Noninvasive In Vivo Measurement of β-Cell Mass in Mouse Model of Diabetes

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Pancreatic β-cell mass (BCM) is a major determinant of the quantity of insulin that can be secreted. BCM is markedly reduced in type 1 diabetes because of selective autoimmune destruction of β-cells. Accurate assessment of BCM in human diabetes is limited to autopsy studies, which usually suffer from inadequate clinical information; thus, the development of noninvasive means of BCM measurement could be important in intervention therapy. The goal of this study was to develop such noninvasive methods for measuring BCM featuring target-specific imaging probes and to investigate whether this technique is feasible, accurate, and predictive of BCM in normal and diabetic states. Using a β-cell–specific monoclonal antibody IC2, modified with a radioisotope chelator for nuclear imaging, we showed that highly specific binding and accumulation to β-cells occurs after intravenous administration of the probe, with virtually no binding to exocrine pancreas or stromal tissues. Furthermore, we observed a direct correlation between accumulation of the probe with BCM in diabetic and normal animals. Nuclear imaging of the animals that received an injection of the radioactive probe showed major difference in signal intensity between normal and diabetic pancreases. The results from this study set the route for further development of imaging probes for measuring BCM that would aid in diagnosis and treatment of diabetic patients in the clinic. *Diabetes* 50:2231–2236, 2001

Pancreatic β-cell mass (BCM) is markedly reduced in type 1 diabetes as a result of selective autoimmune destruction of β-cells (1). Accurate assessment of BCM in human diabetes is limited to autopsy studies, which usually suffer from inadequate clinical information (1). Although repeated blood sampling for determination of glucose, glucagon, tolbutamide, or insulin in response to various secretagogues is presumed to reflect quantitative or qualitative differences in pancreatic BCM or function, the severity of the β-cell loss responsible for abnormalities observed by these in vivo measurements is unknown (2). This loss has to be assessed during the evaluation of therapeutic efficacy of intervention. In addition, such assessment may be extended to evaluate the engrafted islet mass in the liver after islet transplantation. For this reason, noninvasive measurement of BCM could allow longitudinal mapping of the evolution of the disease both in animal models and, more important, in humans.

With the continuing development of magnetic resonance imaging (MRI) and nuclear imaging techniques during the past decade, it became possible to achieve high spatial and functional resolutions (3). During the past decades, we developed a number of imaging ligands for target-specific imaging, including receptor imaging (4–8), antigens (9–11), and lately for imaging of a receptor gene expression (12,13). In parallel, high-resolution imaging systems and techniques that will allow in vivo imaging of mice and rats have been developed recently (13,14).

The present study was aimed at developing noninvasive methods for measuring BCM featuring target-specific imaging probes and investigating whether this technique is feasible, accurate, and predictive of BCM in normal and diabetic states. On the basis of our experience in target-specific imaging, we hypothesized that certain imaging parameters (e.g., % injected dose/g tissue, morphologic signal changes, signal intensity changes) will correlate with BCM. With this goal in mind, we modified the β-cell–specific monoclonal antibody IC2 (15,16) with a radioisotope chelator for nuclear imaging and tested this probe on isolated islets in vitro as well as in a mouse diabetic model in vivo. Our results show that highly specific binding and accumulation to β-cells occurs after intravenous administration of the probe with virtually no binding to exocrine pancreas or stromal tissues, setting an ideal stage for the proposed work. Furthermore, there was a direct correlation between accumulation of the probe with BCM of the given pancreas. In diabetic animals with reduced BCM, the accumulation of the probe was significantly lower than in the normal animals. Nuclear imaging of the animals that received an injection of the radioactive probe showed differences in signal intensity between normal and diabetic pancreases. The results from this study demonstrate proof of principle that it should be feasible to estimate BCM using target-specific imaging probes.

RESEARCH DESIGN AND METHODS

Production, characterization, and modification of IC2 monoclonal antibody. Cells that produce rat monoclonal antibody IC2 specific for the surface of mouse pancreatic β-cells IC2 (15–17) were obtained from Dr. P. Poussier (Royal Victoria Hospital, Canada). Cells were grown in Dulbecco's modified Eagle's medium (Cellgro; Mediatech, Washington, DC) supplemented with

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BCM, β-cell mass; DTPA, diethylenetriamine pentaacetic acid; DTZ, dithizone; MRI, magnetic resonance imaging; ROI, region of interest; STZ, streptozotocin.

