Prolonged In Vitro Exposure to Autoantibodies Against CD38 Impairs the Function and Survival of Human Pancreatic Islets

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Autoantibodies against CD38 (adenosine-5′-diphosphate[ADP]-ribosyl cyclase/cyclic ADP-ribose hydrolase) have been described in 10–12% of patients with type 2 diabetes. In human islets, anti-CD38 autoantibodies (CD38Abs) acutely stimulate insulin release (IR) and increase the cytosolic calcium concentration ($[Ca^{2+}]_i$). Whether CD38Abs affect human islet cell function and survival upon prolonged in vitro exposure is not known. We cultured human islets for up to 7 days in the presence of sera from 10 patients with type 2 diabetes that had neither CD38Ab- nor $[Ca^{2+}]_i$-mobilizing activity (−/−), sera from 6 patients with type 2 diabetes that was CD38Ab-positive and had $[Ca^{2+}]_i$-mobilizing activity (+/+), or no sera (control). At baseline, +/+ sera caused a significant ($P < 0.002$) acute stimulation of IR (IR at 3.3 mmol/l glucose was 45 ± 19, 84 ± 24, and 34 ± 12 μU/ml in control, +/+, and −/− sera, respectively; the corresponding IR at 16.7 mmol/l glucose was 72 ± 25, 204 ± 56, and 80 ± 32 μU/ml). At 3 days, IR at 3.3 mmol/l glucose was 42 ± 18, 27 ± 11, and 43 ± 24 μU/ml ($P = 0.0003$) for control, +/+, and −/− sera, respectively, whereas at 16.7 mmol/l glucose, it was 95 ± 76, 45 ± 35, and 76 ± 42 μU/ml, respectively. After 7 days of exposure, the corresponding IR at 3.3 mmol/l glucose was 40 ± 11, 28 ± 12, and 35 ± 15 μU/ml, respectively, whereas at 16.7 mmol/l glucose it was 79 ± 39, 39 ± 17, and 62 ± 39 μU/ml. At both 3 and 7 days, IR still increased when switching from 3.3 to 16.7 mmol/l glucose ($P < 0.0003$), and incubation with +/+ sera induced a significant decrease in the insulin response ($P < 0.002$). At 7 days, the number of dead cells (as evaluated by an enzyme-linked immunosorbent assay technique) differed significantly between control (1.2 ± 0.3 OD units) cells, islets exposed to −/− sera (1.4 ± 0.1), and islets coincubated with +/+ sera (1.9 ± 0.4, $P < 0.01$). We conclude that prolonged exposure of human islets to sera positive for the presence of CD38Abs with $[Ca^{2+}]_i$-mobilizing activity impairs β-cell function and viability in cultured human pancreatic islets. *Diabetes* **51 (Suppl. 3):S474–S477, 2002**

CD38 (adenosine-5′-diphosphate[ADP]-ribosyl cyclase/cyclic ADP-ribose hydrolase) is a surface and cytosolic enzyme that catalyzes the interconversion of NAD+ and cyclic ADP-ribose (1,2). CD38 is expressed on the surface of monocytes, plateletes, natural killer (NK) cells, T- and B-cells, myeloid cells, vascular endothelium, and in tissues such as brain, cardiac and skeletal muscle, spleen, heart, liver, prostate, and kidney, and it is involved in several cell functions (3). Recent studies suggest that CD38 is also expressed in rat (4) and human pancreatic islets (3). In human B-cells, CD38 activation produces different effects depending on the differentiation stage. Thus, mature B-cells proliferate, whereas the opposite occurs in immature B-cells in the bone marrow. The CD38 signaling pathway in this environment blocks B-lymphopoiesis, mostly by inducing apoptosis (5–10). In pancreatic β-cells, this protein appears to play a role in glucose-induced insulin release (IR) (11,12) through a mechanism that involves its cyclase activity, leading to increased cytoplasmic Ca^{2+} concentrations (13). We and others have recently reported that autoantibodies reacting with CD38 can be found in 10–12% of patients with type 2 diabetes (12–14) or autoimmune thyroid diseases (15). Interestingly, anti-CD38 autoantibodies (CD38Abs) can acutely increase IR from human pancreatic β-cells in vitro (12,13), and this agonistic effect correlates with their ability to mobilize intracellular Ca^{2+} (12). Whether these CD38Abs can directly affect human islet cell function and survival upon prolonged in vitro exposure is not known. To address this issue, we cultured human islets for up to 7 days in the presence or absence of sera typed for CD38 antibodies, and we carried out functional and survival studies at baseline and at 3 and 7 days of incubation.

