and death of peripheral tissues/cells such as cardiovascular cells and neuronal cells by free radicals generated in the diabetic/hyperglycemic conditions may cause or exacerbate diabetic complications (86,87). As shown in the text (see Fig. 6B), accumulating evidence indicates that the mechanism proposed for the $\beta$-cell death, in which PARP activation plays an essential role, is involved in the process of necrotic cell death of many tissues and cells, including cardiovascular cells and neuronal cells. The involvement of the CD38-cADPR signal system in these cells and tissues has also been described (5–8), and the possible involvement of the Reg-Reg receptor system in Schwann cells, neuronal cells, and gastrointestinal cells has also been suggested (63–66). Therefore, the inhibition of PARP activity by PARP inhibitors may result in the improvement of some diabetic complications. Studies evaluating the incidence and severity of diabetic complications in the clinical PARP inhibitor trials will be required.

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REFERENCES


