Evidence exists for an essential role of β-cell apoptosis in the pathology of type 1 and type 2 diabetes. Current methods for diabetes-associated apoptosis detection, however, suffer the drawbacks of relying on situ-based strategies. In this study, we attempted to measure, both in vitro and ex vivo, levels of β-cell apoptosis in diabetic mice using Cy5.5-labeled annexin V. We used streptozotocin-treated BALB/c mice and NOD mice of different ages as models of type 1 diabetes and db/db mice as a model of type 2 diabetes. With annexin V Cy5.5, we established differences in levels of apoptosis between diabetic and control animals. Intravenously administered annexin V Cy5.5 accumulated in pancreata of diabetic mice but not in nondiabetic controls. Furthermore, its localization was specific to apoptotic events within diabetic islets; its selectivity was supported by transferase-mediated dUTP nick-end labeling staining. Because annexin V defines an early marker of apoptosis and the developed probe is suitable for in vivo administration, it may provide a promising tool for real-time identification in intact animals of the earliest stages of diabetes-associated β-cell death and for tracing the events that characterize the pathology of the disease. Diabetes 54:1780–1788, 2005

In recent years, evidence has been accumulating that apoptosis is a central event contributing to type 1 and type 2 diabetes. In fact, the process of β-cell death appears to constitute a major link between the seemingly distinct disease pathways. Whereas type 1 diabetes is characterized by the specific autoimmune recognition and destruction of insulin-producing β-cells, the signals mediating β-cell destruction in type 2 diabetes are less clear but may be metabolic.

Type 1 diabetes–associated autoimmunity appears to be primarily a T-cell–mediated process. Current theories implicate the process of neonatal β-cell remodeling, which is associated with a wave of apoptosis, as a potential source of initiating autoantigen (1,2). Extensive evidence of β-cell death in type 1 diabetes has been gathered using a variety of model systems, including the NOD mouse (3) and the multiple low-dose streptozotocin (MLDS) model (1,4,5).

Transgenic mouse models have been particularly useful in unraveling the complex molecular interactions accompanying β-cell death in type 1 diabetes (6,7). Evidence exists for direct killing of β-cells by T-cells through the perforin/granzyme pathway (8,9) and Fas/FasL interaction (10,11), as well as for β-cell destruction through soluble mediators, the most important being interleukin-1 (12), tumor necrosis factor-α (13), and interferon-γ (14,15).

Insulin resistance, the increasing need for higher levels of insulin in order to have glucose uptake in target tissues, is a hallmark of type 2 diabetes (16). Type 2 diabetes develops when there is inadequate compensation for insulin resistance (16). The main compensatory mechanisms to insulin resistance are enhanced function of the β-cells and an increase in β-cell mass, resulting from a shift in the balance between β-cell renewal (proliferation and neogenesis) and β-cell apoptosis. In animal models, increased β-cell apoptosis has been associated with glucotoxicity (17–19), lipotoxicity (20–23), formation of amyloid deposits (24–26), and/or genetic factors associated with defects in insulin signal transduction (27). Recent evidence from autopsied human pancreata suggests an increased frequency of β-cell apoptosis in cases of diabetes compared with nondiabetic control subjects (28).

Metabolic/environmental factors influence the need for insulin secretion continually throughout the life of an organism. The need to respond to ever-changing energy demands requires insulin secretion by the β-cell to be a tightly regulated process. In type 1 and type 2 diabetes, the delicate balance of cell renewal and cell loss is disrupted. Therefore, the ability to detect the earliest stages of β-cell apoptosis and to quantify β-cell death in vivo would be instrumental in understanding the time course of the disease and in designing new potential therapeutic strategies.

