POTENTIAL FOR IMMUNOPROTECTION OF PANCREATIC ISLETS BY COVALENT MODIFICATION WITH POLY(ETHYLENE GLYCOL)

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ABSTRACT

POTENTIAL FOR IMMUNOPROTECTION OF PANCREATIC ISLETS BY COVALENT MODIFICATION WITH POLY(ETHYLENE GLYCOL)

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Diabetes Mellitus is one of the predominant contributors to morbidity and mortality worldwide. Prior to the advent of insulin therapy, patients suffering from Type I diabetes generally did not survive past childhood. Even with insulin therapy, a physiologically normal insulin response to increased systemic glucose cannot be achieved. Pancreatic islet transplantation has been shown to restore the physiological response to glucose, but risks associated with chronic immune suppression outweigh the benefit of tighter glucose regulation. This study investigates the potential of covalent modification of pancreatic islets with poly(ethylene glycol) (PEG) to abrogate the immune response towards transplanted islets and eliminate the need for chronic immune suppression. Previous studies have shown that PEG can be covalently bound to islet extracellular matrix (ECM) and surface proteins with no adverse affect on islet viability or function. The goal of this study was to determine the effect of covalent PEG modification on binding of islet-specific antibody, and to determine whether or not PEG modification could prolong graft survival *in vivo.* By a novel adaptation of an enzyme-linked immunosorbent assay (ELISA) the amount of islet-specific antibody bound to unmodified or PEG-modified islets was compared semi-quantitatively. Islets treated with 40kD branched PEG-NHS bound significantly more antibody than untreated controls. Based on the student's paired t-test there was no statistically significant change in antibody binding between 5kD PEG-treated and unmodified islets, although 7 of 9 PEG treated groups in this experiment bound less antibody than the corresponding unmodified groups. For *in vivo* islet transplantation , there was no difference in graft survival observed between PEG-treated and untreated grafts. Although PEG treatment did not have an apparent effect on *in vivo* graft survival, the effects observed in the antibody binding experiment suggest that PEG does modify antibody binding and further investigation of this technique is warranted.

FOREWARD

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TABLE OF CONTENTS

FOREWARD iv
1.0 INTRODUCTION 1
1.1 Diabetes Mellitus 1
1.1.1 IDDM 1
1.1.2 NIDDM 2
1.1.3 Gestational Diabetes 2
1.1.4 Secondary Diabetes 2
1.2 Impact of Diabetes 3
2.0 BACKGROUND AND LITERATURE REVIEW 4
2.1 Pancreatic Islets 4
2.1.1 Morphology 4
2.1.2 Metabolic Regulatory Function of Islets 5
2.2 Current Treatment Methods 6
2.2.1 Insulin Therapy 6
2.2.2 Transplantation 7
2.3 Mechanisms of Graft Rejection 7
2.4 Strategies for Prevention or Reversal of Type I DM 8
2.4.1 Interventional Therapy 8
2.4.2 Transplantation of Encapsulated Islets 8
2.4.3 Genetic Manipulation 9
2.4.3.1 Delivery Vehicles 9
2.4.3.2 Strategies 9
2.5 PEG 10
2.5.1 Properties of PEG 10
2.5.2 Previous Studies 11

2.5.2.1 PEG Modification of Synthetic Surfaces 11
2.5.2.2 PEG Modification of Vascular Tissue 11
2.5.2.3 PEG Treatment of Islets 11
2.5.2.4 Islet Viability Following PEG Treatment 12
2.5.2.5 Islet Function Following PEG Treatment 12
2.5.2.6 Complement-Mediated Lysis of PEG-Treated Islets 13
3.0 RESEARCH OBJECTIVES 14
3.1 Is PEG Surface Coverage Complete? Does PEG Enter Islet Cells? 14
3.2 Investigate Ability of PEG to Prevent Ab Attachment 15
3.3 Determine the Ability of PEG-Modification to Prolong Islet Graft Survival *in Vivo*. 15
4.0 EXPERIMENTAL PROCEDURES 16
4.1 Islets Isolation 16
4.2 PEG-Treatment of Islets 17
4.3 Confocal Microscopy 18
4.3.1 Imaging Surface Bound PEG 18
4.3.2 Two Color Imaging of PEG and Antibody Binding 18
4.4 Quantitative Antibody Binding Assay 19
4.5 Islet Allograft Transplantation 20
4.5.1 Induction of Diabetes 20
4.5.2 *In Vitro* Islet Function 20
4.5.3 Islet Transplantation 21
4.6 *In Vivo* Islet Function 21
5.0 RESULTS 22
5.1 Islet Isolation 22
5.2 Confocal Images 22
5.2.1 Effect of Increased Culture Time on Quality of PEG Binding 22
5.2.2 Effect of PEG Molecular Weight 23
5.3 Confocal Microscopy of Bound Antibody 26
5.4 Quantitative Ab-Binding Data 28
5.5 *In Vitro* Islet Function 30
5.6 *In Vivo* Islet Graft Survival 30

6.0 DISCUSSION 32
6.1 Confocal Images of Bound PEG 32
6.2 Quantitative Ab-Binding Assay 33
6.3 *In Vivo* Transplantation Study 34
6.4 Future Directions 35
Appendix A 37
Additional Islet Images 37
BIBLIOGRAPHY 41

LIST OF TABLES

Table 1. Abbreviations and concentrations of various PEG constructs used in this research 17

LIST OF FIGURES

Figure 1. Effect of Culture Time on PEG-Binding. 24
Figure 2. Effect of PEG MW on PEG binding. 25
Figure 3. Determining appropriate Ab treatment for Ab-binding experiments. 25
Figure 4. Simultaneous, two-color PEG/Ab images. 27
Figure 5. Results of Ab-binding assay for 40 kD PEG-treated islets. 29
Figure 6. Results of Ab-binding assay for 5 kD PEG-treated islets. 29
Figure 7. Changes in blood glucose concentration and body mass following pancreatic islet transplantation. 31
Figure 8. Images showing presence of putative necrotic regions. 33
Figure 9. Images of islets modified with 80kD PEG-FITC 37
Figure 10. Images of islets modified with 40kD PEG-FITC, after six days in culture. 38
Figure 11. Images of islets modified with 5kD PEG-FITC. 39

1.0 INTRODUCTION

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is a general term describing one of four metabolic disorders that result in an abnormal elevation in blood glucose concentration. At the root of these disorders is an insufficiency in insulin secretion or an inability of insulin-responsive tissues to sense insulin. The result is inefficient carbohydrate metabolism and accumulation of glucose in the blood.[1]

1.1.1 IDDM

Insulin dependent diabetes mellitus (IDDM, juvenile diabetes, Type I DM) is characterized by almost complete cessation of insulin production. It is caused by autoimmune destruction of the beta-cells of the pancreatic islets, the cells responsible for producing and secreting insulin in response to glucose challenge.[1]

The factors influencing the onset of this disease are not fully understood. It is accepted that there is a genetic predisposition to the disease, but as the probability of two identical twins developing Type I DM is only 30 – 50%, it is clear that environmental factors also play a role.[2] Evidence of a possible environmental trigger is a correlation between exposure to cow's milk in infancy and increased occurrence of Type I DM. It is believed that an immune reaction with

bovine albumin may lead to the production of antibodies reactive with a cell surface protein present on islet beta-cells that resembles bovine albumin.[3]

1.1.2 NIDDM

In non-insulin dependent diabetes mellitus (NIDDM, adult onset diabetes, Type II DM) hyperglycemia results from resistance to insulin action and may be accompanied by hyperinsulinemia and eventually loss of beta-cell mass.[1,4] Risk factors include obesity and a sedentary lifestyle among others. In obesity, a marked overproduction of insulin is observed.[1] This may set the stage for development of insulin resistance. Eventually glucose ceases to be a stimulus for insulin release. This lack of islet activity may lead to beta-cell death.

1.1.3 Gestational Diabetes

Gestational diabetes is a result of overcompensation in insulin resistance during pregnancy. During a normal pregnancy, insulin action is suppressed somewhat in the mother to allow glucose to pass to the developing fetus. If insulin action is suppressed too much the mother will experience hyperglycemia.

1.1.4 Secondary Diabetes

Secondary diabetes refers to a hyperglycemic condition caused by exogenous factors. Cancer, infection requiring pancreatectomy, or exposure to certain chemicals such as

streptozotocin result in loss of islet beta-cells and insufficient insulin production. Other chemicals or drugs may suppress insulin secretion or insulin action.[1]

1.2 Impact of Diabetes

Of the two major forms of DM (Types I & II) Type II DM is generally more severe. Acute symptoms include polyuria, polyphagia, polydypsia, pruritus and excessive weight loss. In the absence of insulin, fats replace glucose as the primary energy source. In severe cases, ketone products of fat metabolism accumulate in the blood resulting in ketoacidosis, coma and death.[1]

The chronic effects of hyperglycemia can also be devastating. Poorly controlled blood glucose has been linked to nephropathy from osmotic overload, loss of eyesight, atherosclerosis and neuropathy.

Based on a survey conducted in 1995 by The American Diabetes Association, ~6% of the population of US suffers from diabetes. Of these, 5 – 10 % have Type I diabetes or IDDM. Thirty thousand new IDDM cases are diagnosed each year. Diabetes is the leading cause of end-stage renal disease, accounting for about 40% of new cases. Diabetes is the leading cause of new cases of blindness in people ages 20-74. Each year, from 12,000 to 24,000 people lose their sight because of diabetes. People with diabetes are 2 to 4 times more likely to have heart disease which is present in 75 percent of diabetes-related deaths. The risk of a leg amputation is 15-40 times greater for a person with diabetes. Each year, more than 56,000 amputations are performed among people with diabetes. All told, diabetes and the complications associated with chronic hyperglycemia constitute one of the largest causes of morbidity and mortality worldwide.

2.0 BACKGROUND AND LITERATURE REVIEW

2.1 Pancreatic Islets

The Islets of Langerhans are endocrine suborgans which reside in the pancreas. They constitute ~1–2% of total pancreas volume. The hormones released by pancreatic islets, insulin, glucagon, somatostatin and pancreatic polypeptide all play important roles in the regulation of metabolism. The anatomical position of the pancreas, just upstream from the liver, reflects this function.[1]

2.1.1 Morphology

Islets are heterogeneous in nature. Each islet is composed of two or three of four possible cell types, alpha-cells, beta-cells, delta-cells, and pancreatic polypeptide-cells. Every islet contains a core of beta-cells surrounded by a mantle of either alpha and/or delta cells or a mantle of delta and PP cells. In some species (rat, human, canine) the mantle is enclosed by a capsule composed of collagenous ECM and a monolayer of fibroblasts. Porcine islets do not possess this collagenous capsule.[5]

2.1.2 Metabolic Regulatory Function of Islets

As mentioned previously, the islet beta-cells are solely responsible for sensing the glucose concentration in the blood and releasing insulin in response. The mechanism is as follows. The glut2 receptor, present on beta-cells, allows diffusion of glucose into the cells, maintaining intracellular glucose concentration at the same level as the surrounding extracellular fluid. An enzyme, glucokinase, acts as a chemical sensor for glucose. Phosphorylation of glucose is the rate limiting step in glucose oxidation in beta-cells. As glucose is oxidized, the intracellular ATP concentration rises. At a critical ATP concentration, ATP sensitive K+ channels open, initiating depolarization of the cell membrane. This depolarization triggers an influx of Ca++ through voltage-gated Ca++ channels. Ca++ is a second messenger that initiates movement of insulin secretory granules along the microtubules and leads to insulin secretion. The primary action of insulin is on muscle and fatty tissue. Insulin signals these tissues to take up and use or store glucose from the blood.

