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AUTOIMMUNE DIABETES

DIANE MATHIS AND JASON GAGLIA

AUTOIMMUNE DIABETES

Type 1 diabetes (T1D), previously encompassed by the terms insulin-dependent diabetes, type I diabetes, or juvenile-onset diabetes, is a classic organ-specific autoimmune disease (for review, see the study by Rose and Mackay[1]). It unfolds in two stages: an occult phase, termed insulitis, when a mixed population of leukocytes invades the pancreatic islets of Langerhans, eventually provoking specific destruction of the insulin-producing β cells; and an overt phase, diabetes, when most β cells have been destroyed or disabled and insulin production is no longer sufficient to regulate blood-glucose levels, resulting in hyperglycemia. Progression from the first stage to the second is not inevitable; nor, when it does occur, it is immediate, taking anywhere from days to decades.

Although autoimmune diabetes is an ancient and increasingly frequent disease, we remain frustratingly ignorant of its etiology and pathogenesis. We do not know what triggers it, have only limited understanding of the genetic and environmental factors that regulate its progression, and have a confused view of the final effector mechanism(s). Consequently, we still do not know how to prevent or reverse T1D in a sufficiently innocuous manner to be therapeutically useful. A major impediment to more rapid progress has been that the location and organization of the pancreas prevent us from readily observing disease initiate and unfold. Consequently, T1D is not diagnosed until very late in pathogenesis when most of the causal events have already played out, and viable options for intervention are much more restricted. A related impediment has been that we have no effective method for monitoring the influence of therapeutic interventions on the course of diabetes, only very indirect and slow indicators, rendering clinical trials long and expensive. It is not surprising that most biotech and pharmaceutical companies do not rush to test their new drug leads on T1D patients and set a very high threshold for entering into clinical trials aimed at preventing or curing this disease.

The Non-Obese Diabetic Mouse Model of T1D and Engineered Derivatives of It

Faced with the difficulties of human experimentation, many investigators have turned to small-animal models of diabetes, in particular, the non-obese diabetic (NOD) mouse strain and simplified derivatives of it. Developed in the late 1970s, the NOD strain is now the most commonly used animal model of autoimmune diabetes (for review, see the study by Anderson and Bluestone[2]). Disease develops spontaneously in these mice, sharing several critical features with the human disorder. As in man, the course of pathology is protracted: insulitis begins at about 4 weeks of age, but diabetes is not evident until 15 to 25 weeks. Again like the human disease, these animals' diabetes is primarily T-lymphocyte-mediated, in both cases, T cells are prominent participants in the insulitic lesion, anti-islet T cell autoreactivity can be demonstrated, and disease is modulated by drugs that interfere with T cells. Finally, both the human and murine disorders are under complex polygenic control, by far the most important contributor being the human leukocyte

antigen (HLA) locus in man and the analogous major histocompatibility complex (MHC) in mice. Importantly, the disease-associated MHC class II molecules in the two species show common structural features, which differ from their non-disease-associated counterparts.[3]

The NOD mouse strain has permitted many of the outstanding issues in the diabetes field to be addressed by direct experimentation, and results on these mice have heavily colored our view of diabetes pathogenesis. Yet, disease is asynchronous and complex in this model, involving complicated genetic and environmental influences and entailing multiple initiating antigens, an array of interacting cellular players and a multiplicity of terminal effector mechanisms. Thus, certain critical questions have remained intractable and diverse strategies have been taken to simplify the NOD disease to highlight particular features by ablation or exaggeration to facilitate experimental dissection.

One such strategy is the generation of T cell receptor (TCR) transgenic (tg) mice to focus on the role of T lymphocytes. The TCR, displayed on the surface of T lymphocytes, is responsible for the recognition of antigens bound to MHC molecules. In such tg animals, the TCR transgenes inhibit rearrangement of endogenous TCR loci, resulting in a T cell repertoire greatly skewed for the transgene-encoded specificity. Several years ago, a tg line carrying the rearranged TCR genes from a diabetogenic T cell clone from a diseased NOD mouse was engineered.[4] The BDC2.5 clone is $CD4^+$, it secretes T_{helper} 1 characteristic cytokines including interferon (IFN)-γ, recognizes its antigen when presented by the MHC class II A^{g7} molecule, and is specific for a β-cell antigen. The precise protein recognized by this clone remains unknown, but it has properties similar to those recognized by several other murine[5,6] and human[7,8] β-cell-reactive T cell clones. BDC2.5 TCR tg mice propagated on the NOD genetic background have a T cell repertoire highly enriched for the transgene-encoded specificity, exhibit rampant insulitis starting around 3 weeks of age, but show diabetes in only 15 to 30% of animals at 20 to 30 weeks.[4,9] Introducing the severe combined immunodeficiency (SCID) mutation or a recombination-activating gene-null mutation into the BDC2.5/NOD line renders the T cell repertoire monoclonal, which results in an even more penetrant and rapid disease—insulitis once again at approximately 3 weeks of age, but now with diabetes in essentially all individuals immediately thereafter[10,11]—nicely demonstrating the critical importance of immunoregulation by other T cells. A TCR tg line corresponding to a second $CD4^+$ T cell clone from a diabetic NOD mouse has also been reported.[12] This clone, NY4.1, also recognizes an unknown β-cell antigen in the context of MHC class II A^{g7} molecules,[13] but it has additional promiscuity.[12,14] The NY4.1 TCR tg line partially backcrossed onto the NOD background shows strong insulitis at approximately 3 weeks of age and highly penetrant diabetes already at 6 to 9 weeks. Finally, three tg lines carrying the rearranged TCR genes from $CD8^+$ T cell clones derived from NOD mice have been described as well.[15–17] Two of them[15,17] express a TCR-α chain that has repeatedly been observed with K^d-restricted, β-cell-specific $CD8^+$ clones from NOD mice.[18–21] All three TCR transgenics develop insulitis and diabetes more intensely and rapidly than regular NOD animals.

Thus, there exists a set of powerful murine models of T1D, each one with its inherent advantages and disadvantages. We do not know at present which features of human diabetes are most faithfully mimicked in which of the models. Indeed, given the heterogeneous symptoms, courses and genetics exhibited by individual patients, it may well be that human diabetes is really a multiplicity of diseases.

A Critical Need for Noninvasive Imaging of Insulitis and the Pancreatic Islets

As alluded to above, a major hindrance in the diabetes field, in both experimental and clinical contexts, has been the inability to directly visualize disease onset, progression, and reversal. Experimentally, investigators are constantly challenged by the scatter in disease parameters and variability in disease course exhibited by individual animals, even individuals derived from inbred strains and housed in pathogen-free conditions, and thus purportedly genetically and environmentally identical. For example, a particular 15-week-old NOD mouse may

develop overt diabetes in days, weeks, or months or, for a significant fraction, may never develop it.

Another issue that constantly confronts researchers is the extent to which events and processes occurring in vivo are mimicked in the in vitro or even ex vivo context. The islets constitute defined structures within the pancreas, and the behavior of β cells is influenced by interactions with both intraislet and extraislet cells in the vicinity, as well as by the unique microenvironment created by the details of the pancreatic vasculature. Removal of islets from the pancreas or, worse, β cells from the islets, is almost certain to perturb β-cell behavior.