To date, the evidence of diabetes-associated β-cell apoptosis has been almost exclusively based on histological detection, namely microscopic identification of morphological changes associated with apoptosis, transferase-mediated dUTP nick-end labeling (TUNEL), and active caspase-3 detection. The first method relies on recognition of events such as membrane blebbing, cell shrinkage, and nuclear condensation, whereas TUNEL identifies DNA fragmentation and can be positive for either apoptosis or necrosis. A recent study explored the possibility for apoptosis detection in a cyclophosphamide-accelerated NOD model of diabetes using caspase-3 labeling (29), but virtually no β-cell death was identified even at the height of β-cell loss, presumably because of the rapid clearing of

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dying β-cells. In addition, the cyclophosphamide-treated 13-week-old NOD mouse model reflects a rather advanced stage of the disease. Many attempts to identify apoptotic events associated with diabetes have been hampered by the rapid clearing of dying β-cells as well as a long time course of the disease. Therefore, novel approaches for the detection of early apoptosis would clearly contribute to our study of the progression of diabetes.

One of the earliest markers of apoptosis is the externalization of phosphatidylserine, an aminophospholipid normally found exclusively on the cytoplasmic leaflet of the plasma membrane. The externalization of phosphatidylserine makes it available for recognition by exogenous annexin V, a naturally occurring calcium-dependent 35-kDa protein that binds with high avidity (Kd = 1 × 10^10 mol/l) to membrane-associated aminophospholipids (30,31). Annexin V labeled with a fluorescent tag is routinely used for histological and cell-sorting studies to identify apoptotic cells (32). In vivo, annexin has been conjugated to technetium-99m (99mTc) for scintigraphy (33–36), superparamagnetic iron oxide nanoparticles for apoptosis detection by magnetic resonance imaging (37), and the Cy5.5 fluorescent dye for near-infrared optical imaging (38,39).

The success of imaging apoptosis using labeled annexin V and the fact that phosphatidylserine externalization is an early apoptotic event prompted us to investigate the potential of a Cy5.5-conjugated annexin V probe for the detection of β-cell death in diabetes. In this study, we demonstrate the successful localization of annexin V Cy5.5 to apoptotic cells in diabetic islets using several model systems of type 1 and type 2 diabetes. Our results establish the feasibility of using fluorescently labeled annexin V for detection of the earliest stages of diabetes-associated β-cell death.

**RESULTS**

We tested the capacity of a Cy5.5-conjugated annexin V probe to detect apoptotic β-cells in the pancreata of diabetic mice. We used four different mouse models: the MLDS model of type 1 diabetes, characterized by β-cell apoptosis; the SHDS model, associated with high levels of cell death (4); the NOD mouse model, in which symptoms develop in an age-dependent manner; and the BKS. Cg−/−Leprdb/J (db/db) model of type 2 diabetes, distinguished by loss of pancreatic β-cells.

**MLDS and SHDS models of type 1 diabetes.** The specificity of annexin V Cy5.5 for apoptotic β-cells was evaluated by flow cytometry of dispersed islet cells with dual channels for annexin V Cy5.5 staining and anti-insulin FITC (FL1). Analysis was performed after gating on cells negative for the vital dye 7-AAD. As expected, a small fraction of cells derived from age-matched untreated BALB/c mice stained for annexin V. In agreement with previous reports, the level of apoptosis in islet cells from MLDS-treated mice was more than twofold higher than controls (41,42), and cell death in isolated islet cells from SHDS-treated mice approached 40% compared with 0.75% in controls (4 Fig. 1A). It is important to note that the apoptotic rates reported by this method may be skewed because of the severe impairment of islet yield as a result of diabetes induction. This is particularly true for the SHDS model, in which our analysis was based on a comparatively low number of cells.

The differential staining was confirmed by fluorescence imaging of STZ-treated versus nontreated isolated intact islets.
islets (Fig. 1B). Nontreated islets produced no signal in the Cy5.5 channel with a few signal-positive foci in islets from MLDS-treated mice. A larger fraction of islets from SHDS-treated mice stained for annexin V.