Islet alpha-cells secrete glucagon. Although not as crucial to survival as insulin, glucagon also plays an important role in fine-tuning blood glucose concentration. Glucagon acts in opposition to insulin at times when glucose must be liberated from endogenous stores. It triggers the release of glucose from carbohydrate stores in the liver and initiates gluconeogenesis, or conversion of proteins and fats to glucose. It also suppresses insulin release. The delta-cells release somatostatin which suppresses both insulin and glucagon release. The PP-cells release pancreatic polypeptide. The role of this hormone is not fully understood.[1]

2.2 Current Treatment Methods

Although therapies have been devised to manage hyperglycemia, complete and permanent reversal of diabetes has only been achieved in certain exceptional cases.

2.2.1 Insulin Therapy

Insulin therapy is, by far, the most common form of treatment for management of Type I DM. When replacement of endogenous insulin with subcutaneous injections of bovine insulin was found to be effective in lowering blood glucose concentration in 1922, it represented the first major breakthrough in the treatment of a previously untreatable disease. Since this initial discovery, methods of producing and extracting insulin have been improved. New formulations for exogenous insulin, have been created to vary the time course of insulin action and provide both for longer-term baseline insulin requirements and transient insulin requirements following meals.[1] Despite these advances, insulin therapy leaves much to be desired. Because of the rapid response of islets to slight variations in glucose concentration, it is impossible to mimic the physiological response by delivery of exogenous insulin.[1] In addition, the quality of glucose control is left entirely up to the patient. Adequate glucose regulation requires frequent monitoring by finger prick and subcutaneous insulin injections three or more times daily.

The advent of the implantable insulin pump eliminates the need for frequent shots, but still requires careful monitoring and input from the patient. Therefore, patient error still comes into play.

2.2.2 Transplantation

Transplantation of insulin producing tissues (whole pancreas or pancreatic islets) seems to be the most logical solution for reversal of diabetes. In successful transplants, regulation of glucose metabolism closely follows the native physiological response. The applicability of these procedures is, however, limited. The morbidity associated with chronic immune suppression outweighs the benefit of tighter glucose regulation. For this reason, pancreas or pancreatic islet transplants are generally only performed along with another more immediately necessary organ transplant. An additional problem associated with pancreas transplant is the chronic release of digestive enzymes into the peritoneal cavity resulting in significant morbidity.[6,7] Even with the most refined peri-transplant protocols, islet transplant success rate at one year does not exceed 20%.[6]

2.3 Mechanisms of Graft Rejection

Rejection of allo-transplanted tissue grafts is most commonly associated with a response by CD8+, cytotoxic, T cells or CD4+, helper T cells or both. Antibodies can also play a role in "second set" rejection, or rejection following prior sensitization.[8] Therefore, in order to eliminate the need for chronic immunosuppression after islet transplant, it is necessary to devise a means to interfere with these immune mechanisms.

2.4 Strategies for Prevention or Reversal of Type I DM

2.4.1 Interventional Therapy

Interventional approaches to treating type I DM fall into three categories. Primary prevention involves identifying and eliminating environmental risk factors (viruses, chemicals, components of diet) from a normal population. Considering the possible correlation between ingestion of bovine albumin in infancy and incidence of type I DM, an example of primary prevention would be a recommendation for mothers to avoid exposing their infant children to cow's milk, especially if there is a family history of type I DM.

Secondary prevention involves early identification of symptoms of the onset, and intervention prior to full development of the disease. Tertiary prevention attempts to deal with clinical complications associated with disease onset.[9]

2.4.2 Transplantation of Encapsulated Islets

The rationale behind this strategy is that a perm-selective membrane surrounding the islet may shield it from attack by humoral components of the immune system, such as antibodies and complement proteins, and the phagocytic and cytotoxic immune cells. Many studies have shown that encapsulation in a perm-selective membrane can prolong allo- or xeno- graft survival in diabetic animals.[10-20]

Choice of material for encapsulation is crucial. First, the material must selectively exclude large molecules such as antibodies while allowing diffusion of insulin, glucose, oxygen and metabolic waste. The membrane must be sufficiently strong to resist breaking. It must also be chemically and biologically compatible to elicit the smallest possible inflammatory response and to ensure islet viability and proper function. Materials used for encapsulation include

complex coacervates of alginate and poly-L-lysine, hydrogels of agarose, photopolymerizable PEG, and PEG, acrylic hollow fibers, tissue-engineered membranes of autologous chondrocytes, and others.[10-20]

The most commonly used encapsulation material for pancreatic islets is a complex coacervate composed of alternating layers of alginate and poly-L-lysine.

The primary failure mode is cellular overgrowth onto the capsule material. This effects the mass transport properties of the capsule and leads to asphyxiation of the encapsulated cells.[21]

2.4.3 Genetic Manipulation

2.4.3.1 Delivery Vehicles. Because the islet beta-cells do not replicate, it is necessary to use a vehicle capable of delivering genes to quiescent cells. The most likely candidates are viral vectors of one of the following types: 1) Replication defective adenovirus, 2) Herpes simplex-1, or 3) lentiviral vectors. At this point however, none of these systems has been proven to be efficacious, stable, or safe enough for clinical use. Adenoviral vectors and the herpes simplex virus are not retroviruses, therefore the gene products they deliver remain episomal and never incorporated into the cell's genome. Additionally, expression of some viral proteins may elicit an immune response and target the transfected cells for removal.[7]

Lentiviral vectors (e.g. HIV-1) do incorporate their genes into the cell's genome for more stable expression, and do not elicit an immune response, but issues of safety have not been addressed.[7]

2.4.3.2 Strategies. Many strategies have been envisaged to modify the immune response towards transplanted islets by gene therapy. For example, islets are extremely sensitive to the oxidative environment in an inflammatory site. A reduction in local oxidant stress by transfection of islet cells with genes encoding antioxidants such as thioredoxin or manganese superoxide dismutase may prolong graft survival.[36-38]

It has been observed that increased tryptophan catabolism may be responsible for maternal tolerance of the fetus, although the mechanism is, as yet, unclear. Transfection with

genes encoding indoleamine 2,3 dioxygenase, a catalyst of tryptophan, may create a local environment with some of the immune properties of the womb during pregnancy.[39]

Finally, expression of anti-inflammatory cytokines or anti-complement agents such as decay accelerating factor may act to undo the immune stimulation caused by foreign islet tissue.[7]

2.5 PEG

2.5.1 Properties of PEG

Poly(ethylene glycol) (PEG) is a polymer derived from ethylene oxide monomers. It is non-toxic and non-inflammatory and has gained FDA approval for internal use. Because the structure of PEG allows water to maintain its hydrogen bonding structure in aqueous solution, it is extremely hydrophilic and is entropically favored to maintain a fully hydrated, extended chain formation. The subsequently large molecular radius gives PEG the ability to sterically repel other molecules.[22-24] These properties have lead to the investigation of PEG as a surface coating to improve the biocompatibility of various biomaterials. It has also been hypothesized that the ability of PEG to reject protein adsorption may decrease the immunogenicity of transplanted tissues by isolating the tissues from access by antibodies and the cellular components of the immune system.[25,26]

2.5.2 Previous Studies

2.5.2.1 PEG Modification of Synthetic Surfaces. Various methods have been used to attach PEG to the surfaces of many materials. The simplest method is to adsorb a PEG-containing surfactant to a surface.[27-29] PEG can also be physically incorporated into a surface by creating a surface physical interpenetrating network (SPIN).[30,31] PEG segments can be included in poly(urethane) block copolymers. The resulting polymer has a surface enriched with PEG when placed in an aqueous medium.[32] Glow discharge treatment with PEG results in a polymer surface covalently modified with PEG.[33] Finally photoreactive PEG may be interfacially polymerized to create a conformal, cross-linked PEG coating.[34] Examples of these surface modification methods have been shown to reduce protein and/or platelet adhesion both *in vitro* and *in vivo*.[27-34]

2.5.2.2 PEG Modification of Vascular Tissue. Using an amine-reactive form of PEG, it is possible to covalently modify the surface proteins of a damaged vascular intima. Treatment with amine-reactive PEG of denuded human placental artery reduced platelet adhesion in an *in vitro* perfusion assay by 89%. In an *in vivo* assay of platelet adhesion in the femoral artery of rabbits following injury by balloon angioplasty showed a significant reduction in platelet adhesion from the untreated controls.[22]

2.5.2.3 PEG Treatment of Islets. Based on previous studies of PEG modification of synthetic surfaces[27-34] and of damaged vascular intima[22], it was hypothesized that covalent modification of pancreatic islet surfaces with PEG might modify the host's immune response towards the islets by preventing specific as well as nonspecific binding of antibodies and other inflammatory mediators at the islet surface. PEG variants can be synthesized to terminate in one or more amine-reactive functional groups such as N-hydroxysuccinimide (NHS) or isocyanate. These functional groups will react with proteins via free amine groups such as are found at the N-terminus or on lysine residues within the protein. Proteins reacted in this fashion will be covalently linked to the PEG chain.

In one study of the efficiency of PEG-binding at the islet surface, the investigator used an avidin-biotin linking system to fluorescently label PEG following PEG treatment. The islets were then imaged using a confocal microscope. Images showed a high degree of fluorescent labeling at the islet surfaces.[25,26] However, the ability of these images to accurately reveal the location of PEG binding came into question due to the nature of the linking system. In short, due to the large molecular weight of avidin, used as a fluorescent tag, it was hypothesized that there may have been PEG throughout the islet volume that was not labeled because the avidin did not diffuse freely to the islet interior.

In subsequent experiments, a directly labeled PEG molecule was used, circumventing the avidin-biotin linking system. Confocal images obtained in this manner showed that PEG was indeed binding in the islet interior as well as the periphery,[35] but due to poor resolution of these images, the nature of PEG binding was still unclear. Islets which showed the highest degree of fluorescent labeling appeared as though PEG may be entering individual islet cells, an undesirable result as PEG that is taken up into cells may have, or indicate, a toxic effect. One goal of this research was to determine conclusively, the nature of PEG binding at the islet surfaces as well as in the interior of islets.