Clinically, a diagnosis of overt diabetes generally implies that most of a patient's β cells have been destroyed or disabled, meaning that the early, autoimmune, phases of the process have already played out and that strategies for disease reversal, in particular immunointervention, are already severely limited. In recognition of this impediment, much effort has been devoted to identify markers that indirectly signal at least the major landmarks of pathogenesis. So far, serum autoantibody titers seem to be the best indicators of islet inflammation, nicely confirmed in the Diabetes and Prevention Trial (DPT)-1 (reviewed in the studies by Devendra and colleagues,[22] Gottlieb and Eisenbarth,[23] and Seissler and colleagues[24]). The appearance of specificities against at least one islet-β-cell antigen is generally considered to reflect insulitis; emergence of specificities against two or more predicts eventual conversion of insulitis to diabetes. Although the technology is certainly moving forward, no T cell assay is currently accepted as a reliable disease indicator, at least not in isolation.[25] Plasma C-peptide levels are today the most accepted indicator of β-cell activity: secreted in equimolar concentration with insulin, C-peptide is a semiquantitative marker of β-cell secretion—a marker, in contrast to insulin, not subject to hepatic extraction.[23,26] However, C-peptide measurements have methodological limitations that compromise their precision under nonsteady state conditions.[27]

Although following autoantibody titers and C-peptide levels certainly provide useful information, a noninterventive means of directly following disease progression would have important clinical applications.

a. Aiding in difficult disease diagnoses
There is mounting appreciation of the heterogeneity of T1D and of the fact that not all patients' diseases present or progress with the classical profile.[28,29] The ability to visualize insulitis and watch it evolve should aid us in diagnosing and treating atypical cases, for example, people with type 1b diabetes, who exhibit severe insulin deficiency in the absence of traditional markers of β-cell autoimmunity and do not have the expected HLA alleles[30,31]; or Latent Autoimmune Diabetes of Adults (LADA) patients, now estimated to constitute 5 to 30% of individuals initially diagnosed as having type 2 diabetes.[32–35]

b. Monitoring disease course
The natural history of T1D in human patients is largely a mystery, our present view being too heavily influenced by results from mouse models. Most of the information available on humans, themselves, derive from either indirect indicators or rare examinations of patient material obtained fortuitously. Recently, pancreas biopsies were laporoscopically "punched" from a set of T1D patients and provided some information on the ongoing inflammatory processes,[29,36] but this procedure is too invasive for anticipatory or widespread employment. Direct visualization of the insulitic lesion in a large patient population should furnish important new insights, perhaps suggesting novel criteria for disease stratification. Sequential visualization of the pancreas in genetically at-risk individuals may permit detection of the earliest signs of autoimmune attack, when immunointervention may have its greatest chance of success, and in autoAb-positive individuals may allow prediction of the conversion to overt diabetes.

c. Evaluating the effects of immunointervention
There is presently no reliable method to monitor diabetogenic T cells in the blood although advances in "tetramer" technology have recently instilled some hope in this regard.[37,38] It seems that the frequency of β-cell-reactive T cells in

the blood is too low to permit direct ex vivo detection over background, likely because they are sequestered in the pancreas, within either the islets or the pancreatic lymph nodes (PLNs).[39] This greatly complicates efforts to follow the effects of therapeutic intervention, presently a priority for those organizing expensive long-term clinical trials. Direct visualization of leukocytic infiltrates in the islets could circumvent this problem, permitting us to detect the disengagement of attacking leukocytes and thereby have an earlier indication of therapeutic benefit.

d. Following the fate of transplanted islets
There is no gold standard for the assessment of islet function post transplant. Although it is known that immunologic and nonimmunologic events can lead to significant graft loss after transplantation, monitoring these processes has proven difficult. Due to lack of the ability to directly measure β cell mass, pancreas and islet transplant recipients have their islet function indirectly monitored via their need for exogenous insulin, glucose control, frequency of hypoglycemia, and measurements of basal or stimulated C-peptide concentration (as a marker of endogenous insulin production). More accurate means of direct, noninvasive monitoring of engraftment and rejection would significantly advance the field.

Unfortunately, imaging the pancreatic islets represents a significant challenge for a number of reasons, meaning that some major problems have been encountered in attempts at in situ visualization. For example, the pancreas is not easily accessible and can be shielded by other major organs, in particular those, such as the liver, that tend to accumulate imaging probes (Figure 1). In addition, islets represent only a few percent of pancreatic tissue are dispersed unevenly throughout the pancreas and lack intrinsic contrast. Lastly, insulitis is chronic, yet asymptomatic, and seems to be very heterogeneous in extent, composition, and aggressiveness.

Recent impressive advances in the field of cellular and molecular imaging offer great hope for overcoming these stumbling blocks. Development

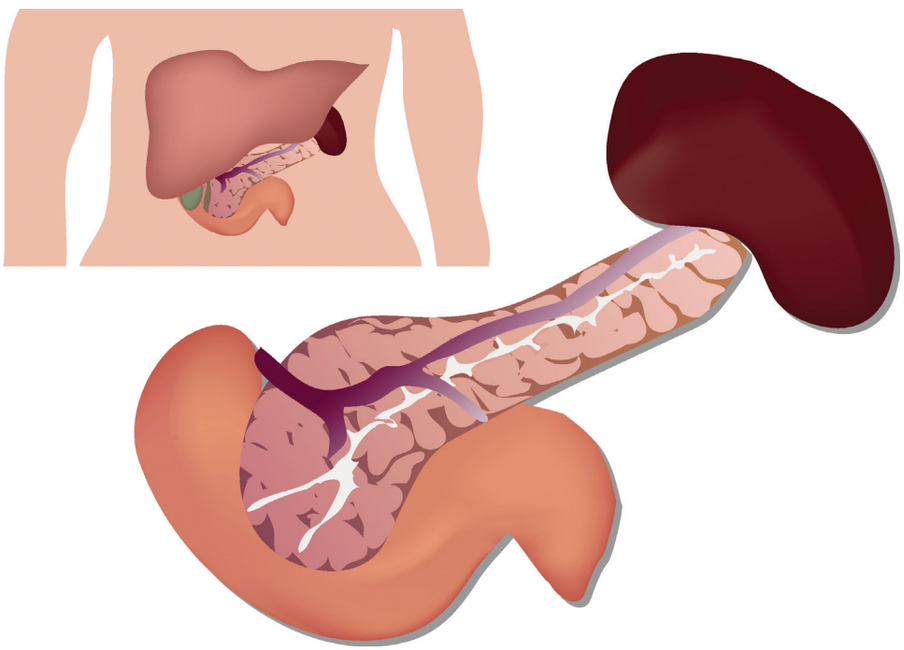

Figure 1. Anatomic position of the pancreas. The pancreas is bordered by the stomach, duodenum, liver, and spleen. The head of the pancreas lies in the curve of the duodenum while the body crosses the midline of the torso and the tail extends toward the hilum of the spleen.

of noninvasive means to visualize the pancreatic islets should allow us to see both the evolution of the disease process and its devolution subsequent to a successful therapy. Two types of imaging methods are imperative: visualization of immune cells invading the islets and visualization of the mass or activity of islet β cells. In other words, we need to evaluate both the immune system attack and its consequences. This chapter will summarize the state-of-the-art on both fronts, considering islet transplantation and T1D itself, in both experimental models and humans.