To investigate the in vivo accumulation of annexin V Cy5.5, pancreata of MLDS- and SHDS-treated mice were removed 6 h after intravenous administration of the probe and imaged ex vivo. There were distinct foci of NIRF signal due to probe binding in the pancreata of STZ-induced diabetic mice. By comparison, the signal associated with pancreata from nontreated animals was at or near background levels (Fig. 2A). Selective accumulation of our probe in islet regions containing apoptotic cells was confirmed by TUNEL assay on frozen sections: considering that the two methods define different stages of apoptosis, regions of annexin localization within islets correlated spatially with apoptotic events detected by TUNEL assay. As expected, in the MLDS model, only a few cells within islets bound annexin V and stained for TUNEL (2.3% and 1.6% respectively), but in the SHDS model, a significant proportion of cells accumulated the probe and were TUNEL positive (26.3% and 17.5%), respectively, \( P < 0.05 \); Fig. 2B and Table 1).

**NOD model of type 1 diabetes.** NOD mice ranging in age from 3 to 12 weeks were used to evaluate apoptosis at different stages during the progression of type 1 diabetes. Average blood glucose levels in NOD mice of all ages were in the normal range and indicated that the animals were pre-diabetic (results not shown). Flow cytometry of islet cells isolated from 3-, 6-, 8-, and 12-week-old mice showed an age-dependent progressive increase in the percentage of annexin V+ cells (ranging from 0.67% at 3 weeks to 3.45% at 12 weeks of age) (Fig. 3A). The majority of annexin V+ cells also stained for insulin, suggesting that the predominant number of apoptotic events represent \( \beta \)-cells. Because infiltrating lymphocytes can be a contributing factor to the total population of apoptotic cells, we analyzed cells from 8-week-old NOD mice double stained with annexin V Cy5.5 and an anti-CD3 antibody. Only 1% of CD3+ cells stained for annexin V, whereas 3.34% of the population fell in the annexin V Cy5.5+, CD3− quadrants. Because this fraction is approximately the same as that determined to be apoptotic and insulin positive, we conclude that the majority of apoptotic cells are \( \beta \)-cells (Fig. 3B). We need to emphasize that our estimate of the percentage of apoptotic cells within each group is only relative and attempts to clarify a trend associated with age. The trend of age-dependent increase in apoptosis was apparent in each of three independent experiments.

The accumulation of in vivo–delivered annexin V Cy5.5 appeared to be selective, because muscle tissue from the same animals produced no signal in ex vivo NIRF images (Fig. 4A). Pancreata from 8- and 12-week-old mice had distinct foci of NIRF signal, consistent with the hypothesis that staining was islet specific. Histological analysis of pancreatic tissue from 3-, 6-, 8-, and 12-week-old mice
confirmed the differential accumulation of annexin V Cy5.5 (Fig. 4B). The fraction of nuclei (DAPI) associated with fluorescence in the near-infrared range (annexin V Cy5.5) clearly increased with age. No annexin V binding was observed in sections derived from 3- and 6-week-old mice, whereas in 8- and 12-week-old animals, 2.6 ± 0.3% for 6-, 8-, and 12-week-old NOD mice, respectively, and reflected a trend similar to the one obtained with annexin V staining (Table 1). TUNEL analysis of the consecutive slice produced a signal associated with apoptotic events, which could be due to dying β-cells as well as infiltrating lymphocytes. Apoptotic rates, as determined by TUNEL, were 0.6 ± 0.1, 1.2 ± 0.2, and 2.3 ± 0.3% for 6-, 8-, and 12-week-old NOD mice, respectively, and reflected a trend similar to the one obtained with annexin V staining (Table 1).

**db/db model of type 2 diabetes.** For this model, we used the BKS.Cg-m^+/+Lepr^db/J strain, in which the db/db mutation is placed on the highly diabetes-prone C57BLKS/J genetic background. The average blood glucose level in the 8-week-old db/db group was 317 ± 36.9 mg/dl (n = 6) compared with 151 ± 6.5 mg/dl in the controls (n = 3) (Fig. 5D). Flow cytometric analysis of islet cells isolated from these diabetic db/db and control mice demonstrated differential binding of the annexin V Cy5.5 probe (Fig. 5A). Significantly more (7.24%) of insulin-positive, 7-AAD-negative cells isolated from db/db mice had a signal in the FL4 (Cy5.5) channel compared with only 0.59% cells derived from control mice.