2.5.2.4 Islet Viability Following PEG Treatment. It is important that the PEG treatment has no effect on islet viability. A standard assay to demonstrate cell viability is a cell proliferation assay. As islet cells do not divide under normal circumstances, an assay that reveals metabolic activity is necessary to prove islet cell viability. Previous studies have used the mitochondrial MTT viability assay. MTT is a straw yellow-colored substance which is cleaved by active mitochondria to form a purple-colored formazan salt in living cells. Results of MTT assays of PEG-treated and untreated islets showed that there is no significant loss of viability after PEG-treatment.[35]

2.5.2.5 Islet Function Following PEG Treatment.

It is also important that the treatment method has no effect on proper islet function. The islets must be able to respond appropriately, by releasing insulin in response to secretogogues,

especially glucose. Previous studies using radioimmunoassay for insulin showed that there was no significant difference in glucose response of PEG-treated and untreated islets.[35]

2.5.2.6 Complement-Mediated Lysis of PEG-Treated Islets. To begin investigation of the immunoprotective effect of a PEG-coating on pancreatic islets, a previous study used an *in vitro*, complement-mediated lysis experiment. In short, islets, either untreated or treated with PEG were incubated in wells of a 96 well microtiter plate overnight in a solution containing rabbit anti-rat islet antiserum. Controls included medium without antiserum, naïve rabbit serum, rat serum and methanol (a negative control). Following incubation, the viability of the islets was tested using the MTT cell viability assay. Results from this experiment were somewhat anomalous. It was expected that in wells that did not contain anti-rat islet antibodies, that viability would be similar between the PEG-treated and untreated groups. However, a significant reduction in viability in the PEG-treated group was observed for groups tested in naïve rabbit serum, rat serum and standard cell culture medium. This suggested to the investigator that there was a toxic effect on the islets during the PEG-treatment process, in contrast to an earlier study.[25,26] In groups of islets that were tested with antiserum, no significant change in viability was found between PEG-treated and untreated groups. Since islet viability was somewhat depressed in all PEG-treated groups, but there was no additional decrease in viability from control in the antiserum tested groups it was hypothesized that for islets that survived the treatment process, the PEG had somewhat of a protective effect.[35] However, the possibility of cross-contamination of all the wells in a plate containing volatile methanol was overlooked. Since methanol was used in some but not all of the runs of the experiment, there is an artificial and unpredictable depression in the viability reported and these data may not be valid.

3.0 RESEARCH OBJECTIVES

Previous studies have shown the potential usefulness of a covalently bound surface PEG layer to prevent rejection of pancreatic islets.[25,26,35] Covalent modification does not reduce viability or function of islets *in vitro*.[25,26] There is also some evidence that the PEG layer may reduce complement-mediated lysis of islet cells exposed to xenogeneic islet antiserum.[35] However, questions still remain regarding the degree of coverage that is achieved by the treatment procedure, and the clinical usefulness of this process. The question as to whether the PEG coating is enough to significantly prolong graft survival *in vivo* has not yet been answered.

3.1 Is PEG Surface Coverage Complete? Does PEG Enter Islet Cells?

Previous studies have suggested the possibility of "hotspots" or areas on the islet surface where PEG coverage is not complete.[25,26] A subsequent study showed that more complete coverage could be achieved, but there was also evidence that some islets were becoming saturated with PEG.[35] Due to low resolution of the confocal images in this previous study, the quality of PEG binding was unclear. One goal of the current research was to answer these questions regarding the quality of PEG binding.

3.2 Investigate Ability of PEG to Prevent Ab Attachment

A previous study showed a possible protective effect of the PEG coating against complement-active antiserum. However, due to possible cross-contamination of test wells by methanol in adjacent, negative control wells, this data is unclear. In this study the ability of PEG to reject islet-specific antibodies will be investigated further.

3.3 Determine the Ability of PEG-Modification to Prolong Islet Graft Survival *in Vivo*.

Although previous studies have investigated the potential of covalent PEG modifcation to prolong pancreatic islet graft survival, none have addressed this issue directly. The final goal of this project was to determine whether or not PEG-modification can prolong graft survival *in vivo*.

4.0 EXPERIMENTAL PROCEDURES

4.1 Islets Isolation

Pancreatic islets were isolated from 300 – 450 g male Sprague-Dawley rats by a modified intraductal collagenase digestion method. The pancreases were distended *in vivo* by injection via the common bile duct of 15 mL of ice-cold collagenase (Collagenase P, Boehringer Mannheim) solution. The collagenase solution was prepared at a concentration of 1.7 mg/mL in cold Hank's Balanced Salt Solution (HBSS) and sterile filtered. The pancreases were then excised and collected in separate 50 mL Falcon tubes. Collagenase solution was added to bring the total volume of each tube to ~7.5 mL. The tubes were placed in a 37 C water bath for ~20 – 25 min. Digestion time varied depending on lot number and activity of collagenase. The tissue digest was vortexed briefly to release the islets. The reaction was quenched by addition of 35 mL cold HBSS (w/out Ca2+ & Mg2+, supplemented with 10% FBS) (HBSS+FBS). The contents of the tubes were washed 3 times with cold HBSS+FBS then filtered through an 800 um wire mesh to remove undigested tissue. The tissue digest was pelleted by centrifugation then resuspended in 15 mL Ficoll-Paque solution (Pharmacia) and overlaid with 13 mL HBSS. The islets were then separated by centrifugation at 700 xg for ten min at 5 C. Islets were removed from the boundary layer and washed three times with HBSS+FBS. The islets were further purified by handpicking under a microscope and cultured in CMRL 1066 medium (Gibco supplanted w/ 1% Penicillin-Streptomycin and 10% FBS).

Table 1. Abbreviations and concentrations of various PEG constructs used in this research

Type	Abbreviation	Concentration
5kD linear PEG-NHS	5kD-PEG	5 mg/mL
5kD linear flourescein-PEG-NHS	5kD-PEG-FITC	5 mg/mL
40kD 8-arm NHS branched PEG	40kD-PEG	25 mg/mL
40kD 7-arm NHS, 1-arm FITC bPEG	40kD-PEG-FITC	25 mg/mL
80kD 7-arm NHS, 1-arm FITC star PEG	80kD-PEG-FITC	50 mg/mL

4.2 PEG-Treatment of Islets

Islets were modified by the following method with one of five PEG molecules. Table I shows the concentration used for each type of PEG.All PEG solutions were prepared with 11mM glucose solution in PBS immediately before addition to islets. Islets were collected into an appropriate vessel half full with HBSS+FBS. Choice of container depended on the number of islets to be modified. The contents of the tubes were washed three times with 4 mM glucose solution. The islets were pelleted by centrifugation and as much supernatant was removed as possible. Freshly prepared PEG solution was added to each tube containing islets. The reaction was allowed to proceed at 37 C for 30 min. Following the incubation the islets were washed 3 times with 4 mM glucose in PBS + 10% FBS. Islets to be used for confocal microscopy were fixed for 20 min at room temperature in 0.1 % paraformaldehyde. Islets used for the transplant study and the quantitative antibody binding assay remained in the reaction tubes for further manipulation.

4.3 Confocal Microscopy

4.3.1 Imaging Surface Bound PEG

To determine, qualitatively, the nature of PEG-binding at the islet surface, islets were treated with 5kD-PEG-FITC, 40kD-PEG-FITC, or 80kD-PEG-FITC as described above then imaged with a Molecular Dynamics confocal microscope. Unless otherwise noted, images were collected at 60x magnification with a pixel size of 0.34um.

After collagenase digestion to separate islets from their native matrix, the islet capsule may be damaged or entirely absent. To determine whether or not the presence of a capsule affected the quality of PEG binding, islets were isolated and treated with PEG after one, four or six days in culture, then imaged as described above.

4.3.2 Two Color Imaging of PEG and Antibody Binding

Islets were collected after six days in culture. Half were treated with 80kD-PEG-FITC as described above. The rest were left untreated. Both groups were then incubated for 2 hours at room temperature in rabbit anti-rat islet antiserum (Chemicon special order). The islets were washed 3 times in 11mM glucose + 10% FBS. They were then incubated with anti-rabbit IgG-Cy3 (Molecular Probes) diluted to 0.05 mg/mL in 11mM glucose. They were washed three times with 11 mM glucose + 10% FBS, then fixed for 20 min at room temperature with 1% paraformaldehyde. The islets were imaged using a confocal microscope operating in two color mode.

4.4 Quantitative Antibody Binding Assay

Islets were collected after six days in culture. Islets from each rat were divided into two equal groups. One group was treated with either 5kD-PEG or 40kD-PEG. The other group was left untreated. Both groups were then incubated with a 1:10 dilution of rabbit anti-rat islet antiserum at room temperature for 2 hours. The islets were washed 3 times with 11 mM glucose + 10% FBS. They were then incubated in a 1:1000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody, also for two hours at room temperature. The islets were washed two times with 11 mM glucose + 10% FBS, pelleted, then transferred to separate Petri dishes. From the Petri dishes, groups of 100 islets were manually counted and transferred to 0.7 mL Eppendorf tubes. The islets were washed once more with 11mM glucose +10% FBS and the supernatant was removed. 200 uL TMB (Mercodia) substrate was added to each tube. The substrate was allowed to react for 15 min at room temperature. The reaction was quenched by addition of 50 uL of 2M H2SO4. 100 uL of supernatant was removed from the reaction tubes and transferred to wells of a 96 well microtiter plate. The absorbances, at 450 nm, of the supernatants were read with a microplate reader with the reference wavelength at 650 nm. Results were analyzed using the student's paired t-test.

4.5 Islet Allograft Transplantation

4.5.1 Induction of Diabetes

Adult, male, 250–350 g Brown Norway rats served as hosts for islet transplants. Diabetes was induced by injection of 60 mg/kg of streptozotocin (STZ) via the tail vein. The STZ solution was prepared at a concentration of 35 mg/mL in ice cold, sterile citric acid/citrate buffer (pH 4.5). Body weight and blood glucose concentration were monitored daily following induction and diabetes was confirmed by blood glucose above 300 mg/dL.

4.5.2 *In Vitro* Islet Function

To ensure that islets were functioning prior to transplant they were tested using a static glucose challenge assay. Islets were collected, counted into groups of twenty, and transferred to separate 0.7 mL eppendorf tubes. Islets were washed three times in 4.4 mM glucose in PBS + 0.25% human albumin (low glucose solution). Islets were incubated for 25 min at 37 C with gentle agitation every 5 min. Islets were pelleted and 50 uL supernatant was removed from each tube and transferred to new 0.7 mL eppendorf tubes. Islets were washed two times with low glucose solution. Then the islets were incubated for 25 min in 16.7 mM glucose in PBS + 0.25% Human albumin (High glucose solution) at 37 C. Again 50 uL aliquots from each tube were collected and transferred to new tubes. The islets were washed twice with low glucose solution then incubated once more for 25 min at 37 C in low glucose solution. The islets were pelleted and supernatants were collected to assay for insulin. Supernatants insulin concentration was determined using a commercially available rat-insulin enzyme-linked immunoassay (ELISA) kit (Mercodia).