LEUKOCYTE INFILTRATION OF PANCREATIC ISLETS (INSULITIS)

Insulitis in Murine T1D

T1D develops in mouse models because of a breakdown in immunological tolerance, at one or multiple levels. A fairly well-accepted scenario for spontaneous disease initiation in NOD mice and derivative models continues to gain experimental support[40,41] and is as follows. Reflecting a normal physiological process, tissue remodeling, a ripple of islet β-cell death occurs between 2 and 3 weeks of age, resulting in the release of β-cell-derived antigens as apoptotic bodies, cell fragments, proteins, or peptides. These antigens are picked up by dendritic cells (DCs) patrolling the area, which further process them, display them on their surface as MHC molecule:peptide complexes, and ferry them to the draining PLNs. There, the DCs displaying MHC molecule:β-cell-antigen complexes are encountered by naïve T cells, which constitutively circulate through the blood and lymph nodes but do not access the other tissues. In the normal state, the T cell repertoire has been tolerized to self-antigens, so there are no pathogenic sequelae to the DC/T cell encounter, but in diabetes-susceptible individuals, β-cell-reactive T cells remain in the repertoire and can be stimulated when they are exposed to their cognate antigen. The now-activated β-cell-reactive T cells take on new migratory properties and begin filtering through the tissues. Within the pancreatic islets, they re-encounter cognate antigen, and they are re-stimulated and retained thereby seeding leukocytic infiltration, or insulitis (Figure 2).

In NOD mice, $CD8^+$ (cytotoxic) T cells are detectable and required at the earliest stage of disease initiation,[42,43] but it is still not known precisely what they do at that point. The earliest leukocyte infiltrate is dominated by $CD4^+$ (helper) T cells, eventually supplemented by substantial $CD8^+$ T and

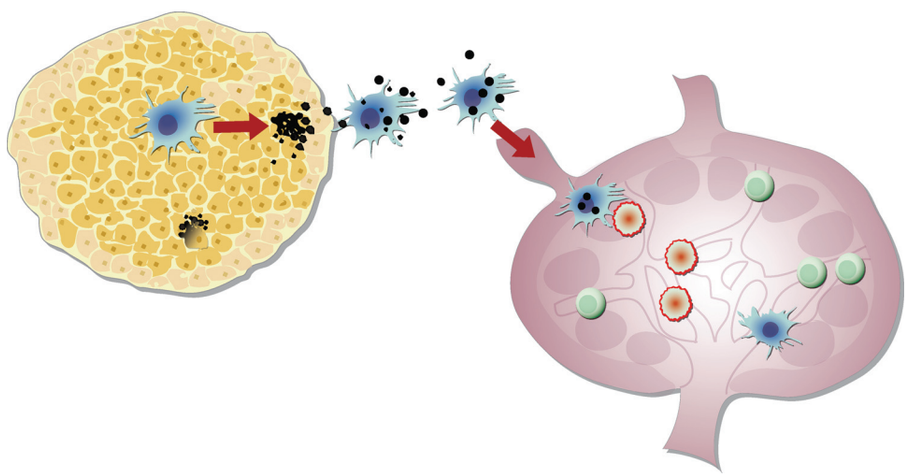

Figure 2. Development of insulitis in susceptible mice. In mice of all strains, a ripple of β-cell death occurs physiologically approximately 2 weeks of age. In susceptible mice, antigens derived from dead β-cells (shown in black) are taken up by dendritic cells (DCs) (blue cells) in the pancreatic islets (left), inducing their migration to the pancreatic lymph node (PLN) (right) with their maturation en route. In the PLN, the DCs present β-cell-derived antigens to naive β-cell-reactive T cells (green cells) in circulation and activate them. Upon recognition of major histocompatibility complex molecule-β-cell peptide complexes, the naive T cells become activated (red cells), acquiring the ability to migrate through the tissues. In the islets, the activated T cells re-encounter cognate antigen are retained and become reactivated, thus initiating insulitis.

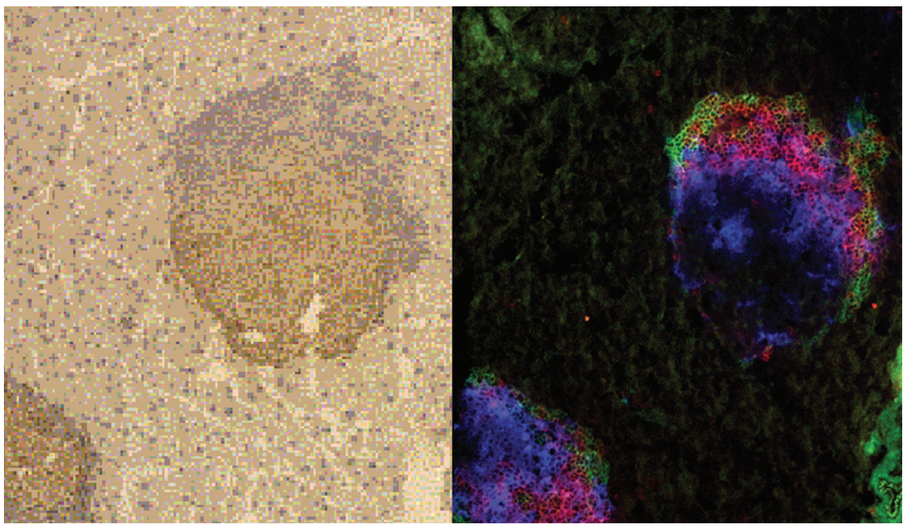

Figure 3. Histology of insulitis. Adjacent pancreas sections from a 16-week-old prediabetic non-obese diabetic mouse are shown. The left section is stained for insulin (brown) with inflammatory infiltrate in blue. The right section is stained for B cells (B220, green), $CD4^+$ T cells (CD4, red), and dendritic cells (CD11c, blue). There is marked inflammatory infiltrate around the insulin positive islets, sparing much of the exocrine pancreas.

B cell populations (Figure 3). Significant numbers of natural killer cells, DCs, and macrophages can also be observed through disease progression and play important roles in diabetes pathogenesis. Debate on the cell type(s) responsible for the death of the bulk of β cells actively continues.

There is great (and experimentally frustrating) mouse-to-mouse variation in the progression from insulitis to diabetes in NOD mice (and most of its derivative models). For example, 100% of males and females show insulitis, so far histologically indistinguishable, but few males (usually ~20%) become clinically diabetic; on the other hand, a high fraction of females (on the order of 80%) develops diabetes, but at ages spanning about 12 to 30 weeks old, with no evident explanation for this broad range.

Insulitis in Human T1D

Today, there is not enough, or good enough, data to be able to definitively judge to what extent the above-described scenario applies to human T1D patients. Our view of human insulitis emanates mostly from rare opportunities to directly assess pancreas samples. These specimens have come primarily from subjects who underwent surgical resection of the pancreas[44] or autopsy.[45,46] It has proven even more difficult to obtain organs from at-risk individuals presumed to have insulitis (due to the detection of autoantibodies) but who have not yet developed clinical disease. Given the critical importance of this issue, efforts are under way by the Juvenile Diabetes Research Foundation to identify such organs from autoantibody-positive donors, with the goal of making these specimens available for study.

In recent-onset diabetes patients, the leukocytic infiltrate was reported to consist mostly of T cells and macrophages, with a predominance of $CD8^+$ over $CD4^+$ T cells.[44–47] Abnormalities in the islet capillary endothelium were observed, including upregulation of MHC class I molecules and ICAM-1, as well as marked dilation.[45,47] It is difficult to evaluate at this point to what extent any apparent divergences in human and murine insulitis reflect pathogenetically relevant differences. Or do they rather reflect artifacts of sampling or sample processing, the advanced stages generally examined in humans, or disease heterogeneity? Clearly, these largely anecdotal observations, though still important, are not adequate; hence, a critical need for a method of noninvasive imaging of leukocytic infiltration of the islets in humans.