RESEARCH DESIGN AND METHODS

Detection of CD38Abs. The clinical characteristics of the population screened for the presence of anti-CD38 autoimmunity and the methods for the detection and characterization of anti-CD38 antibodies have been reported elsewhere (12,13). Briefly, recombinant CD38-maltose binding protein fusion protein (rCD38-MBP, 68 kDa) was obtained from a human CD38 cDNA encoding amino acids 45–300. A volume of 10 μg of recombinant CD38-MBP

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$[Ca^{2+}]_i$, cytosolic calcium concentration; CD38Ab, anti-CD38 autoantibody; fluo-3-AM, fluo-3-acetoxymethyl ester; HBSS, Hanks' balanced salt solution; IR, insulin release; KRB, Krebs-Ringer bicarbonate; KRH, Krebs-Ringer-HEPES; mAb, monoclonal antibody; MFI, mean fluorescence intensity.

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was separated on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membranes were subsequently blocked with a solution of PBS containing 5% nonfat dry milk and 0.15% Tween-20, and then it was incubated for 60 min. Sera from diabetic patients and control subjects were diluted (1:1,000) in the same buffer. After rinsing with PBS containing 0.15% Tween-20, the membranes were incubated for 60 min with a purified rabbit Ig specific for human Ig (G+M), labeled with horseradish peroxidase. The signals were revealed using an enhanced chemiluminescence detection system; the intensity of the bands was measured by densitometry.

Isolation of human islets and preparation of dispersed islet cells. Isolation of pancreatic islets was performed as previously described (13,16,17) from the pancreata of 15 human cadaver donors referred through the local organ procurement organization, with the permission of the institutional ethics committee of the University of Pisa School of Medicine. For the purification procedure, 3 ml of tissue was loaded into 250-ml plastic conicals and resuspended in 50 ml of 80% Histopaque 1.077 (Sigma, St. Louis, MO) and 20% Hanks' balanced salt solution (HBSS), topped with 40 ml HBSS. After centrifugation at 800*g* for 5 min at 4°C, islets were recovered at the interface between the Histopaque and the HBSS layers. The islets were washed with HBSS by centrifugation at 800*g* for 2 min at 4°C, resuspended in M199 culture medium (supplemented with 10% serum and antibiotics), and cultured at 37°C in a CO_2 incubator. Dispersed islet cells were prepared by enzymatic dissociation as previously reported (12). Briefly, human islets were gently resuspended in a calcium-free Krebs-Ringer-HEPES (KRH) buffer with 1 mmol/l EGTA at 30°C. After 8 min, 16.5 μg/ml trypsin and 2 μg/ml DNase were added, and a further 8–10 min incubation at 30°C was allowed. Islet cell dissociation was monitored by observing aliquots of the suspension under an inverted light microscope. The procedure was stopped when most cells were single, or in small groups of 2–3 cells, by adding KRH buffer containing 1.67 mmol/l calcium and 1% calf serum. At the end of the dissociation, cells were washed with PBS, resuspended in M199 culture medium, and eventually placed at 37°C in a CO_2 incubator.