4.5.3 Islet Transplantation

After confirmation of diabetes in the host rats, islet transplants were performed within three days. The host animal was anaesthetized with a cocktail of ketamine (~0.35 cc) and xylazine (0.04 cc). The abdomen was shaved and washed with 70% ethanol. A small midline incision was made in the skin of the abdomen and the underlying abdominal wall. The graft of ~4,000 islets was transferred to the peritoneal cavity via pipette in 0.5 mL carrier (PBS + 0.25% human albumin). The albumin was utilized to reduce adherence of the islets to hydrophobic surfaces during the procedure. Four rats received untreated islet grafts. Four received grafts treated with 5kD-PEG. One rat was sham-operated as a negative control. Blood glucose and body weight were monitored daily until graft failure, indicated by return of blood glucose concentration to above 300 mg/dL. Host rats were euthanized following graft failure by exsanguination under anesthetic.[16,17,40]

4.6 *In Vivo* Islet Function

Three to four days following graft placement, the ability of the grafts to respond to a spike in blood glucose was tested. Food was withheld 90 minutes prior to the experiment. The rats were anesthetized by intramuscular injection of ketamine (0.35 cc). Xylazine could not be used during this procedure as it elevates the resting blood glucose level and thus would have impacted the results. A baseline measurement of the blood glucose level was taken. The rats were given an IV bolus of 1.5 g/kg glucose solution (0.5g/mL in PBS). Blood glucose measurements were then taken at 0, 15, 30, 60, 90 and 150 min.

5.0 RESULTS

5.1 Islet Isolation

After collagenase digestion, the collagenous islet capsule was partially or completely destroyed, though after several days in culture the capsule was capable of regenerating. Initially an isolation method was used that included two serial density gradient separations. It was found that the purity of islets prepared this way was not consistently high enough to allow culture for more than one night. For this reason modifications were made to the isolation procedure. By eliminating the second density gradient separation and instead handpicking the islets from the remaining exocrine tissue, purity was improved to allow longer culture time, and yield was improved three to four times over the previous method.

5.2 Confocal Images

5.2.1 Effect of Increased Culture Time on Quality of PEG Binding

Originally, it was hypothesized that if PEG was bound to the islet capsule, it may act as a perm-selective barrier and prevent cellular and molecular components of the immune system

from access to the islet cells. It was also believed that this would be a longer-lasting modification than if PEG was bound to islet cell surface markers which might be rapidly recycled. Considering the questions surrounding the issue of PEG binding in previous research, and after learning that the capsule is destroyed during collagenase digestion, we felt that it was important to clarify these issues and to determine the effect of the capsule on the PEG modification process.

Figure 1 shows images of three islets from groups isolated one, four and six days prior to modification with 80 kD FITC-PEG. The images are maps of the intensity of light collected at 535 nm portrayed in pseudocolor. Red and white indicate high intensity, blue is low intensity and black represents signal below the threshold. Before interpreting the images obtained by confocal microscopy, it is important to note that the signal collected varied from experiment to experiment. Factors affecting the overall signal include: increased background fluorescence from FITC-PEG in solution, photobleaching from exposure to light, reduced activity of amine-reactive PEG due to slow dissolution of PEG powder and hydrolyzation of the amine-reactive moiety in water. For these reasons, quantitative comparisons of images from different experiments are not valid. Images have been selected to reflect trends observed.

It is important to note from Figure 1, that as the islets are allowed time in culture, the islet periphery becomes smoother. This indicates regeneration of a layer of extracellular matrix surrounding the islet. The images also show that when this protein layer is present, it does react strongly with amine-reactive PEG.

5.2.2 Effect of PEG Molecular Weight

During the course of the research, three structural variants of the PEG molecule were employed: a small linear molecule (5 kD PEG), a medium-sized branched molecule (40 kD PEG), and a larger star-shaped molecule (80 kD PEG). The qualitative variation in the binding of these PEG variants was investigated with fluorescence microscopy. Figure 2 shows images of three islets all from one isolation group, modified with one of the three different PEG molecules. It was hypothesized that as PEG molecular weight increased, the amount of PEG that was able to

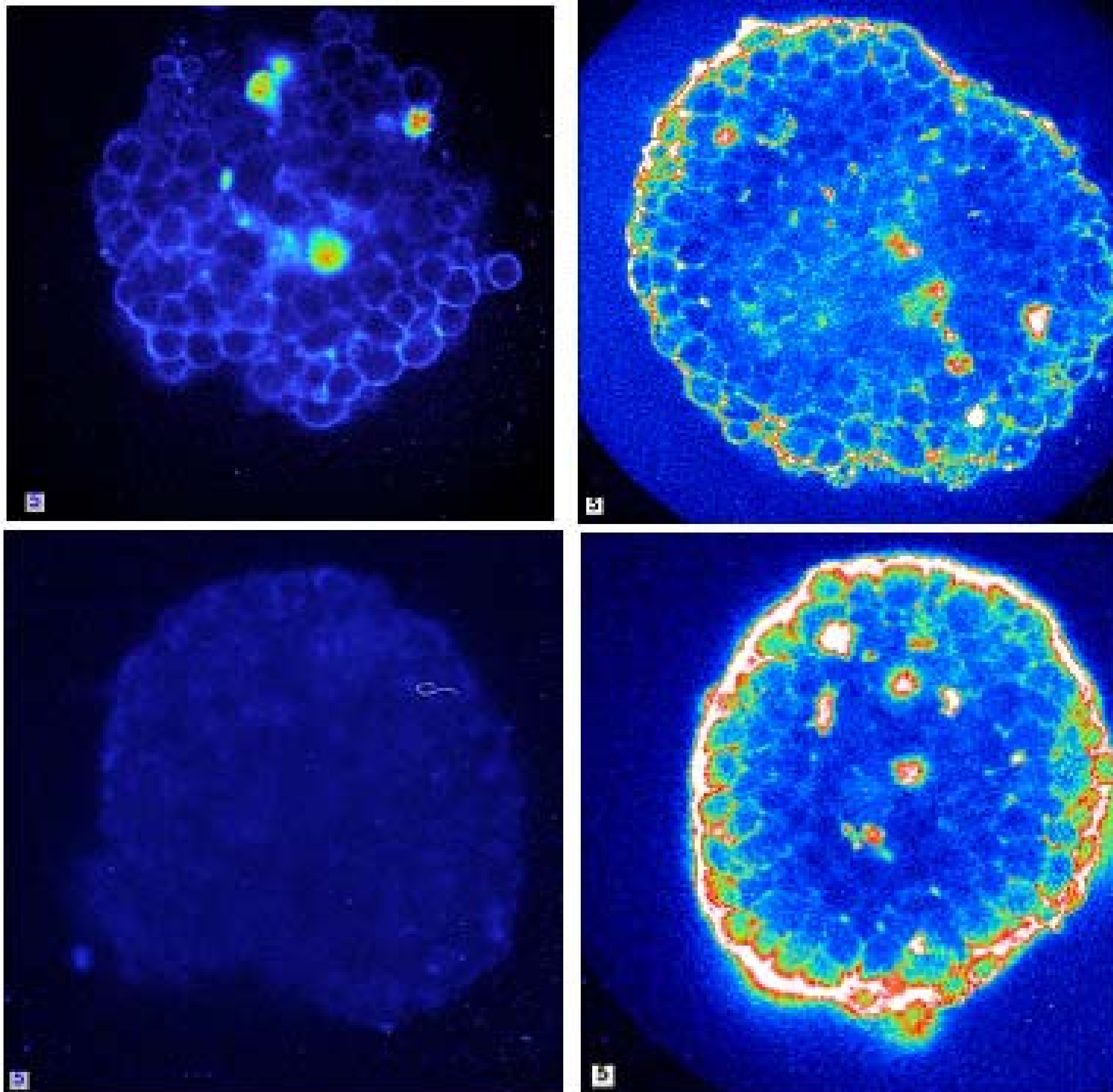


Figure 1. Effect of Culture Time on PEG-Binding.

Image in lower left is 6-day-old untreated islet for reference. Remaining islets were treated with 80 kD FITC-PEG after varying time in culture. Clockwise from upper left, islets were treated 1, 4, and 6 days following isolation. The width of the scale box in the lower left of each image equals 5 um.

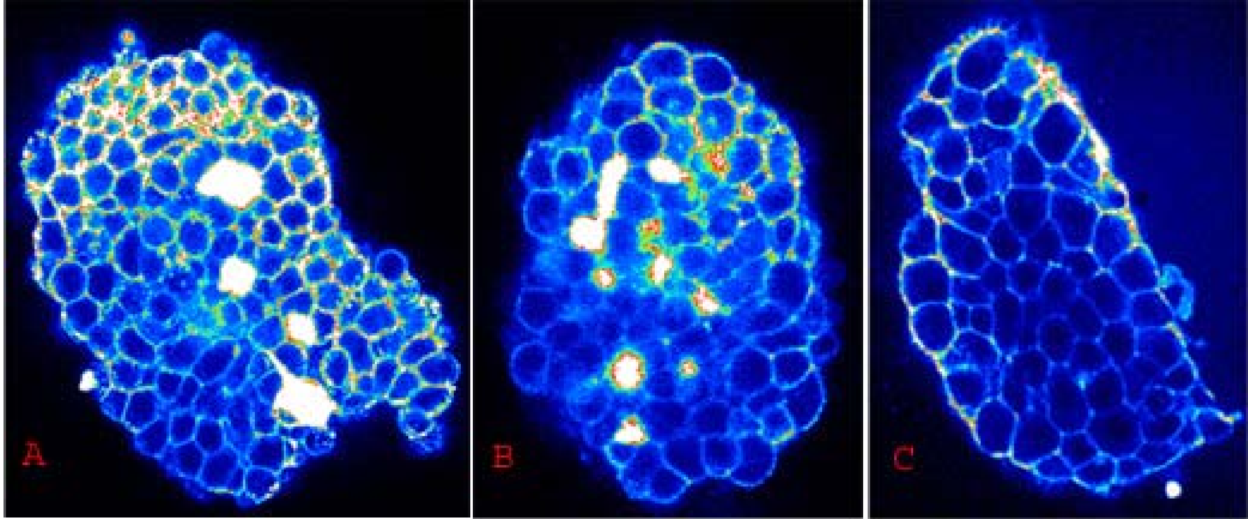


Figure 2. Effect of PEG MW on PEG binding.

Three images of 6-day-old islets modified with three different PEG-molecules. Islets were treated with A) 5 kD FITC-PEG, B) 40 kD FITC-PEG and C) 80 kD FITC-PEG.