Imaging Insulitis

To date, several general approaches to in vivo visualization of insulitis have been exploited, either experimentally in mice or clinically in humans: targeted molecular probes, cells marked either genetically or with extrinsic labels, or vasculature imaging techniques.

Targeted Molecular Probes

This strategy entails intravenous injection of a labeled molecule followed by visualization. A given molecular probe is chosen because, for one reason or another, it concentrates in the islet infiltrate, reflecting some feature of the pathological processes playing out.

Up to now, the most encouraging results along these lines have been obtained with ^{123}I-labeled interleukin (IL)-2, a cytokine that functions primarily as a T-cell growth factor. In theory, cytokines have significant potential as imaging probes because they are small molecules, can have high affinity for their receptor(s), and tend to have a favorable half-life and blood clearance. IL-2 seemed a propitious choice in the T1D context because its receptor is known to be upregulated on activated lymphocytes, which predominate in the insulitic lesion; indeed, lymphocyte expression of the IL-2 receptor could be detected histologically in rodent insulitis.[48] Signore and colleagues validated ^{123}I-(IL-2) as an imaging probe for lymphocytic infiltrates[49,50] and reported that it bound preferentially to insulitic versus normal pancreata from both NOD mice and the biobreeding (BB) rat model of T1D.[49–52] This result was later confirmed by another group, using ^{125}I-(IL-2), but their biodistribution studies failed to demonstrate pancreas-specific probe concentration,[53] challenging the utility of this technique in the absence of simultaneous anatomic localization, for example, by computed tomography (CT) or magnetic resonance imaging (MRI).

As concerns humans, preliminary findings on prediabetic individuals, that is, before clinical manifestation of disease, have been promising, but the method has proven less sensitive for recent-onset diabetes. Five of ten T1D patients, within 90 days of insulin initiation, and five of five first-degree relatives showing insulin autoantibodies and an impaired first-phase insulin response had significant probe accumulation within the pancreas, compared with zero of ten normal controls.[54] In the newly diagnosed T1D patients, no correlation was found between ^{123}I-(IL-2) uptake and anti-GAD or anti-IA-2 antibody titers. Although very few patients have been studied with this method so far, the lack of correlation between the imaging signals and known markers of anti-pancreas autoimmunity implies worse sensitivity than current serologic methods of disease staging/prediction.

Another molecular probe with reported use in the diabetes context is technetium-99m-labeled polyclonal human immunoglobulin (Ig)-G. Scintigraphy of ^{99m}Tc-(hu-IgGs) has been successfully applied to several inflammatory diseases, including osteomyelitis[55] and rheumatoid arthritis.[56] It is believed that the radiolabeled Igs accumulate at sites of inflammation by binding to Fc receptors on infiltrating macrophages or by nonspecific mechanisms such as increased macromolecular leakage through inflamed endothelium[57] (see below). Although Ig deposition has been identified in histology of infiltrated human islets,[45] results to date on visualization of radiolabeled IgG in the T1D context have not been very impressive. In a study of 15 recently diagnosed patients, within 4 weeks of presentation, ^{99m}Tc-(hu-IgGs) were found to concentrate in the pancreas in less than half of the cases, and accumulation did not correlate with the titer of autoantibodies (anti-GAD or anti-IA-2) or with metabolic parameters.[58]

There has been increasing interest in the potential for exploiting positron emission tomography (PET) of molecular probes to monitor inflammation. 2-[^{18}F]fluoro-2-deoxy-D-glucose (18-FDG) is a radiolabeled glucose analog wherein one hydroxyl group is replaced with fluorine-18. 18-FDG is transported into cells, phosphorylated there like glucose, and trapped. In cells harboring phosphatase activity, probe washout is eventually noted. 18-FDG is a useful tumor detection agent due to the enhanced glucose utilization and low phosphatase activity in many neoplasms.[59,60] This agent is also taken up very effectively by several inflammatory cell types, with uptake associated with inflammation often showing washout on delayed imaging due to intrinsic phosphatase activity.[61] Although the uptake of 18-FDG

by infiltrated islets from diabetic mice was enhanced compared with that of normal islets, this technique has yet to be used for whole-body imaging, in either mice or humans, as the relatively small differences in 18-FDG intensity that occur, combined with the low resolution of PET, have been limiting.[62]

The general approach of imaging molecular probe accumulation has the advantage of relative ease of application in both experimental and clinical settings. But it also has several clear disadvantages. First is the requirement for an easily detectable but innocuous label. Second is the issue of specificity: the biology of the probe's target is often more complex than anticipated. For example, ^{123}I-IL-2 turns out that IL-2 binds to low-, medium-, and high-affinity receptors, which are represented to varying degrees on different leukocyte subsets. In particular, the high-affinity IL-2 receptor is now known to be prominent on regulatory T cells, in addition to activated T and B cells, probably explaining the reported probe accumulation in many tissues in both normal and diabetes-prone rodents.[53] Together, these two caveats render it extremely difficult for such a method to distinguish infiltrated islets from the closely associated PLNs. Yet, the potential ease of application ensures that the general approach of imaging molecular probes will continue to be actively pursued. As more and more mechanistic information accumulate, novel molecular targets will be identified, perhaps one with ideal properties.

Imaging Labeled Cells

Another general approach to visualize insulitis is to monitor the pancreatic islet accumulation of tagged leukocytes. Activated lymphocytes are a reasonable option because they represent substantial populations within the islet infiltrate, and, unlike naïve lymphocytes, they traffic through tissues rather than being confined to the blood and lymph nodes.[63] An even better alternative is activated T lymphocytes that specifically recognize a β-cell antigen because upon recognition of cognate antigen in the islets they will be re-stimulated and retained thereby amplifying their islet-specific accumulation. The lymphocytes can be equipped with any tag that can be encoded in the genome and expressed efficiently or bound or taken up stably and innocuously and can be imaged effectively.

Multiple modalities for labeling cells for tracking in the NOD mouse system (or derivative TCR-tg systems) have been reported over the past few years. All of the reports described tracer accumulation in the pancreas region, either ex vivo[64] or in vivo,[65–67] subsequent to probe injection into lymphocyte-depleted NOD animals. Moore and colleagues[64] exploited the tat membrane-translocation signal from HIV-1 to enhance lymphocyte uptake of cross-linked iron oxide magnetic nanoparticles (CLIO-Tat), followed by MRI. The methodology used by Billotey and colleagues[67] was quite similar, except that they used a novel class of magnetic nanoparticles (MNPs), rendered anionic due to external treatment of the iron core with dimercapto succinic acid (AMNPs), and introduced them into T cells. Internalization and MRI visualization of the AMNPs was augmented due to their negative charge. Srinivas and colleagues[66] chose their imaging platform to optimize probe localization and quantitation. T cells were labeled with a perfluoropolyether (PEPE) nanopartide tracer agent and were detected in vivo via spin density-weighted ^{19}F MRI. The ^{19}F MRI was very selective for the labeled cells, while anatomical context was provided by concomitant ^{1}H imaging. The nonspecificity of lymphocyte-tracking experiments was addressed by the innovative strategy of Moore and colleagues.[65] These investigators prepared CD8^{+} T cells from a TCR-tg mouse line that were highly enriched for those displaying a TCR specific for a particular β-cell peptide. The T cells were loaded with MNPs derivatized with a "tetramer" of MHC molecule: β-cell-peptide complexes and tracked by MRI. Although the results from this set of studies suggest that it might be possible to visualize large numbers of tagged lymphocytes in the mouse pancreas by MRI, the eventual utility of this method is not entirely evident. In these experiments, the cells were introduced into lymphocyte-deficient NOD mice carrying the SCID mutation or irradiated, which have no T or B cells, lymph nodes, insulitis, or diabetes. It is not known whether enough cells can localize to the pancreas of mice with a full lymphocyte complement to be readily detectable or how much interference there

will be from signals emanating from the other organs harboring lymphocytes, above all the PLNs, spleen, and liver.