In vivo–administered annexin V Cy5.5 specifically accumulated in the pancreata of db/db mice, as evidenced by the significantly higher NIRF signal yielded by the tissue from db/db compared with control C57BLKS/J tissue (Fig. 5B). Histological examination of pancreata from diabetic mice showed Cy5.5^+ foci due to accumulation of the probe (Fig. 5C), with no Cy5.5 signal in sections derived from control pancreata. TUNEL staining was consistent with the Cy5.5 staining. Relative apoptotic rates as determined by annexin V Cy5.5 and TUNEL were comparable (2.3 ± 0.3 and 2.0 ± 0.12%, respectively) (Table 1). The higher levels of apoptosis detected in db/db versus control mice correlated with differences in blood glucose levels between the two groups.

**DISCUSSION.**

Diabetes is a major health care issue that is reaching epidemic proportions. Currently, the clinical diagnosis of diabetes is based on measurement of glucose levels. However, abnormal blood glucose levels are a relatively late marker of the disease. Being able to identify early events in the progression to diabetes as diagnostic markers could enhance the potential for successful therapeutic intervention and lead to better understanding of the natural history and the pathology of the disease.

In our study, an annexin V Cy5.5 probe was assessed for its potential to detect diabetes-associated β-cell death in mouse models of type 1 and type 2 diabetes, both in vitro and ex vivo, as a first step in noninvasive imaging of dying β-cells. However, our current concept of an expected low frequency of apoptosis over a fairly long time span increases the difficulty in measuring apoptosis in most models of diabetes.
The time points used for assessment of apoptosis in the MLDS and NOD models of type 1 diabetes were early in the progression to diabetes, as reported for both models: in the MLDS model immediately after 5 days of STZ treatment (1,41) and in 3- to 12-week-old NOD mice (3). Whereas occasional intraislet lymphocytes were detected at 3–4 weeks of age (43–47), extensive lymphocytic infiltration is usually seen by 8 weeks of age. As expected from previous studies, our analysis using this new probe found elevated β-cell death in pancreata of STZ-induced diabetic mice (1,4,41,48) and increasing levels of apoptosis in NOD mice between 3 and 12 weeks of age. This latter finding is consistent with the observed differential priming of diabetogenic CD8+ T-cells in the pancreatic lymph nodes of NOD mice between 4 and 8 weeks of age (5) and parallels the trend described by O'Brien et al. (3). In addition, we identified increased apoptosis in pancreata of diabetic db/db mice (49). Remarkably, in the latter model, apoptosis was detected in both endocrine and exocrine tissue. This observation suggests the contribution from lymphocytic cell death to the total pool of apoptotic events, a possibility that, to our knowledge, has not been addressed systematically. The detection of immunological markers and inflammatory mediators in type 2 diabetes (50–52), however, has engendered speculations of immune cell involvement (53,54). Apoptosis itself can induce an immune response (53,55), and it is certainly feasible that depending on the magnitude and duration of the initial pathology, secondary phenomena, including insulinitis, may appear with different timing and severity. Generally, levels of apoptosis, as determined by FACS of isolated primary pancreatic β-cells, were higher than the ones reported by ex vivo annexin V Cy5.5 staining. This is possibly due to the somewhat damaging isolation procedure, which has likely resulted in overestimation of the percentage of dead or dying cells by FACS.

We validated the specificity of in vivo-administered annexin V Cy5.5 for apoptotic cells. The feasibility of using labeled annexin V as an in vivo probe is well supported by previous research. In humans, 99mTc-labeled annexin V has been used to detect apoptosis in the hearts of patients with acute myocardial infarction (34) as well as during acute cardiac rejection (56) and in an intracardiac tumor (57). More recently, 99mTc-labeled annexin V was used in a quantitative tumor apoptosis imaging study using single-photon emission computed tomography in patients with head and neck carcinoma (35,36). Biodistribution and dosimetry measurements of various forms of 99mTc-labeled annexin V in humans point to the safety of this tracer and its suitability for use in a clinical setting (58–60). In rodents, 99mTc-labeled annexin V was tested as a marker of apoptosis in a murine model of immune arthritis (61) and autoimmune myocarditis (62) as well as of chemotherapy-induced apoptosis in breast cancer (63) and hepatoma xenograft models (64). Annexin V labeled with the fluorophore Cy5.5 (Cy) has been used as a probe for imaging of tumor apoptosis using NIRF in nude mice (38,39). Consistent with our observations, no toxicity was observed at doses up to 2.5 mg/kg, which is the highest reported dose (38). In addition, the small size of annexin V (35 kDa) allows for its rapid renal clearance (39).