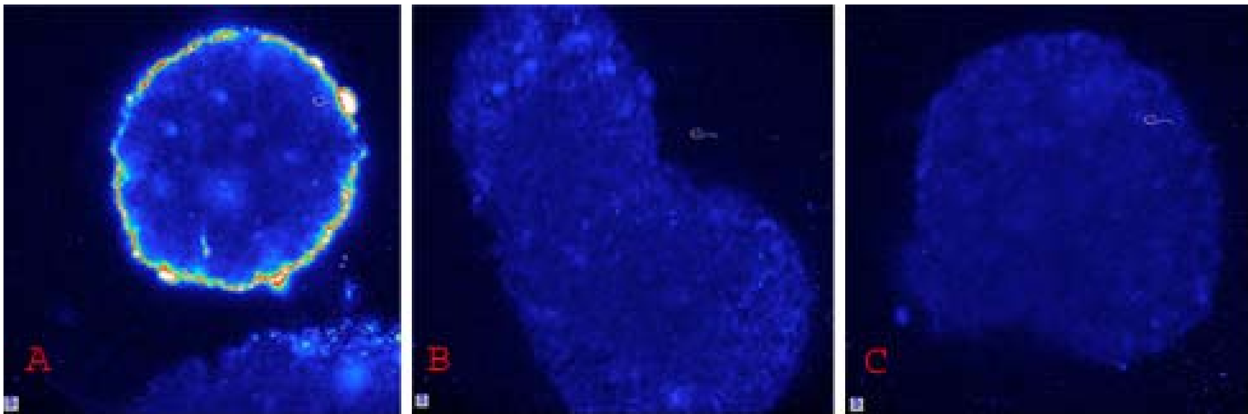


Figure 3. Determining appropriate Ab treatment for Ab-binding experiments.

Images are: A) u*nmodified islet incubated with anti-islet antiserum and FITC-secondary Ab,* B) *unmodified islet incubated with control non-clonal primary Ab and FITC-secondary Ab,* C) *unmodified islet no Ab treatment.*

diffuse to the center of the islet would decrease. The images show, to the contrary, that in the range investigated, there was little if any relationship between diffusion and molecular weight. In all three images, staining of individual islet cells is apparent throughout the islet volume. Compare these images to the negative control in Figure 1. The gain for the negative control image was increased to the point that it was possible to discern the islet background autofluorescence. In contrast, the gain for the images of PEG-modified islets was much lower. In these images, the circumference of each islet cell is discernible, and there is very little staining in the centers of individual cells. This indicates that for all three PEG configurations, a significant amount of PEG is diffusing throughout the islets.

5.3 Confocal Microscopy of Bound Antibody

Prior to completing any qualitative or quantitative antibody binding assays, it was necessary to find an antibody treatment that would label the islets strongly and act as a suitable positive control. Strong labeling was achieved after incubation first with rabbit anti-rat islet antiserum then with donkey anti-rabbit IgG – FITC. Figure 3 shows images of typical islets. The top left image is an un-modified islet incubated with full strength rabbit anti-rat islet antiserum followed by the fluorescein-labeled secondary antibody. Note the strong staining at the islet periphery. The remaining two images are negative controls. The upper right image is an unmodified islet incubated with a solution of non-clonal rabbit IgG followed by the secondary antibody. The final image is an unmodified islet not incubated with any immunological reagents. The negative control images show that non-specific staining and the islet background autofluorescence do not account for the staining observed in the antiserum group.

Figure 4 shows images of three islets that were modified first with 80 kD PEG-FITC then treated with anti-islet antiserum followed by Cy3 labeled secondary Ab. The green channel represents areas where PEG is bound. The red signal represents bound Ab. The PEG coating in these images appears patchy, but this may be due to the operating mode of the microscope used to capture the images. Specifically, the microscope that captured single color images scanned

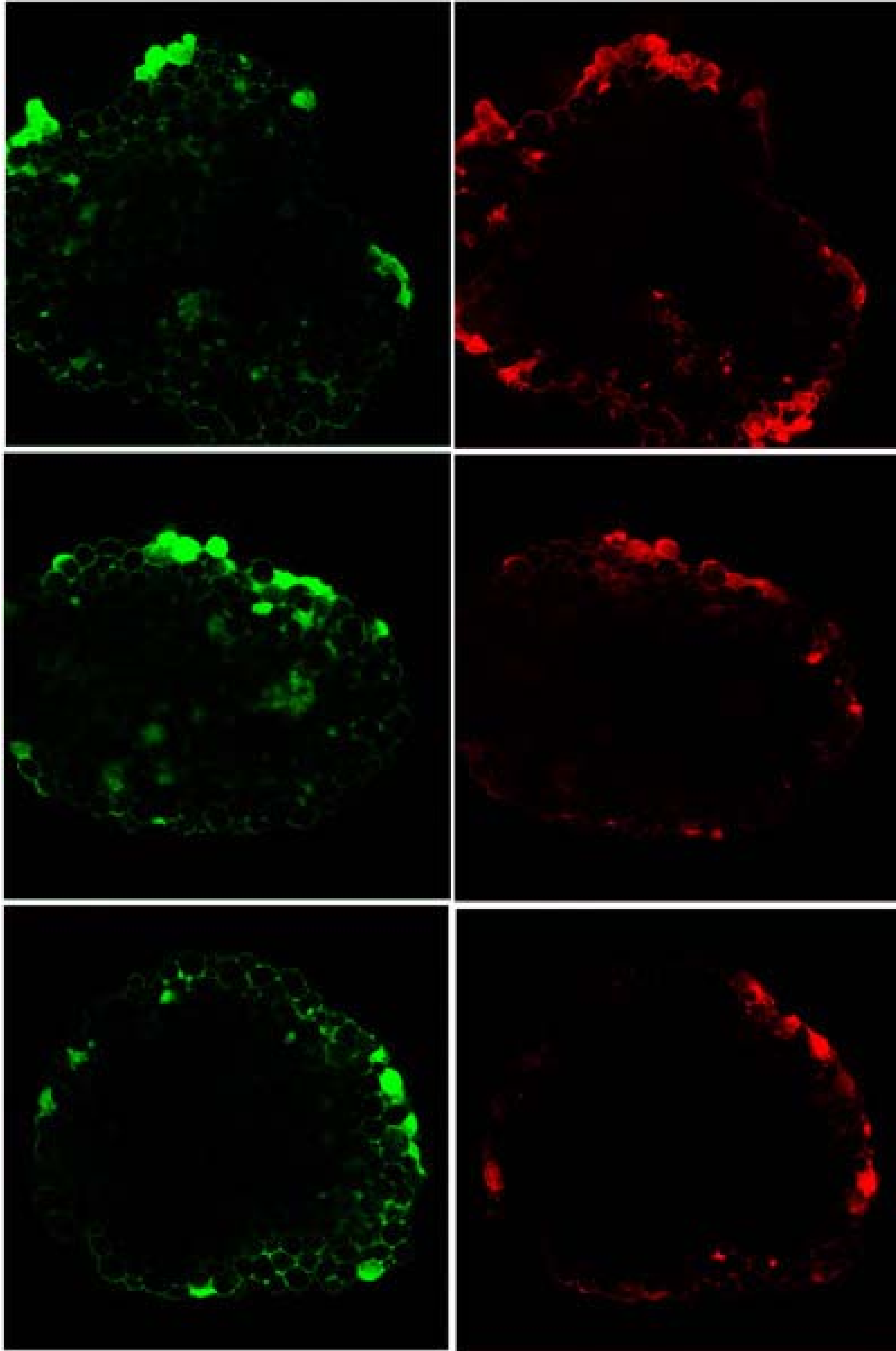


Figure 4. Simultaneous, two-color PEG/Ab images.

Images of 6-day-old islets modified with 80 kD FITC-PEG then incubated with anti-islet antiserum followed by Cy3 labeled secondary Ab. Red represents Ab, Green represents PEG.

the islets twice and averaged the signal from the two scans. The microscope that captured the two-color images scanned the islets a total of eight times per image. Since fluorescein is less photostable than Cy3, the green signal was likely attenuated by photobleaching. Nonetheless, it should be apparent that there are regions on the islet surface where PEG and bound Ab coincide.

5.4 Quantitative Ab-Binding Data

To determine quantitatively, the ability of PEG to prevent Ab binding at the islet surface, a modified ELISA assay was used. Figure 5 shows the results of the binding assay using untreated and 40 kD PEG modified islets. The data is presented in pairs with the untreated groups in the left column and the treated groups in the right. Groups of islets isolated on the same day and from the same animal were paired. Absorbance measurements are directly related to the concentration of converted chromogenic substrate, which is in turn related to the amount of Ab bound to the islet surfaces. It is clear from the chart that 40 kD PEG treatment does not reduce Ab binding. In fact a statistical increase in Ab binding was found for PEG-treated islets, suggesting that by some mechanism, this treatment increases the amount of bound antibody.

The assay was also run using 5 kD PEG treated islets. Figure 6 shows the results of this experiment. Although this is not a significant result, the data does suggest that reacting the islet with this type of PEG had some effect on the quantity of Ab that is able to bind. In seven of the nine trials, the PEG group bound less antibody than the untreated group.

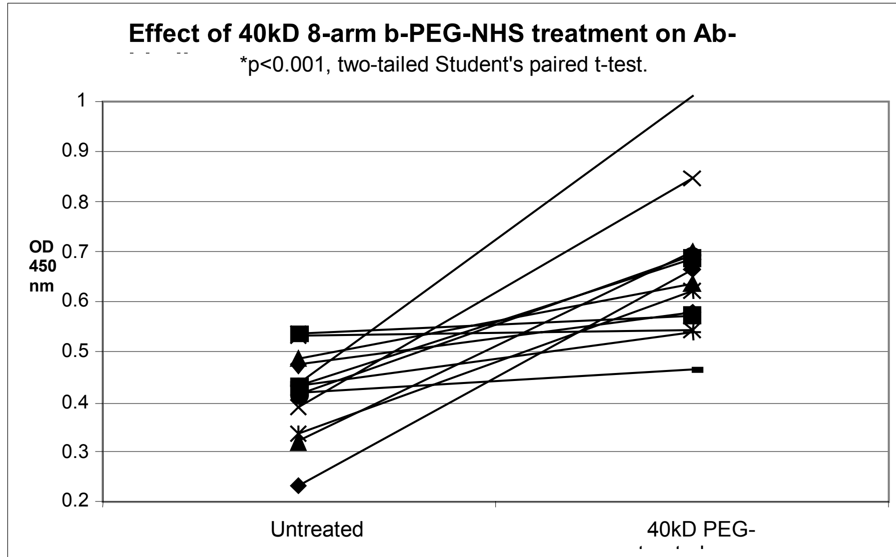


Figure 5. Results of Ab-binding assay for 40 kD PEG-treated islets.

Absorbance at 450 nm is directly related to quantity of Ab-bound. Data appear as paired groups.