10% fetal bovine serum (Cellgro, Mediatech). Four million cells were injected intraperitoneally into *nu/nu* mice (Charles River Breeding Laboratories, Wilmington, MA) primed with 0.2 ml of Pristan (Sigma, St. Louis, MO); 2 weeks later, ascitic fluid was withdrawn from the peritoneal cavity. Antibody was purified on the affinity column with goat anti-rat IgM (Pierce, Rockford, IL) immobilized on activated agarose gel (Pierce). The purity of the antibody was confirmed by 12% SDS-PAGE under reducing and nonreducing conditions, and the class specificity was confirmed by Western blot with goat anti-rat IgM-biotin (Pierce) followed by incubation with avidin-peroxidase (Bio-Rad, Richmond, CA) and diaminobenzidine staining (Sigma).

For pharmacokinetic studies, IC2 antibody was labeled with Na ^{125}I (NEN Life Science Products, Boston, MA) by the IodoGen method (Pierce) with subsequent purification on a Sephadex G-25 column (Sigma).

To label antibody with radionuclide for nuclear imaging, we first modified it with a chelate group. One milligram of IC2 monoclonal antibody was dialyzed against 10 mmol/l borate buffer with 150 mmol/l NaCl (pH 8.7) overnight and centrifuged at 5,000 rpm for 15 min. A 6 molar excess of diethylenetriamine pentaacetic acid (DTPA) cyclic anhydride (Pierce) dissolved in DMSO was added to the antibody, and the mixture was incubated for 4 h at 4°C. After incubation, antibody was dialyzed against 10 mmol/l sodium citrate buffer with 150 mmol/l NaCl (pH 6.0) overnight. IC2-DTPA was then labeled with $^{111}InCl_2$ (NEN Life Science Products) for 1.5 h and subsequently purified on Bio-Spin columns (Bio-Rad).

Characterization of the IC2 antibodies by fluorescence microscopy in vitro. Pancreatic islets were isolated from C57BL/6 mice (n = 10, Charles River Breeding Laboratories) as described by Montana et al. (18) and plated on glass coverslips placed in 12-well plates with RPMI 1640 medium supplemented with 10% fetal bovine serum immediately before the experiment. For fluorescence microscopy, islets were incubated for 1 h with purified IC2 antibody followed by 1 h of incubation with goat anti-rat IgM conjugated with FITC (1:100 dilution; ICN-Cappel Pharmaceuticals, Aurora, OH). All incubations were carried out at 4°C. After final washing, the islets were fixed with 4% formalin, washed with phosphate-buffered saline, and mounted on histological slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Islets were flattened with coverslips (Fisher Scientific, Pittsburgh, PA) and examined with an inverted fluorescence microscope (Axiovert 100TV; Zeiss, Wetzlar, Germany). Images were collected using a cooled charge-coupled device (Photometrics, Tucson, AZ) with appropriate excitation and emission filters (Omega Optical, Brattleboro, VT). Two control reactions were performed: *1*) pancreatic islets with secondary antibody without IC2 antibody and *2*) unrelated cell line (9L gliosarcoma) with IC2 antibody.

Characterization of the IC2 antibodies in vivo. Pancreases from C57BL/6 mice (n = 3) were used as experimental tissues. For control tissue, 9L gliosarcoma cells were injected into *nu/nu* mice 2 weeks before experiment to obtain a tumor. To evaluate intrapancreatic distribution of IC2 antibody in vivo, experimental and tumor-bearing mice received an intravenous injection of original or DTPA-modified IC2 antibody and were killed 24 h later. The tumor tissue and pancreases were excised, snap-frozen, cut into 8-μm sections, and subjected to colocalization experiments to detect the presence of the probe in the tissue.