We propose that this probe has potential as an in vivo imaging tool because of its accumulation in pancreata of diabetic mice after delivery through the circulation. We were able to observe clusters of dying cells in excised pancreata in different diabetic animal models after intravenous injection of the probe. This study was limited to ex vivo imaging, although advances in optical imaging technology that will be essential for progress toward noninvasive in vivo measurement are anticipated. Issues such as tissue scattering, limited depth penetration, low spatial resolution, and inadequate capacity for quantitative esti-
mation of probe accumulation need to be addressed in order to use in vivo optical imaging to detect and characterize discrete events such as isolated clusters of apoptotic cells within an internal organ.

Despite these shortcomings, optical imaging has several attractive features such as the use of nonionizing low-energy radiation, high sensitivity with the possibility of detecting micrometer-sized objects, continuous data acquisition, and the development of potentially cost-effective equipment. Fast and relatively easy imaging procedures make this modality attractive for potential clinical use. Recently, several technical advances in developing highly sensitive detection devices have led to the biological use of cooled charged-coupled device cameras capable of imaging very low levels of light emitted from internal body organs of rodents. At the near-infrared region between 700 and 900 nm, absorption by intrinsic photoactive biomolecules is low and allows light to penetrate into the tissue to a depth sufficient for applications in small animals (59). Therefore, imaging in the near-infrared region has the advantage of minimizing tissue autofluorescence and dramatically improving the target-to-background ratio (60). Fluorescence imaging can be carried out at different resolutions and depth penetrations. For example, some progress in resolving the above-mentioned problems has already been made by the application of fluorescence-mediated tomography, which delivers tomographic reconstruction of the image by mathematical modeling of

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**FIG. 4.** Ex vivo apoptosis detection using annexin V Cy5.5 in pre-diabetic NOD mice. A: Ex vivo NIRF imaging of pancreata derived from 8- and 12-week-old NOD mice. Muscle tissue defined background signal. Strongly fluorescing foci were visible in NOD mouse pancreata. Results are representative of three animals. B: Fluorescence microscopy of adjacent pancreatic tissue sections derived from 3-, 6-, 8-, and 12-week-old NOD mice. Pancreatic islets were identified on H&E-stained sections. One adjacent section was observed under the Cy5.5 filter to evaluate annexin V accumulation. The other adjacent section was subjected to TUNEL staining (FITC channel). There was a good correlation between TUNEL and annexin V Cy5.5 staining. Arrows point to cells staining with annexin V Cy5.5 and with TUNEL.
diffusion and scattering and has achieved resolution of 1–2 mm and nanomolar sensitivity (65). Alternatively, the application of a recently described multimodality strategy (66), combining the sensitivity of optical imaging methods with a modality delivering higher spatial resolution and tomographic capabilities, would represent a step toward in vivo use of similar probes.

In vivo–delivered probes for the detection of apoptosis in intact animals could potentially allow real-time evaluation of cell death and thus circumvent the problems associated with using “frame-by-frame” in situ methods to measure apoptosis, a process that occurs over time. Such in vivo–delivered probes would permit the tracking of the time course of diabetes-associated β-cell loss and would expand the range of tools available for study and ultimately for early detection of the disease. Furthermore, this technique could be used for evaluation of islet viability after transplantation, a therapy for patients with type 1 diabetes that has become more effective in the past few years (67,68). The utility of our approach in this scenario is underscored by the evidence that even in optimal conditions and in the absence of graft rejection, ~60% of transplanted islet tissue is lost 3 days after transplantation (69,70). Still, we need to emphasize that this study only represents a first step toward a potentially novel and valuable imaging strategy, and its true utility as a diagnostic tool remains to be validated.

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