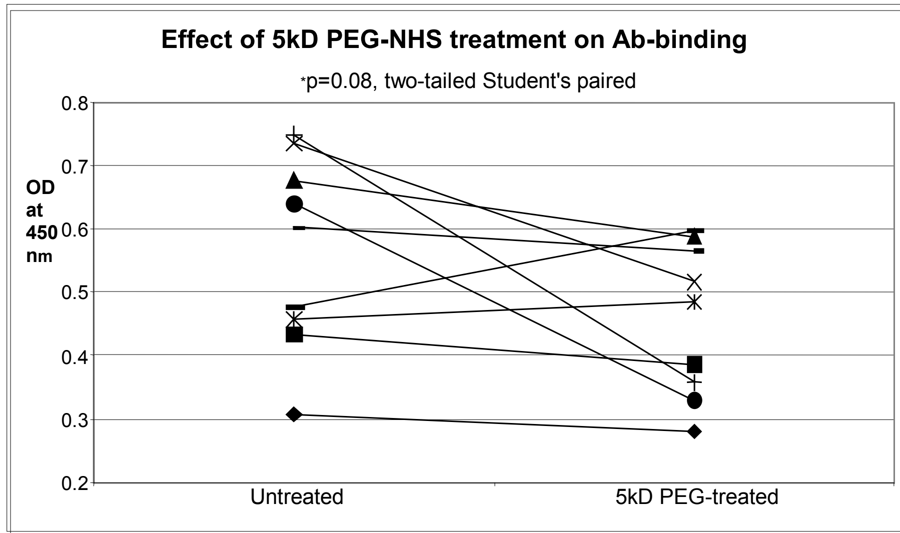


Figure 6. Results of Ab-binding assay for 5 kD PEG-treated islets.

Absorbance at 450 nm is directly related to quantity of Ab-bound. Data appear as paired groups.

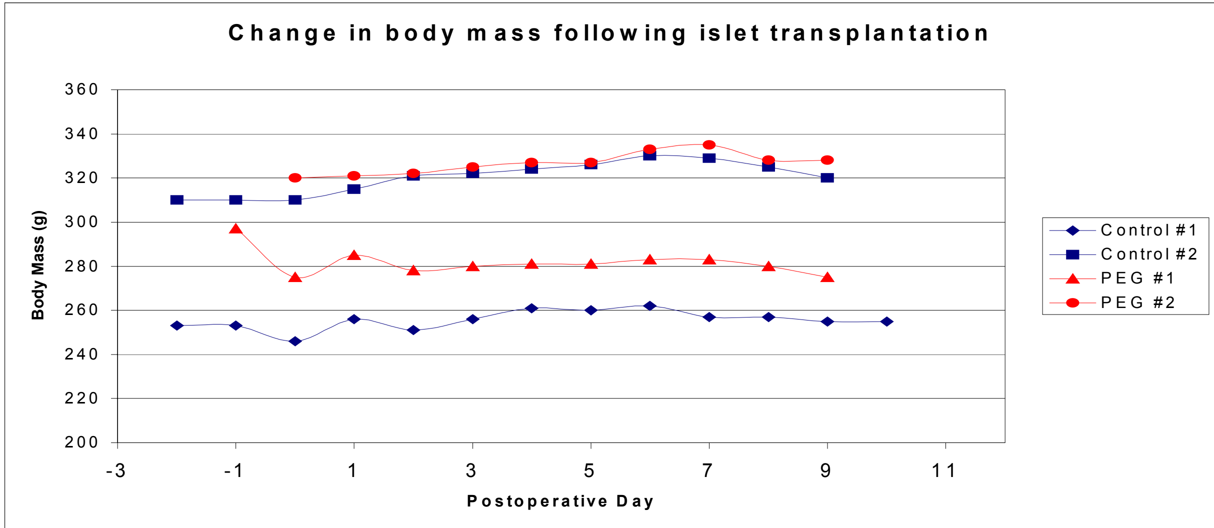
5.5 *In Vitro* Islet Function

To verify that islets were still functioning and could respond to a spike in ambient glucose concentration, a static glucose challenge assay was performed. It was hypothesized that islets would release more insulin in the high concentration glucose solution than in the low concentration glucose solution. The results of the first run of this experiment bore out the hypothesis. High glucose supernatants contained ~5ug/mL more insulin than the low glucose supernatants (data not shown). After this positive result and evidence from a pilot islet transplantation study that the islets were functioning properly *in vivo*, this *in vitro* experiment was considered unnecessary and isolated islets were instead reserved for the transplants.

5.6 *In Vivo* Islet Graft Survival

As a final test of the effectiveness of PEG modification for immunoprotection of pancreatic islets, this strategy was investigated *in vivo*. Figure 7 shows the results of the transplant study. Similar responses were observed from both the untreated and the PEG-treated islet grafts. Blood glucose concentration and body weight were tracked from the day before the transplant until graft failure was confirmed. In all animals, significant reduction of blood glucose concentration was observed after 2–4 days. Weight loss was reversed sometimes after one day and always before 3 days following transplant.

Elevation of blood glucose concentration was generally observed after seven days and full return of the hyperglycemic state always occurred between eight and nine days following the transplant. Due to lack of any observable effect, the experiment was discontinued.



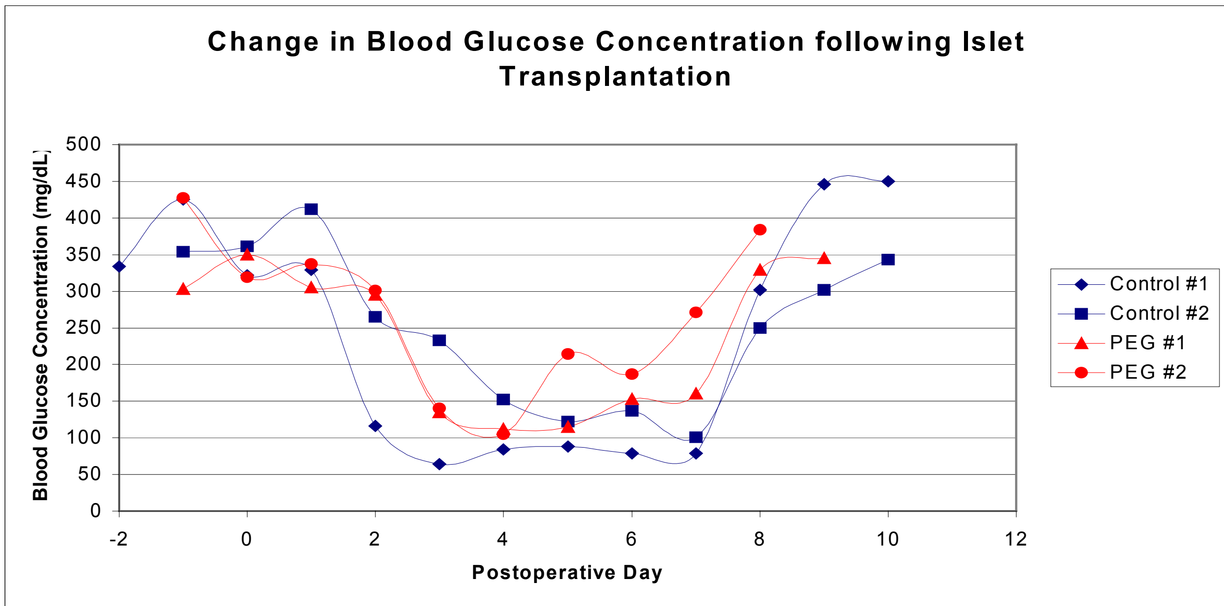


Figure 7. Changes in blood glucose concentration and body mass following pancreatic islet transplantation.

Elevation of blood glucose concentration above 300 mg/dL and reduction in mass at day 8 were considered evidence of graft failure in all animals.

6.0 DISCUSSION

6.1 Confocal Images of Bound PEG

After making adjustments to the previously applied imaging protocol employed by Susan Dadd[35], namely by scanning with greater lateral resolution and capturing with a more powerful objective lens, it was possible to obtain higher resolution images and to discern individual islet cells within the islet volume with confocal microscopy. From these images it became clear that although the collagenous capsule does provide a good substrate for covalent modification, it does not prevent diffusion of large molecules into the islet interior. This suggests that treatment of just the capsule is not sufficient to prevent acute rejection of the pancreatic islets, as islet specific antibodies and inflammatory cytokines may still infiltrate into the islets. However, as the PEG was able to penetrate the islets, good labeling of individual islet cell surfaces was observed, and presence of PEG here may be beneficial, since although permeation of immunologic molecules may not be prevented, their binding might be blocked.

6.2 Quantitative Ab-Binding Assay

After observation of confocal images of islets treated either with fluorescent PEG or with a fluorescent secondary antibody, it was obvious that in some instances the fluorophore was present inside individual islet cells. This is evidence that these cells are dead and thus are more permeable to large molecules (figure 8). This is important to keep in mind when interpreting data from the quantitative antibody binding assay. Islet cell necrosis would depend on such factors as time in culture, purity of the islet preparation and degree of collagenase digestion, and would be difficult to predict. Due to the fact that necrotic regions of the islets were likely to become saturated with secondary antibody, and that a relatively low number of islets contained large necrotic regions, this may have artificially elevated measurements of islet-specific, bound antibody in some but not all experimental groups. Specifically, in the antibody binding assay for

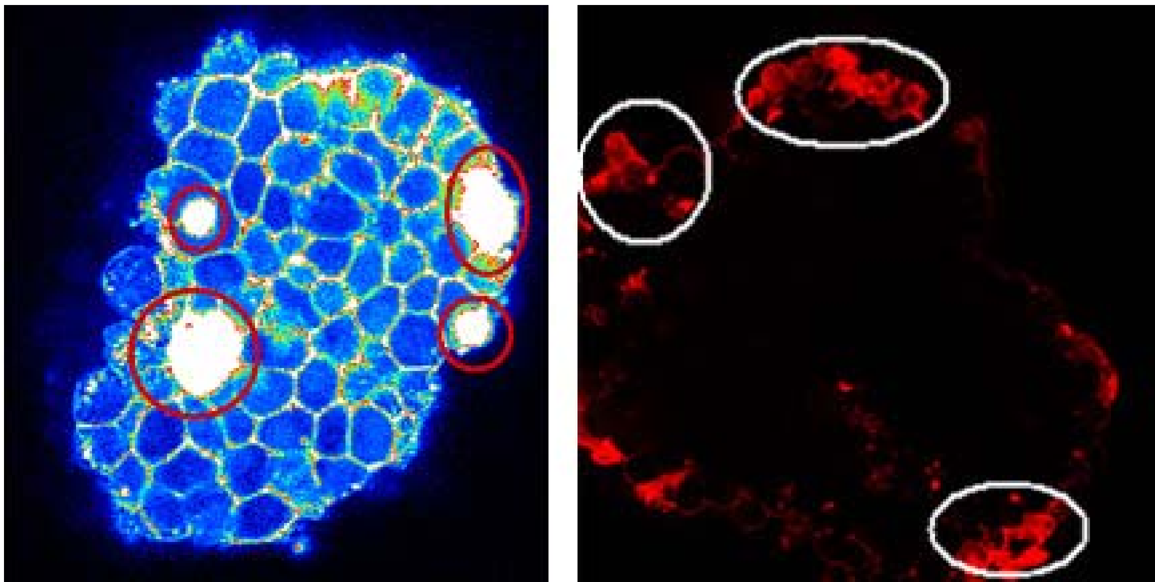


Figure 8. Images showing presence of putative necrotic regions.