Double-channel fluorescence microscopy was used to colocalize injected antibodies with insulin-producing cells in the pancreas. Sections were incubated with primary rabbit anti–porcine insulin antibodies (1:50 final dilution; ICN-Cappel Pharmaceuticals) followed by incubation with the mixture of goat anti–rat IgM-FITC (1:50 final dilution) and rhodamine-labeled goat anti–rabbit IgG antibodies (1:100 final dilution; Pierce). Slides were covered with coverslips in Fluoromount-G and examined with an inverted fluorescence microscope as described above. The pancreatic tissue sections from an animal that did not receive an injection and tumor tissue sections of an unrelated cell line (9L gliosarcoma) were used as control tissues.

Determination of BCM. BCM was quantified by point-counting morphometry as described by McCulloch et al. (2) using dithizone solution (DTZ) (19–21). Mouse β-cells are clearly defined within the islets with DTZ, whereas in rat islets, both β- and non–β-cells were stained with DTZ (S. Bonner-Weir, unpublished observation). The whole mount of pancreatic tissue was carefully spread with the lobes spread out on the large (35 × 75 mm) histological slide, covered with the coverslip, and lightly pressed to flatten the tissue but so that the islets in the tissue would not be damaged. With the point-counting method, intercepts of a 90-point grid were counted at final magnification 420× on fields that covered the tissue without overlap. A minimum of 50,000 points/animal, usually with considerably more points, was counted. The BCM was then calculated with correction for pancreas weight for included nonpancreatic tissue (lymph nodes, fat, etc.).

Animal model. C57BL/6 mice (12–13 weeks of age, 35–40 g) received an injection of 160 mg/kg streptozotocin (STZ) intravenously 4–5 days before experiment. Blood from tail snip was sampled in a fed state at 10:00 A.M. daily, and the glucose level was measured using Glucometer Elite XL (Bayer, Eikhart, IN). Animals that had a glucose level of 350 mg/dl were used for experiments.

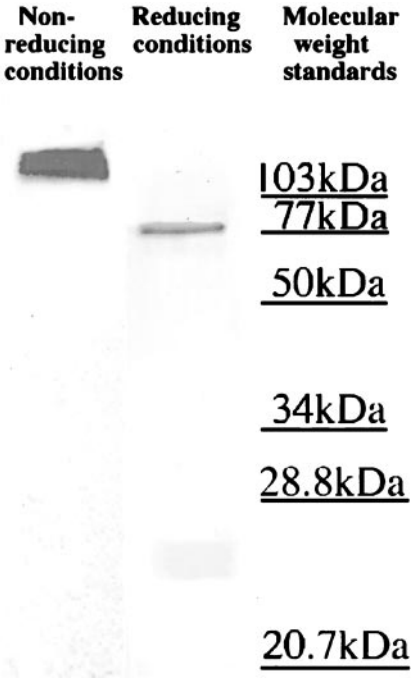

FIG. 1. Western blot under reducing conditions of IC2 antibodies shows two bands, corresponding to heavy and light chain.

Biodistribution. The iodinated probe was injected intravenously (50 μg protein; 20 μCi/animal) in diabetic (n = 5) and normal (n = 5) mice. Twenty-four hours after injection, the animals were killed and perfused intracardially first with 20 ml of 0.9% NaCl and then with an additional 6 ml of DTZ solution, prepared as described above. Pancreases were removed, weighed, and counted in a γ-counter. Accumulation of the probe in the pancreas was expressed as a percentage of injected dose per gram of pancreatic tissue (%ID/g). BCM measurements were performed immediately after biodistribution on unfixed DTZ-stained whole tissues as described above. Biodistribution of the probe to the pancreas (%ID/g) was further correlated with BCM.

Nuclear imaging. For nuclear imaging, normal (n = 3) and diabetic mice (n = 3) received an intravenous injection of 20 μCi of IC2-DTPA-^{111}In (50 μg protein). Twenty-four hours later, mice were killed and perfused as described above, pancreases were removed and weighed, and the whole organ was placed on the bottom of plastic tubes (1 cm diameter). Imaging was performed on Radioisotope Camera Series 100 (Ohio-Nuclear, Cleveland, OH) with pinhole camera acquisition and 30 min of acquisition time. Imaging signal was collected from the whole organ. After imaging, each digital image was analyzed by drawing a region of interest (ROI; 1 cm diameter) around the whole organ and estimating radioactivity in this region. BCM measurements were performed immediately after imaging on unfixed DTZ-stained tissues as described above. Signal intensity (in arbitrary units of radioactivity), corrected for the background on the nuclear images, was correlated with the BCM of a given pancreas.