Several groups have used optical imaging techniques, particularly bioluminescence, for tracking lymphocytes (reviewed in a study by Hardy and colleagues[68]). Lee and colleagues exploited luciferase-expressing $CD4^+$BDC2.5 T cells after adoptive transfer into NOD/SCID recipients to examine the mechanism by which non-$CD4^+$ splenocytes contribute to accelerated disease.[69] Because the reporter is encoded in the genome in this system, the signal is not diluted by cell division; however, local positional effects including scatter, rotation, and attenuation as well as limited photon penetration may all complicate photon-counting measurements in vivo. Although the results from these experiments suggest that it may be possible to track and monitor the expansion of a large number of antigen-specific T cells in a mouse model via bioluminescence, this rather artificial system, using an otherwise immunodeficient recipient, suffers from the same limitations as described above in translation to a more physiologic model.

Some attempts have also been made at fluorescence imaging of insulitis with confocal or 2-photon microscopy techniques to gain a better understanding of the processes that lead to the development of T1D on a cellular basis. These techniques are only applicable to animal models and are inherently invasive, requiring surgical exposure of the pancreas. Many of the problems with these approaches, including difficulty visualizing islets, limited field of depth, changes in the pancreas with externalization, and some potential solutions are reviewed in the study by Martinic and von Herrath.[70]

As concerns the human arena, a classic nuclear medicine technique to visualize inflammation is the so-called "white blood cell scan." In this test, a small number of peripheral blood lymphocytes are isolated from a patient and labeled with In^{111} or Tc99m ex vivo, followed by their re-infusion into the same subject, that is, autologous transfer.[71] Application of this technique to insulitis was explored in the 1980s with In^{111}-labeled peripheral lymphocytes.[72] After redistribution from the liver and lungs, a small amount of radioactive tracer did localize to the tail of the pancreas in two out of three patients with newly diagnosed T1D, whereas not in control individuals. However, not unexpectedly, more than 90% of the radioactivity localized to the spleen. These preliminary findings were of limited value as the large, unfavorable difference in tracer localization between the pancreas and adjacent spleen render any calculations of pancreas uptake rather unreliable. Other practical limitations of this technique include the relative radiosensitivity of lymphocytes and they need to be used with caution in the setting of autoimmune disease. Furthermore, subsequent animal experiments in the BB/Worcester rat failed to show localization of In^{111}-labeled lymphocytes to the pancreas.[73]

Because of the variety of methodological options, considering both probe choice and imaging modality, the general approach of imaging marked cells has potential for permitting one to visualize the pancreas in either experimental or clinical contexts. However, there are some significant theoretical limitations. First, there will be extensive nonspecific accumulation of cells in many organs unless the antigen specificity of particular β-cell-reactive T or B cells can be exploited. However, current knowledge about these specificities in humans remains woefully meager. Second, even if specific accumulation of tagged leukocytes in pancreatic islets can be achieved, a great deal of work will need to be invested before we have a sense of what pathogenetic parameters this accumulation actually reads out. Lastly, approval for and implementation of cell-based human protocols can be challenging.

Imaging Alterations in the Vasculature

This general approach relies on changes in blood vessel properties as a marker for insulitis. Alterations in vascular parameters such as transient vasoconstriction, vasodilation, increased blood flow, and vascular leakage precede inflammation and are necessary, helping to orchestrate the influx of diverse leukocyte subsets.[74] So, given that insulitis is basically an inflammation of the islets, modifications of the pancreatic microvasculature are likely to accompany its initiation and evolution. Changes in the intraislet vasculature have been reported to

precede streptozotocin (SZ)-induced insulitis,[75–80] and vascular swelling and modifications of endothelial cell morphology in the para-islet region signal imminent insulitis in NOD mice.[81] In both of these diabetes models and others, increased blood flow and edema ensue.[80,82–84] Such alterations may be detectable by some of the powerful new methodologies for imaging the microvasculature in rodents and humans have been described in recent years.[85,86]

One strategy has been to use biocompatible nanoparticles with prolonged intravascular circulation and macrophage uptake. Results from a series of experiments on the NOD mouse strain and engineered variants of it argue for the utility of probing vascular alterations with fluorescent or magnetic nanoparticles. Exploiting the fluorescent properties of a magnetofluorescent probe, imaged by ex vivo confocal microscopy and flow cytometry, Denis and colleagues[87] demonstrated an enhancement of its accumulation when insulitis was present, resulting from both an increase in microvascular permeability, promoting probe leakage, and an influx of phagocytic macrophages, permitting probe uptake and, thereby, inhibiting its diffusion. The fluorescent probe was able to portray the degree of insulitis aggressivity in the exaggerated BDC2.5/NOD TCR tg model, both during the unfolding of the insulitic lesion over time, and with the differentially destructive lesions of BDC2.5 variants carrying diverse mutations or on a different genetic background. The probe also permitted monitoring of insulitis progression in standard NOD mice, but the signal differential was much less impressive than with the BDC2.5 model.

Noninvasive in vivo imaging of vessel changes and associated probe accumulation could be achieved through MRI of magnetic nanoparticles (MNP-MRI, Figure 4). Although it was possible to distinguish BDC2.5 insulitis from no insulitis at an early stage (ie, at 4 weeks of age), at later times (beyond 6 weeks), this property was lost, which actually matched previous histological data on the lesion "settling down" at advanced ages in this system.[87] Pancreas T2 values 24 hours after MNP injection, in comparison with control muscle T2 values, proved the best indicator. Improvements to the imaging protocol[88] allowed excellent discrimination of both incipient diabetes in BDC2.5 TCR tg mice and recent-onset diabetes in straight NOD animals. Most importantly, it was possible to noninvasively predict within several days of treatment whether mice treated with anti-CD3 monoclonal antibody immune therapy would be cured or not some weeks after the treatment.