A)Image of islet modified with flourescent PEG, B)Image of islet modified with PEG then treated with anti-islet antiserum and flourescent secondary antibody. Circled areas appear to be permeabilized islet cells, which have become saturated with the respective flourescent marker

5kD PEG-treated islets, of nine paired groups, seven showed reduced antibody binding from the untreated control. It may be that in the two groups that did not follow the trend, a greater number of PEG-treated islets contained significant regions of necrosis, in which case the reading taken would not accurately reflect the amount of islet-specific bound antibody. Although the results of this experiment were not statistically significant, it seems likely that there is some potential for PEG to reject specific antibody binding to pancreatic islets.

6.3 *In Vivo* Transplantation Study

The results from the *in vivo* islet transplantation study suggest that covalent modification with 5kD PEG does little to prevent acute rejection of transplanted allotypic pancreatic islets. It may be that the surface PEG layer is simply not a robust enough barrier to prevent access to the islet by the various components of the host immune system. However, certain factors for successful islet transplant could not be satisfied given the limited resources available for this research. For instance, choice of implant site was governed by the technical limitations of the researcher. Sites more suitable for islet transplant might be the portal vein or under the kidney capsule.[41,42] Additionally, it has been observed that transplantation of a greater number of islets often leads to a more successful result.[43,44] Due to limitations of time it was not possible to prepare a sample of more than 4,000 islets for each transplant. Although this graft size was sufficient to reverse diabetes initially, the rapid reversion to the diabetic state may have been due to a less than ideal number of islets per graft. This research thus does not clearly eliminate covalent modification of pancreatic islets with PEG as a way to shield this transplanted tissue from the host immune system, but the transplant protocol as it was performed does not clearly suggest that this approach would be a clinically feasible solution to IDDM.

6.4 Future Directions

Perhaps the most fruitful approach involving covalently bound PEG will be to combine this technique with other chemical or biological means to modify the immune response. One can envision PEG as a molecular tether to immobilize slow release vesicles or genetically altered cells that would release anti-inflammatory cytokines locally at the islet surface. Polyfunctional PEG could also serve as a foundation for a more extensive PEG microstructure, which could provide a diffusion barrier as well as containing cells or drug-delivery vehicles. As it has been shown to have little effect on viability or function of pancreatic islets, [25,26] and since it may have the effect of limiting antibody binding, this technique warrants further attention as a means of immunoisolating pancreatic islets.

APPENDIX

Appendix A

Additional Islet Images

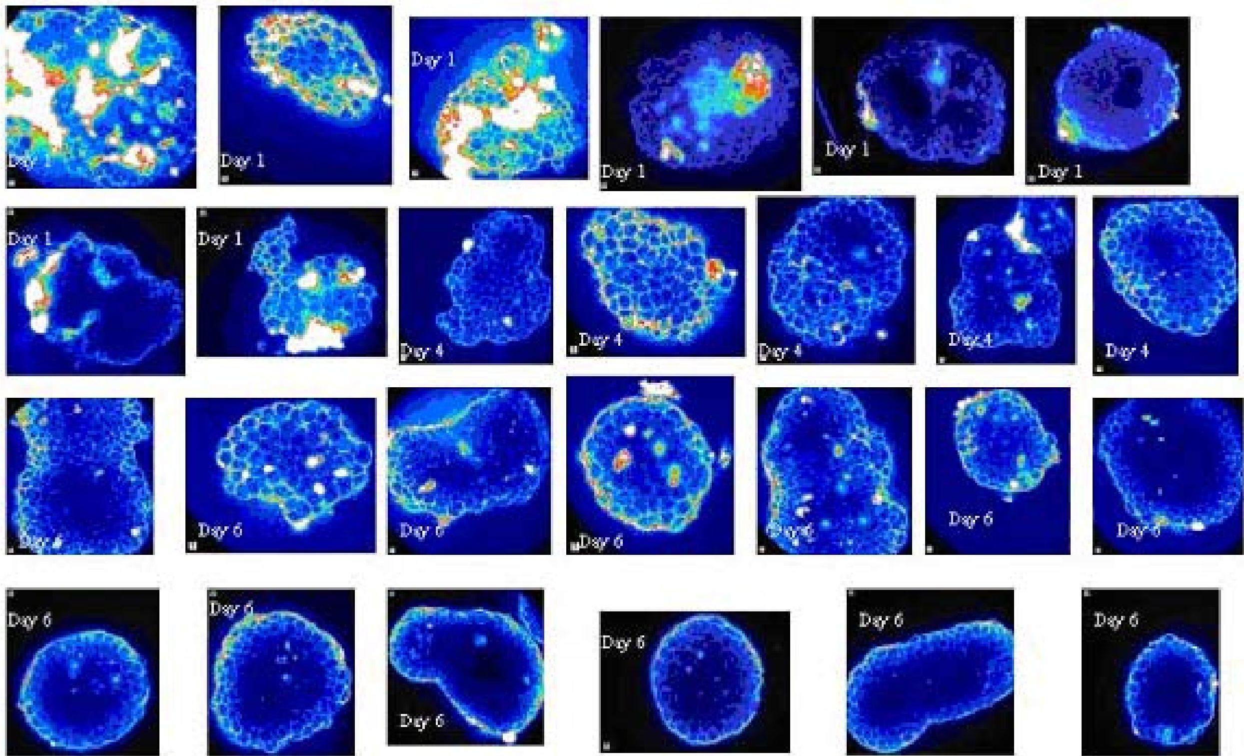


Figure 9. Images of islets modified with 80kD PEG-FITC

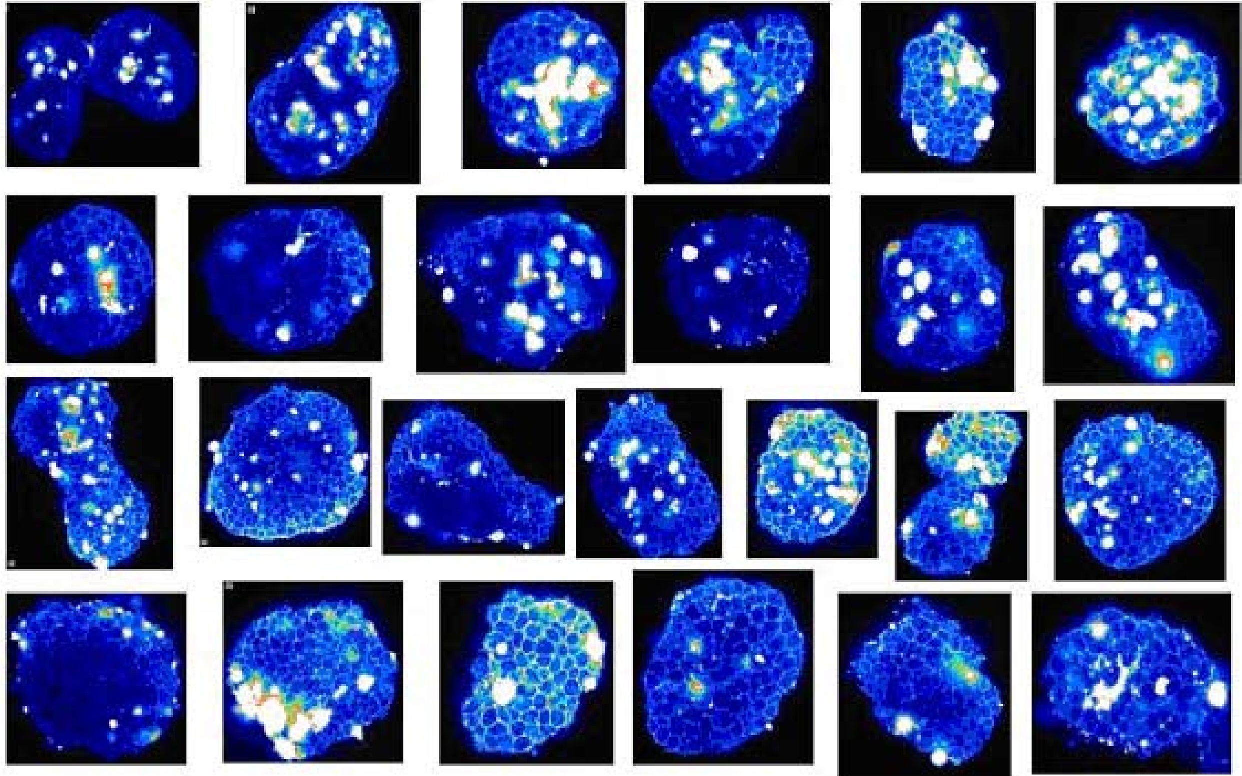


Figure 10. Images of islets modified with 40kD PEG-FITC, after six days in culture.

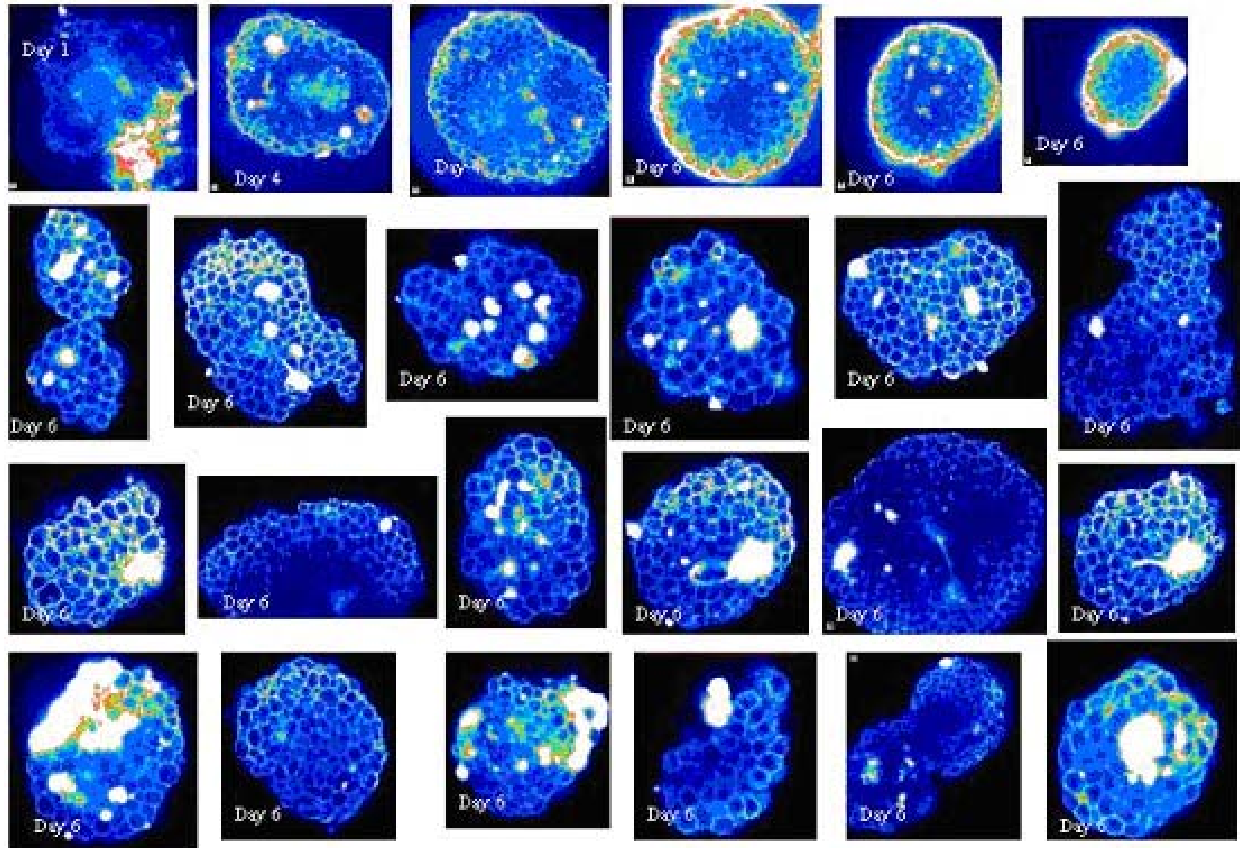


Figure 11. Images of islets modified with 5kD PEG-FITC.