The animal protocol was approved by the MGH Institutional Review Committee on Animal Care and was conducted in accordance with National Animal Welfare guidelines.

Statistical analysis. Linear regression analysis was used to correlate data between BCM and the %ID/g for normal and diabetic animals. Two-sample unequal variance Student's t test was used for comparisons of biodistribution data between normal and diabetic animals. $P < 0.05$ was considered significant.

RESULTS

Characterization of IC2 antibody. The purity and class specificity of obtained antibody were confirmed in SDS-PAGE under reducing and nonreducing conditions and Western blot. As shown in Fig. 1, under reducing condi-

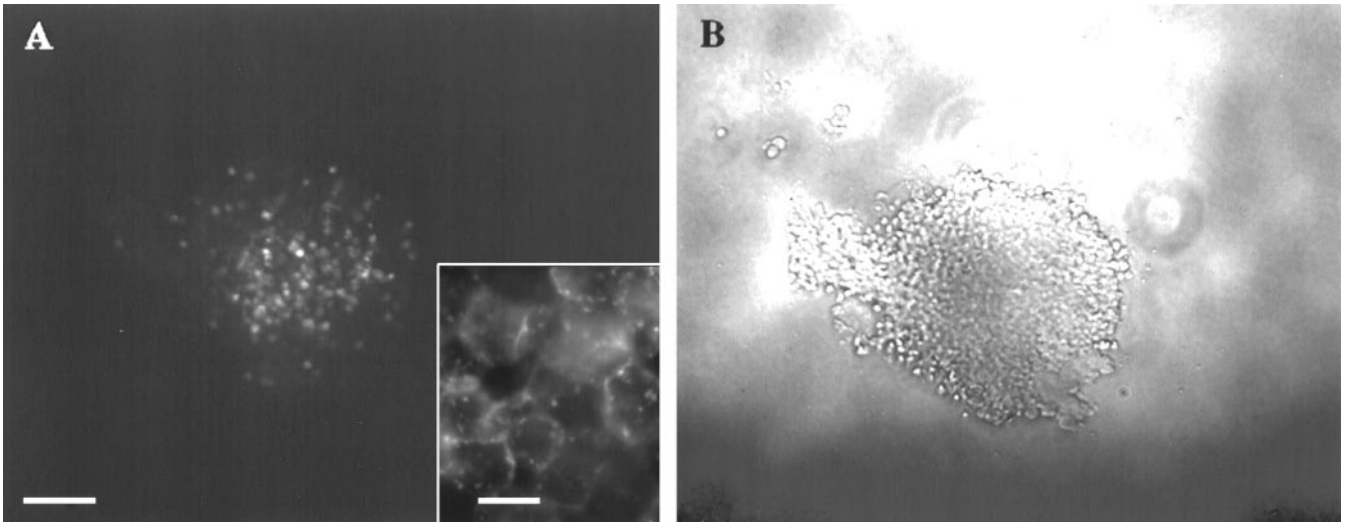

FIG. 2. Micrograph of an islet, flattened between coverslips, showing positive binding with IC2 antibodies on fluorescent (*A*) and bright field (*B*) images. Insert (*A*) shows at higher magnification the cell surface distribution of the probe after 1 h of incubation (magnification bars: *A*, *B* = 20 μm; insert = 10 μm).

tions the heavy chain migrated with a relative molecular weight of ~70,000–80,000 Da and the light chain showed a band at ~25,000 Da, which is characteristic for IgM class antibodies. Staining with anti-IgM antibodies on Western blot confirmed their class specificity to be that of IgM.

IC2 specificity in vitro. Specificity of purified and DTPA-modified antibodies was analyzed in vitro by fluorescence microscopy with mouse pancreatic islets. The images (Fig. 2) showed specific binding of IC2 antibodies to the islets (Fig. 2*A*) with the fluorescence distributed throughout the cell surface (Fig. 2*A*, insert). The same results showing cell surface binding of IC2 were demonstrated previously by Aaen et al. (16). Comparison of these pictures with the bright field images of the islets (Fig. 2*B*) showed that the specific reaction with antibodies was mostly pronounced within the core of the islets. This observation is in accordance with the common structure of the islets where β-cells are surrounded by non–β-cell mantle (22,23). Control reactions with unrelated cells (9L gliosarcoma) and without primary antibodies were negative (data not shown).