To date, imaging MNP accumulation is considered the best validated general approach for

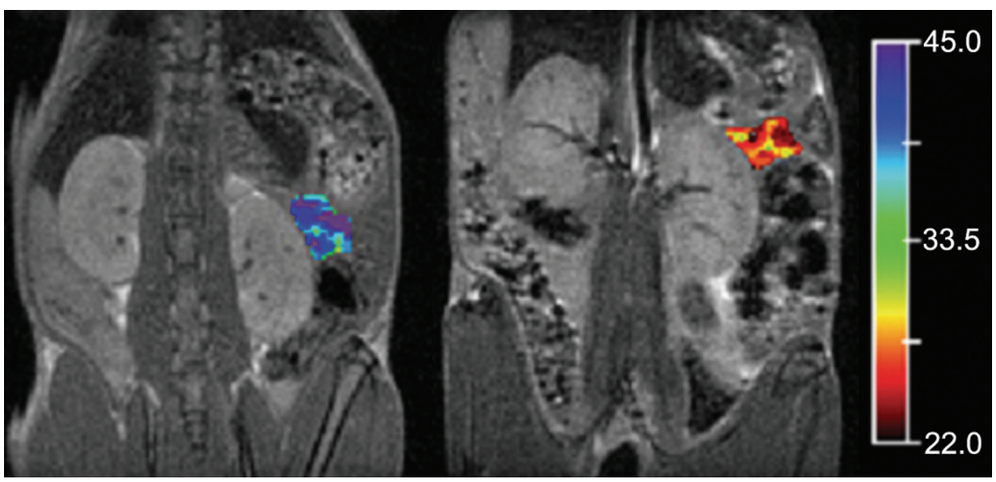

Figure 4. Destructive insulitis corresponds to decreased T2 on MNP-MRI. Female BDC2.5/NOD mice were imaged 24 h after magnetic nanoparticle administration. Images with T2 pseudocoloring (scale corresponding to T2 in msec to the right) over the region of the pancreas are shown. The animal on the left is an untreated nondiabetic control, whereas the animal on the right had been treated with cyclophosphamide 6 d prior, causing a destructive insulitis. The lower T2 in the animal with destructive insulitis reflects increased probe accumulation. Adapted from Turvey SE et al.[88]

visualizing pancreatic insulitis and its conversion to diabetes. Particularly exciting, as mentioned above, is "proof of principle" that this methodology is able to predict clearance of insulitis subsequent to therapeutic intervention.[88] This, together with the fact that analogous probes and imaging protocols have already been used in cancer patients,[89] argue for expedited translation to the clinical context. The major disadvantage of the approach appears to be that, although it does signal inflammation and its evolution, it is an indirect measure, and therefore its pathogenetic correlates need to be carefully established.

Medarova and colleagues[90] sought to confirm the utility of imaging microvascular leakage as an approach to monitoring the unfolding of T1D in mice. They explored the utility of MRI of a long circulating paramagnetic contrast agent, protected graft copolymer covalently linked to gadolinium-diethylenetriamine pentaacetic acid residues labeled with fluorescence isothiocyanate (PGC-GdDTPA-F). They did report vascular changes, presented in a "semiquantitative" fashion, in diabetic mice. However, the model they used, Balb/c mice treated with high-dose SZ is not a model for autoimmune diabetes because there is generally not a leukocytic infiltrate in the islets, rather, they just do not have many β-cells after treatment from a direct toxic effect. So it is not really clear what the relevance the vascular changes signaled in this study has to true T1D.

ENDOCRINE PANCREAS (β-CELL MASS)

Development and Structure of the Pancreas and Islets

The pancreas is a complex organ with both endocrine and exocrine components. During development, it arises from dorsal and ventral buds from the duodenal part of the primitive gut endoderm near the foregut/midgut junction. One ventral bud, or two which quickly fuse into one, becomes the inferior portion of the head of the pancreas, whereas the dorsal bud gives rise to the body and tail. In humans, the head lies in the curve of the duodenum, the body crosses the midline of the torso, and the tail extends toward the spleen. The endocrine cells are embedded within the exocrine pancreas in small groups termed the islets of Langerhans. Islets typically range in size from 40 μm to 400 μm in diameter. They are not evenly scattered throughout the organ,[91,92] with a greater concentration in the tail than the head of the pancreas.[91] Islet architecture differs between mice and humans (Figure 5). In mice, there is a β-cell-rich

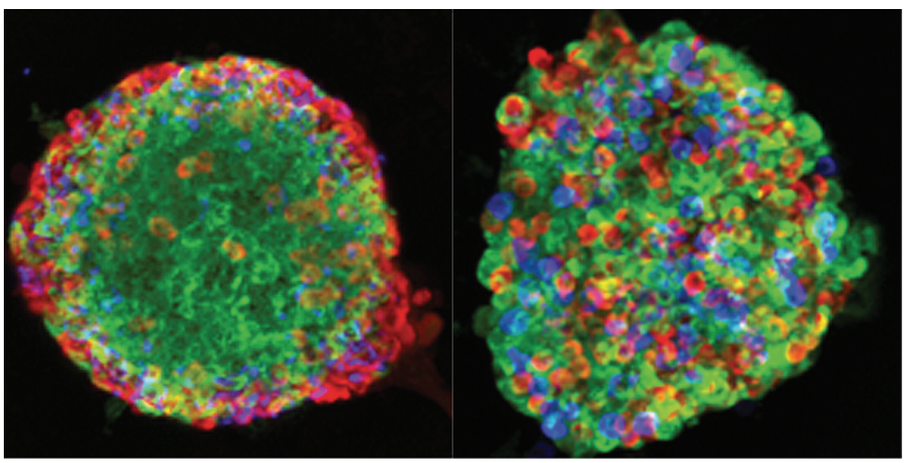

Figure 5. Islet architexture varies between species. 3-D reconstructions with 0° projection with respect to the y-axis on entire isolated islets acquired a 1-μm intervals in the axial (z) axis are shown (× 40 original magnification; β-cells, green; α-cells, red; δ-cells, blue). The image on the left is of a mouse islet, with a typical β-cell core and non-β-cell mantle. The image on the right is of a human islet, with α-cells and δ-cells intermingled with β-cells. Adapted from Brissova M et al.[93]

core with a non-β-cell mantle, whereas in human islets, non-β cells are not confined to the periphery, with a more heterogeneous distribution of the different endocrine cell types throughout the islets.[93,94] In the murine pancreas, islets are typically composed of 70 to 80% insulin-producing β cells, approximately 5% somatostatin-producing δ cells; the remaining 15 to 20% are glucagons-producing α cells or pancreatic-polypeptide-producing (PP) cells, with the relative percentages of these two cell types depending upon embryologic derivation. The head of the pancreas (the duodenal lobe of mostly ventral origin) is enriched for PP cells, whereas the body and tail (the splenic lobe of dorsal origin) for α cells.[93,95] In humans, islets are more variable in composition with generally lower percentage of β-cells (approximately 55%, range 30–75%) and higher percentages of α-cells (approximately 35%, range 10–65%) and δ-cells (approximately 10%, range 1–20%).[93,94] These relative differences must be kept in mind when developing imaging techniques designed to measure islets or β cells.

In newborn children, islets comprise approximately 20% of pancreatic tissue[96] but this fraction declines to 1 to 2% of pancreatic mass by adulthood through a more rapid postnatal growth rate of exocrine tissue. By adulthood, islet mass has increased about 5-fold from that at birth, whereas exocrine tissue increases about 15-fold over the same time period. Ultimately, there are about 1 million islets comprising approximately 1 g of tissue in a normal adult human pancreas, containing around 8 mg or 200 units of insulin at any given time.[97]

Islets are innervated and highly vascularized. Although only representing 1–2% of the pancreatic mass, they receive at least 10% of the pancreatic arterial blood flow.[98] In the rat, arterioles penetrate the islet through discontinuities in the mantle into the core and branch into a number of fenestrated capillaries, forming a glomerular-like network. The efferent vessels coalesce either at the periphery of the islet or into an overlying extensive finger-like postcapillary venule network, depending upon islet size.[99] Although human islets do not have the same structural organization as their murine counterparts, the majority of the endocrine cells in human islets are still within close proximity to vascular cells.[94] As noted above, this rich vascular network may offer a potential imaging target.