BIBLIOGRAPHY

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1. Berne RM, Levy MN. Physiology. 4th ed. New York: Mosby: pp. 772, 796, 807, 822-846, 1010. 1998.

2. Green A. "Epidemiology of IDDM." In: Leslie RDG, Robbins DC (eds). Diabetes Clinical Science in Practice. Cambridge: Cambridge University Press: 106-16, 1995.

3. Leslie RDG, Gale EAM. "Aetiology of IDDM." In: Leslie RDG, Robbins DC (eds). Diabetes Clinical Science in Practice. Cambridge: Cambridge University Press: 134-56, 1995.

4. "Glucose Homeostasis." In: Leslie RDG, Robbins DC (eds). Diabetes Clinical Science in Practice. Cambridge: Cambridge University Press: 103, 1995.

5. Carroll PB, "Anatomy and Physiology of Islets of Langerhans." In: Ricordi C (ed). Pancreatic Islet Cell Transplantation. Austin: R.G. Landes Company: 7-18, 1992.

6. White SA, James RFL, Swift SM, Kimber RM, Nicholson ML. "Human islet cell transplantation – future prospects." Diabetic Medicine. 18: 78-103. 2001

7. Giannoukakis N, Rudert WA, Robbins PD, Trucco M. "Targeting autoimmune diabetes with gene therapy." Diabetes. 48: 2107-2121. 1999.

8. Janeway CA, Travers P, Walport M, Capra DJ. Immunobiology: The Immune System in Health and Disease. 2nd ed. London: Elsevier Science Ltd. 1999.

9. Pozzilli P. "Strategies for the prevention of Insulin-Dependent Diabetes." In Leslie RDG, Robbins DC (eds). Diabetes Clinical Science in Practice. Cambridge: Cambridge University Press: 392-404, 1995.

10. O'Shea G, Sun A. "Encapsulation of Rat Islets of Langerhans Prolongs Xenograft Survival in Diabetic Mice." Diabetes. 35: 943-946. 1986.

11. Fan M, Lim Z, Fu X, Levesque L, Tai L, Sun A. "Reversal of Diabetes in BB Rats by Transplantation of Encapsulated Pancreatic Islets." Diabetes. 39: 519-22. 1990.

12. Mazaheri R, Atkison P, Stiller C, Dupre J, Vose J, O'Shea G. "Transplantation of Encapsulated Allogeneic Islets into Diabetic BB/W Rat." Transplantation. 1: 750-4. 1991.

13. Lum Z, Tai I, Krestow M, Norton J, Vacek I, Sun A. "Prolonged reversal of diabetic state in NOD mice by xenografts of microencapsulated rat islets." Diabetes. 40:1511-16. 1991.

14. Lacy P, Hegre O, Gerasimidi-Vazeou A, Gentile F, Dione K. "Maintenance of normoglycemia in diabetic mice by subcutaneous xenografts of encapsulated islets." Science. 254: 1782-84. 1991.

15. Iwata H, Takagi T, Amemiya H, Shimizu K, Yamashita K, Kobayashi K, Akutsu T. "Agarose for a bioartificial pancreas." Journal of Biomedical Materials Research. 29: 967-77. 1992.

16. Lanza R, Borland K, Staruk J, Appel M, Solomon B, Chick W. "Transplantation of encapsulated islets into spontaneously diabetic BB/Wor rats without immunosuppression." Endocrinology. 131:637-42. 1992.

17. Cruise GM, Hegre OD, Lamberti FV, Hager SR, Hill R, Scharp DS, Hubbell JA. "*In vitro* and *in vivo* performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes." Cell Transplantation. 8: 293-306. 1999.

18. Lim F, Sun AM. "Microencapsulated islets as bioartificial endocrine pancreas." Science. 210: 908-10. 1980.

19. Pollok JM, Kolln PA, Lorenzen M, Torok E, Kaufmann PM, Kluth D, Bohuslaviski KH, Gundlach M, Rogiers X. "Islets of Langerhans encapsulated with a tissue-engineered membrane of rat chondrocytes maintain insulin secretion and glucose-insulin feedback regulation for at least 30 days in culture." Transplantation Proceedings. 33: 1713-4. 2001.

20. Duvivier-Kali VF, Omer A, Parent RJ, O'Neill JJ, Weir GC. "Complete protection of islets against allorejection and autoimmunity by a simple barium-alginate membrane." Diabetes. 50: 1698-1705. 2001.

21. De Vos P, Wolters GHJ, Fritschy WM, van Schilfgaarde R. "Obstacles in the application of microencapsulation in islet transplantation." International Journal of Artificial Organs. 16(4): 205-12. 1993.

22. Deible CR. Molecular Barriers to Thrombosis Formed with Protein Reactive Poly(ethylene glycol). PhD. Dissertation, University of Pittsburgh, 1998.

23. Kjellander R, Florin E. "Water structure and changes in thermal stability of the system poly(ethylene glycol)-water." Journal of the Chemical Society, Faraday Transactions. 77: 2053-77. 1981.

24. Knoll D, Hermans J. "Polymer-protein interactions." The Journal of Biological Chemistry. 258(9): 5710-5. 1983.

25. Panza JL. Strategies for covalent modification of pancreatic islet capsules with polymers for possible immunoisolation. M.S. Thesis, University of Pittsburgh. 1998.

26. Panza JL, Wagner WR, Rilo HLR, Rao RH, Beckman EJ, Russell AJ. "Treatment of rat pancreatic islets with reactive PEG." Biomaterials. 21: 1155-64. 2000.

27. Maechling-Strasser C, Galin JC, Schmitt A. "Preadsorption of polymers on glass and silica to reduce fibrinogen adsorption." Journal of Biomedical Materials Research. 23: 1385-93. 1989.

28. Maechling-Strasser C, Galin JC, Schmitt A, Housse-Ferrari V, Sebille B, Mulvihill JN, Cazenave JP. "Synthesis and adsorption of a poly(n-acetylethyleneimine)-polyethyleneoxide-poly(n-acetylethyleneimine) triblock-copolymer at a silica/solution interface. Influence of its preadsorption on platelet adhesion and fibrinogen adsorption." Journal of Biomedical Materials Research. 23: 1395-1410.

29. Lee JH, Kopecek J, Andrade JD. "Protein-resistant surfaces prepared by PEO-containing block copolymer surfactants." Journal of Biomedical Materials Research. 23: 351-68. 1989.

30. Desai NP, Hubbell JA. "Solution technique to incorporate polyethylene oxide and other water-soluble polymers into surfaces of polymeric biomaterials." Biomaterials. 12: 144-53. 1991.

31. Desai NP, Hubbell JA. "Tissue response to intraperitoneal implants of polyethylene oxide-modified polyethylene terephthalate." Biomaterials. 13: 505-10. 1992.

32. Brunstedt MR, Ziats NP, Schubert M, Stack S, Rose-Caprara V, Hiltner PA, Anderson JM. "Protein adsorption and endothelial cell attachment and proliferation on PAPI-based additive modified poly(ether urethane ureas)." Journal of Biomedical Materials Research. 27: 499-510. 1993.

33. Sheu MS, Hoffman AS, Ratner BD, Feijen J, Harris JM. "Immobilization of polyethylene oxide surfactants for non-fouling biomaterial surfaces using an argon glow discharge treatment." Journal of Adhesion Science and Technology. 7(10): 1065-76. 1993.

34. Tseng YC, Park K. "Synthesis of photoreactive poly(ethylene glycol) and its application to the prevention of surface-induced platelet activation." Journal of Biomedical Materials Research. 26: 373-91. 1992.

35. Dadd SJ. Characterization of pancreatic islet modification with amine-reactive polyethylene glycol. M.S. Thesis, University of Pittsburgh, 1999.

36. Hotta M. Tashiro F. Ikegami H. Niwa H. Ogihara T. Yodoi J. Miyazaki J. "Pancreatic beta cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes." Journal of Experimental Medicine. 188(8):1445-51, 1998.

37. Moriscot C. Pattou F. Kerr-Conte J. Richard MJ. Lemarchand P. Benhamou PY. "Contribution of adenoviral-mediated superoxide dismutase gene transfer to the reduction

in nitric oxide-induced cytotoxicity on human islets and INS-1 insulin-secreting cells." Diabetologia. 43(5):625-31, 2000.

38. Sentman ML. Jonsson LM. Marklund SL. "Enhanced alloxan-induced beta-cell damage and delayed recovery from hyperglycemia in mice lacking extracellular-superoxide dismutase." Free Radical Biology & Medicine. 27(7-8):790-6, 1999.

39. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C, Mellor AL: "Prevention of allogeneic fetal rejection by tryptophan catabolism." Science 281:1191-1193, 1998.

40. De Vos P. De Haan B. Pater J. Van Schilfgaarde R. "Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts." Transplantation. 62(7):893-9, 1996.

41. Mythili MD. Ramakrishna B. Gunasekaran S. "Reduction of immunogenicity by cyclophosphamide pretreatment and culture on xenograft survival of monkey pancreatic islets transplanted into rats." Pancreas. 24(3):235-41, 2002 Apr.

42. Ikebukuro K, Adachi Y, Yamada Y, Fujimoto S, Seino Y, Oyaizu H, Hioki K, and Ikehara S. "Treatment of streptozotocin-induced diabetes mellitus by transplantation of islet cells plus bone marrow cells via portal vein in rats." Transplantation. 73(4):512-8, 2002 Feb 27.

43. Wennberg L, Song Z, Bennet W, Zhang J, Nava S, Sundberg B, Bari S, Groth CG, and Korsgren O. "Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days." Transplantation. 71(8):1024-33, 2001 Apr 27.

44. Jain K, Asina S, Yang H, Blount ED, Smith BH, Diehl CH, and Rubin AL. "Glucose control and long-term survival in biobreeding/Worcester rats after intraperitoneal implantation of hydrophilic macrobeads containing porcine islets without immunosuppression." Transplantation. 68(11):1693-700, 1999 Dec 15.