Intrapancreatic specificity in vivo. Twenty-four hours after intravenous injection of the probe, dual-channel fluorescence microscopy was performed on frozen sections of excised pancreas. Within the pancreas, the accumulation of the probe was specific to β-cells, as was confirmed by localization of the probe in insulin-stained β-cells (Fig. 3). The surrounding endocrine, as well as exocrine tissues, showed no accumulation of the probe. These results indicate that the probe could be used specifically for the identification of β-cells, setting an ideal stage for imaging where the signal/noise ratio is a crucial parameter. It seems that after 24 h, the probe was taken up by the cells and accumulated within the cytoplasm in contrast to in vitro experiments in which the surface binding was observed after only 1 h of incubation.

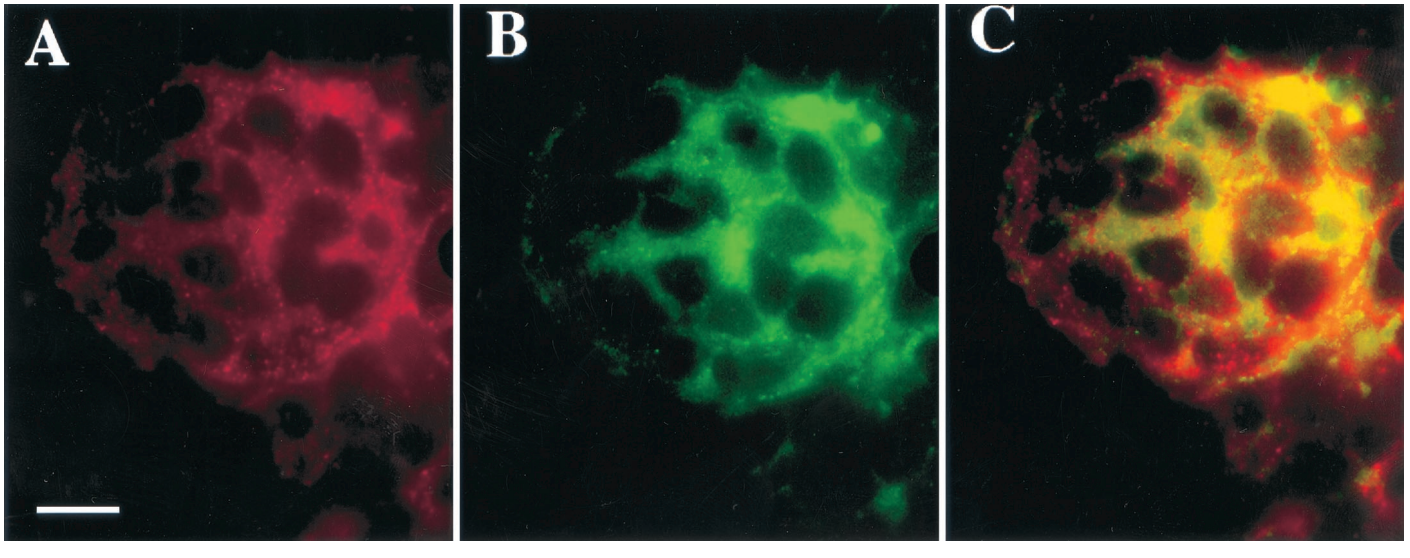

FIG. 3. Fluorescence micrograph of an islet showing localization of IC2 antibodies within insulin-positive β-cells 24 h after intravenous injection of IC2 antibody. *A*: Rhodamine channel shows β-cells stained for insulin. *B*: FITC channel shows accumulation of the probe within the cytoplasm (notice the negative space of the nuclei). *C*: Composite colocalization image of two channels. (Magnification bar = 10 μm).