Measuring β Cells

Fluorescence Imaging

Molecular imaging techniques have already greatly increased our understanding of the development and function of the pancreas in animal models. Hara and colleagues have described the imaging of β cells and β-cell progenitors labeled with fluorescent proteins under control of the mouse insulin I promoter (MIP) and neurogenin3 (Ngn3) promoter.[92] β cells so marked did not appear to be uniformly distributed, aligning based on embryologic origin, along large blood vessels, and the pancreatic and common bile ducts (Figure 6). Similar findings were reported by Alanentalo and colleagues using ex vivo optical projection tomography with insulin staining of segmental pancreases; they also described preliminary findings on islet distribution with the development of diabetes.[100] Chaillet and colleagues chose another approach to fluorescently label β cells. Using targeted mutagenesis, they developed a mouse with a modified insulin II gene allele, InsIIEGFP, that expresses a proinsulin-enhanced green fluorescent protein (EGFP) fusion peptide. This protein is processed in β cells into insulin and an EGFP-tagged C-peptide, labeling the insulin secretory granules.[101] Although valuable information has been gained from these and related techniques, none of these genetic strategies are translatable to clinical study.

Measurements of Pancreatic Volume

Its minor contribution to pancreas mass and volume as well as its lack of intrinsic contrast makes noninvasive measurements specifically of the endocrine portion of the pancreas difficult, much akin to identifying the proverbial needle in a haystack. However, it has been previously noted on pathologic evaluation of pancreas tissue from individuals who died soon after initial presentation that exocrine atrophy may accompany the development T1D, with these exocrine changes believed to be related to islet-exocrine vascular connections and the loss of the anabolic effects of various islet hormones, particularly insulin.[102] This observation has led to attempts

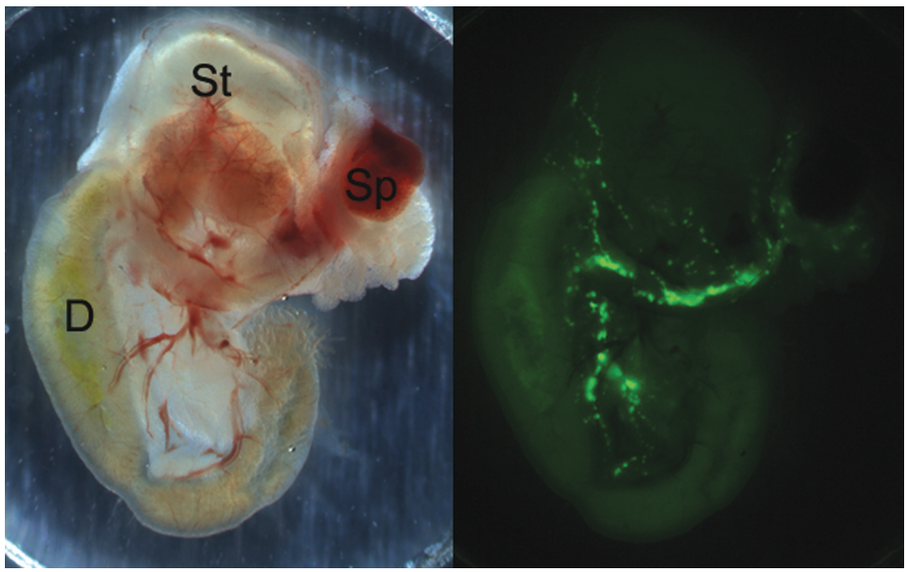

Figure 6. Imaging of the pancreatic β-cells in the intact whole pancreas. The pancreas from a newborn (PO) MIP-GFP mouse is shown with the surrounding tissues attached (D, duodenum; St, stomach; Sp, spleen). A white light image is shown on the left and the corresponding fluorescent image on the right. In the fluorescent image, there appear to be 2 major streaks of β-cells visible; the streak along the body and tail of the pancreas to the spleen and the streak along the duodenum, which are believed to arise from the dorsal and ventral buds, respectively. Photographs courtesy Dr Manami Hara, University of Chicago.

to measure pancreatic volume in individuals with long-standing T1D by ultrasound,[103] CT,[104] and MRI[105] to monitor pancreatic atrophy. Although these studies have found a significant decrease in pancreatic volume, approaching a 50% loss in individuals with long-standing T1D relative to normal controls, there has not been good correlation between pancreatic volume and duration of illness, C-peptide, or autoantibody titers. This finding is not surprising, since on pathologic study, although exocrine atrophy and insulin deficiency are associated, the extent of pancreatic atrophy has not correlated with duration of diabetes, residual insulin content, autoantibody status, or microangiopathy.[106,107]

Biochemical Measurements of β-Cell Mass

Instead, biochemical assays of β-cell function have become the experimental standard in longitudinal studies and clinical research on T1D. The insulin secretory responses, measured either directly or via the co-secreted C-peptide, to various β-cell secretagogues including oral or intravenous glucose, mixed meals, glucagon, sulfonylureas, and arginine have all been used.[108–110] Responses to different stimuli can be highly discordant, as typified by loss of intravenous-glucose-induced insulin secretion but retention of response to intravenous arginine "early" in T1D.[109]

There is also some debate as to how well these functional assays correlate with true β-cell mass as opposed to reflecting temporal dysfunction related to factors such as glycemia. Correlative studies in humans after islet transplantation have been attempted, resulting in correlation coefficients of between 0.65, $p < .05$[111] and 0.789, $p = .011$[112] having been reported for acute insulin response to arginine ($AIR_{arginine}$) versus transplanted islet mass after autotransplantation or allotransplantation, respectively. Although reassuring in finding a significant correlation, these studies have the inherent limitation of variable and unknown engraftment rates precluding direct application to endogenous β-cell mass estimates.

To address this issue in a more physiologic system, McCulloch and colleagues performed an analysis in a nonhuman primate model of diabetes, with controlled β-cell destruction using the toxin SZ. They compared various insulin-secretory responses with pancreatic insulin content and morphometric

analysis of β-cell mass by histology at necropsy. Initial studies were done after low-dose SZ administration, when the animals remained normoglycemic with normal fasting-insulin levels. Despite the normoglycemia, there was a slight reduction in the $AIR_{arginine}$, a larger reduction in the acute insulin response to intravenous glucose ($AIR_{glucose}$), and a still larger reduction in the ability of glucose to potentiate the acute insulin response ($\Delta AIR_{arginine}/\Delta G$).[113] This pattern closely matches that seen clinically in "early" T1D.[109] With further doses of SZ, $AIR_{glucose}$ and $AIR_{arginine}$ were further reduced and hyperglycemia developed.[113] In this model, the correlation coefficient between $AIR_{arginine}$ and β-cell mass determined morphometrically ($r = 0.29$) was not statistically significant, but $AIR_{arginine}$ did correlate with pancreatic insulin content ($r = 0.61, p < .02$). Although both $AIR_{glucose}$ and $\Delta AIR_{arginine}/\Delta G$ showed a linear correlation with morphometrically estimated β-cell mass by morphometry ($r = 0.63$, $p < .02$ and $r = 0.58$, $p < .02$, respectively), these values intercepted the β-cell mass axis at approximately 0.2 gm. This indicates that reductions in pancreatic insulin content are disproportionate to decreases in β-cell mass, and there is still 40 to 50% of β-cell mass detectable histologically when biochemical measures of β-cell function approach zero.[114]

Targeted Molecular Probes

Besides the usual imaging barriers with targeted imaging agents, such as nonspecific retention and clearance from the rest of the body, noninvasive in vivo islet imaging strategies have been severely hindered by the low fraction of β cells embedded within the exocrine pancreas. Several approaches have been used to specifically target β cells over exocrine tissue in developing imaging probes. Moore and colleagues used the β-cell-specific monoclonal antibody IC2 for β-cell mass determinations in excised pancreata from normal and SZ -treated mice,[115] but have yet to successfully translate this method into an in vivo imaging technique. Hampe and colleagues abandoned a similar approach using the β-cell-specific antibody K14D10 after they determined that it lacked the required specificity to overcome the low β-cell percentage within the pancreas.[116] Others have attempted peptide-mediated targeting via phage display with both in vivo[117] and ex vivo[118] selection techniques. The Arap group identified phage that home to blood vessels within the islets.[117,119] Although these sequences may be useful for localized drug delivery, binding to the vasculature instead to the islets themselves limits their use for direct imaging of β cells. Samli and colleagues have identified a phage that preferentially accumulates in the islets over the exocrine portion of the pancreas,[118] but is unlikely to have the requisite specificity for imaging according to the reported immunohistochemistry results.