Cytosolic calcium concentration mobilization experiments. For these experiments, fluo-3-acetoxymethyl ester (Fluo-3-AM) and 4-bromo A23187 ionophore, HBSS, FCS, RPMI-1640 medium, pluronic F-127, and DMSO were purchased from Sigma. IB4 (an agonistic anti-CD38 monoclonal antibody [mAb], IgG2a), as the intact molecule or F(ab′)2, was used as positive control. IB6 (a nonagonistic anti-CD38 mAb, IgG2b) and OKT-10 (a nonagonistic anti-CD38 mAb, IgG2b) were used as CD38-blocking tools (18). CB-gp120 (anti–HIV-1 gp120 mAb, IgG2a), and CB-ICAM-1 (anti–CD54/ICAM-1, IgG1) were used as internal controls. The F(ab′)2 fragmentation of IB4 and goat anti-human Ig (GAHIg) mAbs were obtained as described. All mAbs used in the cytosolic calcium concentration ($[Ca^{2+}]_i$) mobilization experiments were produced in the laboratory and were purified by both affinity chromatography on protein-A Sepharose and high-performance liquid chromatography on hydroxylapatite (18). Experiments on $[Ca^{2+}]_i$ mobilization were performed on Jurkat T-cells and isolated human pancreatic β-cells, as described in detail in previous work from our laboratory (12). Changes in $[Ca^{2+}]_i$ levels were monitored by flow cytometry after loading cells with the Ca^{2+}-sensitive fluorescent dye Fluo-3-AM. Briefly, Jurkat T-cells or human β-cells (12) were washed twice in HBSS (pH 7.0) and 5% FCS, resuspended in the same medium at a concentration of 2×10^6 cells/ml, and incubated for 30 min at 37°C with 5 mmol/l Fluo 3-AM in the presence of 0.01% pluronic F127. Cells were then washed twice and resuspended in test tubes at 10^6 cells/ml and used within 1 h. After calibrating the base level, 50 μl of serum was added to 650 μl of cells (10^7 cells/ml in complete medium). The effects on $[Ca^{2+}]_i$ were analyzed continuously at 37°C on a FACSort flow cytometer (Becton Dickinson) with Cellquest software. Cells were gated for size and side-scatter to eliminate debris and dead cells from the analysis. Standard controls included incubation with sera from patients with type 2 diabetes with no evidence of CD38Abs, sera from anti–CD38-negative healthy control subjects, and the A23187 ionophore. Dynamic changes in $[Ca^{2+}]_i$ were monitored by plotting continuously the shift in the Fluo 3-AM fluorescence over a 540-s time period.

Insulin secretion studies. Within 3 days of isolation, aliquots of the islet preparations were studied in terms of: *1*) the acute effect of CD38Abs on glucose-stimulated insulin secretion and *2*) the effect of prolonged (after 3 and 7 days) culture in the presence of CD38Abs on glucose-stimulated insulin secretion. In these experiments, we selected sera negative for CD38Abs as well as sera positive for both CD38Abs and $[Ca^{2+}]_i$-mobilizing activity. For group 1 experiments (12,13), batches of islets of comparable size (~30/tube) were preincubated at 37°C for 45 min in Krebs-Ringer bicarbonate (KRB) solution, supplemented with 3.3 mmol/l glucose and 0.5% BSA. The islets were then washed and incubated at 37°C for 45 min in KRB solution containing 3.3 or 16.7 mmol/l glucose, either with or without the addition of 10% serum from patients negative or positive for the presence of CD38Abs. At the end of the incubation period, aliquots of the medium were collected for the measurement of insulin concentration. For group 2 experiments, islet aliquots were placed into 60-mm petri dishes and maintained in suspension culture up to 7 days in either control medium or medium containing 10% serum from patients. In these experiments, we selected sera that had all previously tested for both anti-CD38 antibody positivity and $[Ca^{2+}]_i$-mobilizing activity. The islets were studied after 3 and 7 days from the beginning of the incubation. For the assessment of glucose-stimulated insulin secretory function, aliquots of ~30 islets per tube were preincubated at 37°C for 45 min in KRB solution, supplemented with 3.3 mmol/l glucose and 0.5% BSA. The islets were then washed and incubated at 37°C for 45 min in KRB solution containing 3.3 mmol/l glucose and then washed and incubated for additional 45 min in the presence of 16.7 mmol/l glucose. At the end of each incubation period, aliquots of the medium were collected for the measurement of insulin levels.

Evaluation of islet cell death. The effect on human islet cell death/survival of 7-day coculture with human sera was assessed by the cell death detection ELISAplus assay (Roche, Basel, Switzerland), implemented as detailed previously (19). Briefly, aliquots of ~15 islets of comparable size were incubated for 30 min with a lysis buffer at room temperature and then centrifuged at 200*g* for 10 min at 4°C. Aliquots of the supernatant (20 μl) were placed into microtiter plate wells coated with streptavidin. A volume of 80 μl of a mixture containing anti–histone-biotin antibody and anti–DNA-peroxidase antibody was then added, and incubation was allowed for 120 min at 37°C. Preparations were then washed, and 100 μl of a solution containing ABTS (2,2′-Azino-di[3-ethylbenzthiazolin-sulfonate]; the substrate for peroxidase) was added. At the end of a 15-min incubation, absorbance of samples was read at 405 nm on a Molecular Devices spectrophotometer.