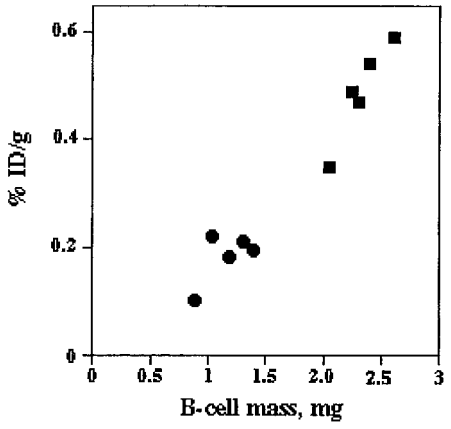

FIG. 4. Linear regression analysis showing the correlation between BCM and accumulation of iodinated probe in pancreas of normal (■) and STZ-induced (●) diabetic mice ($y = 0.236x - 0.071$; $r^2 = 0.936$). Each data point represents one animal. Accumulation of the probe is expressed as percentage of injected dose per gram of tissue (%ID/g).

Control experiments, which included incubations with appropriate combinations of secondary and anti-insulin antibodies, showed negative reactions. After incubation with anti-insulin antibody followed by incubation with the mixture of secondary antibodies to the probe (FITC channel) and to the primary anti-insulin antibodies (rhodamine channel), control pancreas from animals that did not receive an injection showed reaction in the rhodamine channel only. Frozen sections of control tumor (9L gliosarcoma) from animals that received an injection showed negative reactions in all experimental setups. Experiments performed with the DTPA-modified antibodies showed the same intrapancreatic accumulation as the unmodified antibodies (data not shown).

Characterization of the probe in STZ diabetic mice. Accumulation of the probe in the pancreas of normal and STZ-induced diabetic mice was expressed as a percentage of injected dose per gram tissue (%ID/g tissue) and plotted against BCM of the given pancreas. In normal animals, BCM was higher than in diabetic mice, as was the accumulation of the probe (Fig. 4). Animals with STZ-induced diabetes showed ~50% loss of BCM with significantly lower accumulation of the probe than normal mice ($P < 0.005$). There seemed to be a good correlation between BCM and probe accumulation ($r^2 = 0.936$) within the two subgroups. The overall accumulation of the probe in the islet β-cells has a tendency to decrease with reduction of BCM.

Nuclear imaging. To find out whether imaging technique could be used to estimate BCM, we performed nuclear imaging of the pancreas of normal and diabetic animals that received an injection of IC2-DTPA-^{111}In. Figure 5 shows representative images of the organs with clear difference in signal intensity between normal and diabetic pancreases, although the weight of these organs was approximately the same. The amount of radioactivity acquired by these organ, as assessed by ROI analysis of the images, revealed major differences in the amount of radioactivity associated with the pancreas of normal and diabetic animals, depending on the BCM in these organs (Fig. 5). These results demonstrate proof of principle that it is feasible to estimate BCM using accumulation of radiolabeled probe.

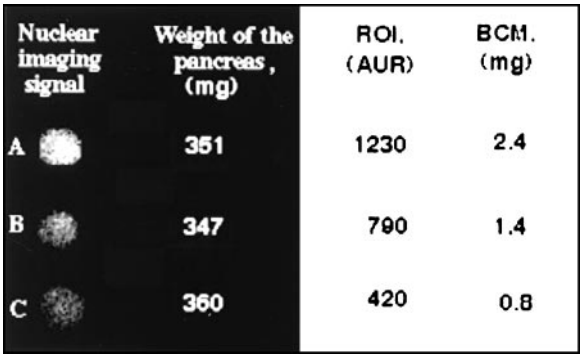

FIG. 5. Nuclear imaging of representative normal (*A*) and diabetic (*B*, *C*) pancreases with corresponding weights of these organs (Each group had $n = 3$). Animals received an intravenous injection of IC2-DTPA-^{111}In, and excised organs were imaged 24 h later. Imaging signal was acquired from the whole organs. After imaging, each digital image was analyzed by drawing an ROI (1 cm diameter; the diameter of the sample tube) around the whole organ and estimating radioactivity in this region. Note higher signal intensity (amount of radioactivity in arbitrary units of radioactivity [AUR]) in the pancreas of the normal animal with the higher BCM.