Other investigators are trying to identify unique β-cell targets using available protein and mRNA expression data. One such target has been the sulfonylurea receptor, a subunit of the adenosine triphosphate (ATP)-sensitive potassium channel (K_{ATP}) present on the plasma membrane of pancreatic β cells. Activation of this channel leads to insulin release; drugs targeting this molecule have been available since 1950s for use in the treatment of type 2 diabetes. There have been numerous attempts to develop fluorine-18 or carbon-11 derivatives of these agents for use in PET imaging.[120–123] Unfortunately, successful use of these compounds in the measurement of β-cell mass in humans is yet to be reported, with many of these agents having been found to have a relatively low signal-to-noise ratio, resulting from high plasma-protein binding, and relatively low uptake in the pancreas compared with other abdominal organs.[120,123]

Using a functional genomics approach, Maffei and colleagues recently identified a new potential imaging target, vesicular monoamine transporter type 2 (VMAT2). This molecule is present on β-cells but absent in the exocrine pancreas, liver, and kidney.[124] VMAT2 is expressed in the neurons of the sympathetic nervous system, monaminergic neurons in the enteric and central nervous systems, as well as subpopulations of cells in the adrenal medulla and gastrointestinal tract.[125] Fortuitously, the VMAT2 ligand [11C]dihydrotetrabenazine (DTBZ) is already available as a PET neuroimaging agent and has been used as an objective measure of Parkinson's disease severity and progression.[126]

Regarding β-cell imaging, preclinical studies in rats and baboons have reported PET-based β-cell visualization.[127,128] Although PET imaging of individuals using [11C] DTBZ has been undertaken and preliminary data show apparent differences between a normal control subject and patients with long-standing T1D,[129] correlation with functional β-cell markers has yet to be reported. In practice, VMAT2 expression in adjacent tissues and local neurons as well as changes in VMAT2 expression independent of insulin secretory ability may all complicate DTBZ PET interpretation. For example, VMAT2 has been identified on sympathetic fibers within the exocrine pancreas and not just the endocrine pancreas.[130,131] Furthermore, VMAT2 expression does not appear to correlate with β-cell function in samples from patients with insulin-hypersecretory states including hyperinsulinemic hypoglycemia or insulinomas.[131] Because there remain little information regarding the regulation and function of VMAT2 within the islets or the factors that affect its density on β cells, there is a need for further substantial exploration before VMAT2 imaging can be used as a diagnostic tool.

IMAGING ISLET TRANSPLANTATION

Islet transplantation is a promising experimental therapy that attempts to treat diabetes with transplantation of only the endocrine portion of the pancreas, as isolated organelles, as opposed to transferring the entire, mixed endocrine plus exocrine, pancreas (for a review, see the study by Gaglia and colleagues[132]). Given the ongoing clinical islet transplantation trials, the potential for ex vivo labeling of islets, and many unanswered questions regarding islet engraftment and immune responses against them, there has been a recent emphasis on imaging in islet transplantation. Multiple imaging modalities including bioluminescence imaging (BLI), PET, x-ray, and MRI have been applied to imaging islet transplantation and rejection in rodent models (reviewed in a study by Gaglia[133]).

Limited photon penetration through tissue and local positional effects profoundly limit the utility of BLI measurements. These constraints have restricted the use of BLI in monitoring endogenous β-cell mass, but this technique has been applied to monitoring transplanted islets,[134,135] which are often located closer to the surface of the animal and contained within a smaller total volume than endogenous islets. Using constant emission isotropic light-emitting beads with spectral emission similar to firefly luciferin oxidation, Virostko and colleagues have defined the local tissue characteristics including absorption and scatter at the renal and hepatic transplant sites in a mouse model. Although the beads have constant emission, dynamic changes in photon measurements were identified postimplantation,[136] which may complicate quantification of islet mass by BLI.

Although a low signal-to-background ratio has plagued PET imaging of endogenous islets,[137] the ability to exogenously label or genetically modify islets prior to transplantation has led to the successful use of PET imaging in several transplant models. For example, early post-transplant events have been monitored with islets labeled ex vivo with FDG just prior to transplantation. However, rapid washout and the relatively short half-life of fluorine-18 has limited imaging to the first 6 hours after transplantation.[138] Several groups have developed a more durable approach, permitting in vivo longitudinal studies using a PET reporter gene technique.[139–141] By transfecting islets prior to transplantation with a mutant form of the gene encoding the herpes simplex virus 1 thymidine kinase (HSV1-*sr39tk*), Kim and colleagues were able to quantitatively measure 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl)-guanine (FHBG) trapping, which is dependent on thymidine kinase activity. The PET signal was directly proportional to the number of transplanted islets. Using this technique, they were able to demonstrate the protective effect of expressing IL-10 on islets transplanted into NOD mice.[139]

Microencapsulation has been used to decrease the immunogenicity of islets for transplantation by creating a semi-permeable membrane around the islets to decrease immune recognition and prevent passage of antibodies and complement factors that might otherwise lead to graft rejection. Such membranes also provide a medium for embedding imaging agents. Islets in alginate microcapsules containing barium or bismuth sulfate, heavy metals

detectable via x-ray, or Feridex, an iron oxide nanoparticle detectable by MRI, have been imaged in islet transplantation models.[142,143]

Iron oxide nanoparticles have also been used to directly label islets ex vivo for subsequent imaging via MRI after transplantation. Moore and colleagues reported uptake of dextran-coated nanoparticles primarily by β cells,[144,145] whereas in contrast Saudek and colleagues have found significant uptake of carbodextran-coated nanoparticles initially by resident islet macrophages,[146,147] but by β cells only after longer incubations.[148] Several techniques including antibody coupling and poly-L-lysine complexing with electroporation have been used to facilitate nanoparticle uptake into islets over incubation alone.[149,150] Because some of these exploratory animal experiments used commercially available nanoparticles already being used in human investigations, including Feridex and Resovist, rapid translation to human clinical islet transplant trials is expected.

CONCLUSIONS

Despite a pressing need, techniques to accurately measure insulitis or β-cell mass have remained elusive. Recent advances in molecular imaging modalities including BLI, MRI, and PET have started to yield promising results in animal models, greatly expanding our understanding of the underlying disease processes. Some of these techniques may be translatable to human clinical study. Ultimately, continued advances in the field should improve diagnostic accuracy, further increase our understanding of the disease processes, improve monitoring of response to potential therapies, and lower barriers to the development of novel therapeutics.

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