Statistical analysis. Sera were considered to be positive for CD38Abs if the optical density on the Western blot was ≥3 SD above the mean of the previously reported control group (i.e., ≥1.9 units) (12,13). In the $[Ca^{2+}]_i$ mobilization experiments, basal mean fluorescence intensity (MFI) averaged 69 ± 1 units in a total of 32 incubations. An increment in MFI of ≥20% of the mean basal MFI (i.e., ≥83 MFI units) was taken to be a positive $[Ca^{2+}]_i$ response.

Data are given as the means ± SD; insulin concentrations were log-transformed for use in statistical analysis. A comparison of group values was performed by two-way ANOVA, with glucose concentrations (3.3 or 16.7 mmol/l in the incubation medium) and treatment (control and negative and positive sera) as main factors; Bonferroni-Dunn post hoc testing was then used for individual group comparisons.

RESULTS

To test whether CD38 positivity and $[Ca^{2+}]_i$-mobilizing activity were associated with acute stimulation of IR in isolated human islets, six sera that were CD38 positive and had $[Ca^{2+}]_i$-mobilizing activity and 10 sera that had neither CD38 positivity nor $[Ca^{2+}]_i$-mobilizing activity were selected, and the results were compared with those obtained from islets exposed to neither sera. A total of 45 separate incubation batches were analyzed (18 control, 12 for the 6 positive sera, and 15 for the 10 negative sera). At baseline, IR at 3.3 mmol/l glucose was 45 ± 19, 84 ± 24, and 34 ± 12 μU/ml in control islets and islets exposed to positive or negative sera, respectively. At 16.7 mmol/l glucose, the corresponding IR was 72 ± 25, 204 ± 56, and 80 ± 32 μU/ml. By two-way ANOVA, positive sera caused a significant ($P < 0.002$) acute stimulation of IR at both low and high glucose concentration, whereas no significant effect was observed with negative sera, confirming previous results (12).

The results obtained after 3 or 7 days of incubation are given in Tables 1 and 2. At 3 days, IR still increased when switching from 3.3 to 16.7 mmol/l glucose ($P < 0.0001$). However, incubation with positive sera induced a significant decrease in the insulin response ($P = 0.0003$). Likewise, after 7 days of exposure (Table 2), IR increased when switching from 3.3 to 16.7 mmol/l glucose ($P < 0.0001$), but positive sera induced a decrease in insulin response ($P = 0.02$).

Islet cell death/survival. At 7 days of incubation, the

TABLE 1
Insulin release in response to an acute glucose challenge from control islets and islets pre-exposed for 3 days to sera with ($CD38^+$, Ca^{2+}-positive) or without ($CD38^-$, Ca^{2+}-negative) anti-CD38 positivity and $[Ca^{2+}]_i$-mobilizing activity

	Insulin (μU/ml)	
Glucose	3.3 mmol/l	16.7 mmol/l
Control	42 ± 18	95 ± 76
$CD38^+$, Ca^{2+}-positive	27 ± 11*	45 ± 35*
$CD38^-$, Ca^{2+}-negative	43 ± 24	76 ± 42

*$P < 0.0001$ vs. control and $P = 0.0014$ vs. negative sera by Bonferroni-Dunn test.

amount of dead cells differed significantly between islets cultured in control medium (1.2 ± 0.3 OD units), islets cocultured with negative sera (1.4 ± 0.1 OD units), and islets cocultured with positive sera (1.9 ± 0.4 OD units, $P < 0.01$ vs. control) (Fig. 1).

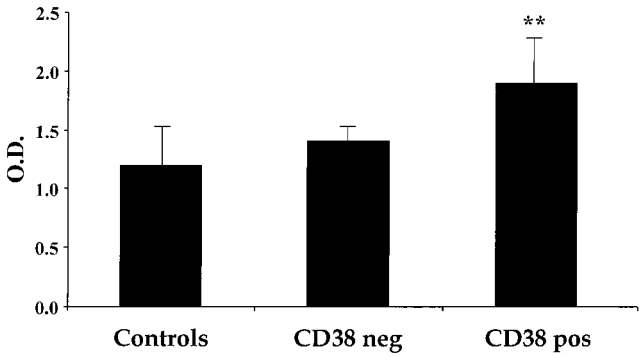

FIG. 1. Number of dead cells among control islets, islets exposed to anti–CD38-negative sera (CD38 neg), and islets exposed to anti–CD38-positive $[Ca^{2+}]_i$-mobilizing sera (CD38 pos) after 7 days of coincubation. **$P < 0.01$ vs. control.