DISCUSSION

The development of imaging techniques and reagents that lead to noninvasive assessment of pancreatic BCM in vivo may have a significant impact in managing pancreas and/or islet transplantation, in the understanding of the pathogenesis in islet engraftment, and for assessing the efficacy of modulations in therapy of type 1 diabetes. Unfortunately, such noninvasive techniques are not available. Here we report on the development and testing of a target-specific probe that specifically bound to islet β-cells after intravenous injection, and the signal intensity of the probe on nuclear images was directly proportional to BCM in the pancreas of normal and diabetic animals.

For establishing a BCM quantitation technique, it was necessary to find a suitable candidate for the marker (ligand) on the surface of β-cells and evaluate its expression. The successful candidate should meet several conditions: *1*) it should be expressed specifically by β-cells and not by any other pancreatic cells; *2*) it should be expressed in a sufficient amount to be available for imaging probe; and *3*) tissues surrounding the pancreas should be devoid of this marker to avoid high background signal from these organs during imaging. Many of the β-cell markers/ligands known so far, such as insulin (24), glutamic acid decarboxylase (25), carboxypeptidase H (26), GM2-1 pancreatic islet ganglioside (27), ICA 69 autoantigen (28), imogen 38 (29), and several others are expressed on other pancreatic and nonpancreatic cells. Therefore, we focused our attention on IC2 monoclonal antibody (15) for an islet cell surface antigen. This antibody was shown to be β-cell–specific (15–17). Binding of IC2 to the RIN5F insulinoma cell line was not affected by neuraminidase treatment but was substantially reduced by proteases. Electron microscopy of IC2-labeled islet β-cells showed exclusive binding to the surface membrane (16).

Therefore, these results suggested that IC2-specific islet surface antigen is a protein. It was also shown that IC2 did not react with insulin and therefore would not interfere with its secretion. At the same time, we did not observe any specific reaction of IC2 antibody with non–β-cell tissue. The availability of the marker to the probe was

confirmed in both in vitro and in vivo experiments in the current study. These data suggest that the marker for IC2 antibody meets the three requirements for target-specific imaging, and IC2 antibody could be an ideal candidate for imaging such a marker on the surface of β-cells.

Our results demonstrated that after chemical modification, the antibody retained its ability to bind to β-cells both in vitro and in vivo. When radiolabeled probe was used in animals with STZ-induced diabetes, a positive correlation was observed between the probe accumulation and BCM. The nuclear imaging signal also correlated directly with the BCM in normal and diabetic animals. Two factors contributed to the accumulation of the probe within the islets. First, high specificity of the probe to β-cells was shown in this and previous studies (15–17). Second, islet vasculature consists of numerous capillaries that are fenestrated and, hence, highly permeable (23,30,31). These capillaries, resembling a glomerulus, course through the islet in a tortuous manner that is ideal for cell-blood and blood-cell interactions. In addition, the blood flow to the islets has been found to be disproportionally large (10–20% of the pancreatic blood flow) for the 1–2% of pancreatic volume (32–34). These features create a favorable environment for delivery of the imaging probe from the blood. The efficiency of this delivery needs to be explored in future studies.

Despite the very promising results, more work is needed to investigate further the antigen for IC2 antibody and to modify the probe. For example, the question on the exact location of IC2 antigen on the β-cell surface remains uncertain. If it is being secreted to the plasma membrane along with insulin, it is unclear whether degranulated β-cells would express enough of it to be detected by IC2 antibodies. Further modification of the probe for practical clinical applications would include labeling with ^{99m}Tc, which is widely used in nuclear medicine because of the shorter half-life (6 h) compared with indium (2.8 days). We also plan to modify this probe so that it would be possible to use it with MRI. This technique has higher spatial resolution than the techniques involving the use of isotopes (micrometers rather than several millimeters) (35,36). In comparison with isotope techniques, however, MRI is several magnitudes less sensitive (millimolar rather than picomolar) (3); therefore, all possible techniques should be exploited to achieve our goals. We expect that after conjugation of the probe with superparamagnetic iron oxide, previously used by us for target-specific imaging (4), it would be possible to image a single islet by MRI. Given the ability to spatially encode the imaging signal, it should thus be feasible to measure BCM. Therefore, we believe that further development of these probes for measuring BCM would aid in evaluating new treatments and interventions used in therapy for diabetes.

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