DISCUSSION

The present study confirms that sera from type 2 diabetic patients with CD38Abs and calcium-mobilizing activity can acutely stimulate IR from cultured human islets. However, prolonged incubation of human islets with these sera impairs insulin secretion and increases the rate of apoptosis.

In human purified B-cells, CD38 activation gives rise to different effects according to the differentiation stage. In fact, mature B-cells proliferate in response to CD38 ligation, whereas the opposite occurs in immature B-cells in the bone marrow (5), mostly as a consequence of increased apoptosis (6,7). In tonsillar B-cells, ligation of CD38 protects cells from apoptosis by upregulating *bcl-2* expression (8). These contrasting results may be explained by assuming tissue-specific ligands for CD38, which could modulate the molecule's ability to transduce signals in association with other surface receptors and hence modulate its biological effects. In immature B-cells, the transduction pathway triggered by T16 (an anti-CD38 mAb) appears to overlap with the one driven by CD19, since the two molecules induce similar patterns of protein tyrosine phosphorylation. Moreover, CD38 ligation results in phosphorylation of CD19 and induction of its association with *lyn* and phosphatidylinositol-3-kinase (9,10). Recently, the effect of CD38 ligation on myeloid differentiation has been assessed using stroma-supported myeloid cells (20). The addition of anti-CD38 (T16) to the cultures induced a profound reduction of the most mature cell population (promyelocytes, myelocytes, metamyelocytes, etc.); mean cell recovery was 13% of that in parallel cultures with an isotype-matched control antibody. Cell recovery was also reduced by F(ab)′ (2) or Fab fragments of anti-CD38. The authors of this work conclude that CD38 mediates signals that culminate in suppression of myeloid cell growth and survival.

The present study is the first to evaluate the effect of sera from diabetic patients with anti-CD38 antibodies and calcium-mobilizing activity on the survival of human islets; the results suggest that in human islets, CD38 ligation by human anti-CD38 antibodies accelerates the rate of apoptosis. Previous work has shown that activation of CD38 on the nuclear membrane is associated with an endonuclear calcium rise, which is in turn regarded as a proapoptotic signal (21). With regard to this observation, it is of interest that the positive human sera we selected all had intracellular calcium-mobilizing activity; the observed acceleration of apoptosis in human islets thus resonates with the sequence of events seen in splenic cells (21).

In parallel with this proapoptotic effect, insulin secretion at both low and high glucose concentrations was markedly impaired, in contrast to the stimulatory effect observed in freshly isolated islets acutely challenged with these sera. Thus, prolonged incubation with stimulatory sera leads to a degree of functional exhaustion of the β-cell, which is already fully evident after 3 days of incubation. Whether this β-cell failure is only functional (i.e., reversible) or whether it may result from β-cell apoptosis cannot be determined from the current data. The simultaneous occurrence of enhanced apoptosis and decreased IR does suggest that β-cell loss may contribute to the secretory deficit, but quantitation of this mechanism, namely, true functional incompetence, or stunning, of β-cells is not possible.

The significance of these calcium-mobilizing autoantibodies in the pathogenesis and natural history of diabetes remains elusive. Superficially, the development and persistence of autoantibodies with organ-directed biological activity would fit in a paradigm of β-cell exhaustion after prolonged overstimulation. Many critical aspects of such a sequence, however, remain to be investigated.

TABLE 2
Insulin release in response to an acute glucose challenge from control islets and islets pre-exposed for 7 days to sera with ($CD38^+$, Ca^{2+}-positive) or without ($CD38^-$, Ca^{2+}-negative) anti-CD38 positivity and $[Ca^{2+}]_i$-mobilizing activity

	Insulin (μU/ml)	
Glucose	3.3 mmol/l	16.7 mmol/l
Controls	40 ± 11	79 ± 39
$CD38^+$, Ca^{2+}-positive	28 ± 12*	39 ± 17*
$CD38^-$, Ca^{2+}-negative	35 ± 15	62 ± 39

*$P < 0.0001$ vs. control and $P = 0.02$ vs. negative sera by Bonferroni-Dunn test.

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