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Serial Analysis of Biomarkers of Acute Pancreas Allograft Rejection

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Abstract

Pancreas transplant recipients experience graft loss in spite of improvements in immunosuppressant therapies and diagnostic technologies. Therefore, a method to improve detection and management of acute rejection is needed. This longitudinal study investigated the usefulness of three biomarkers, granzyme B, perforin, and HLA-DR, measured by real-time PCR on peripheral blood mononuclear cells, for their ability to detect acute rejection and its resolution in 13 recipients of pancreas allograft. Data demonstrated that pre-transplant baseline expression of biomarkers decreased following the initiation of immunosuppression. Throughout follow-up (range 3–27 months), individuals without acute rejection episodes had little variation in their biomarker levels. Recipients with biopsy-proven rejection had a significant increase in the levels of biomarkers as early as 5 weeks before clinical rejection diagnosis. Furthermore, all seven patients with biopsy-proven rejection demonstrated a decrease in the levels of granzyme B and perforin following the increased immunosuppression for the treatment of rejection. This is the first clinical serial measurement of biomarkers in pancreas patients. The data demonstrate that upregulation of granzyme B, perforin, and HLA-DR in peripheral blood mononuclear cells are sensitive to changes in the immune environment and could possibly be used to identify those patients at higher risk of rejection.

Keywords

Biomarkers; Pancreas Transplantation; Rejection; Pancreas Allograft

Introduction

Acute pancreas allograft rejection is a major obstacle that contributes to both short and long-term consequences of transplantation, despite advances in immunosuppressant therapies and diagnostic technology (1–5). During the first year following transplantation over 20% of pancreas allograft recipients lose their graft and by 5 years posttransplant the rate of graft loss is over 50% (6). Each year in the United States an increasing proportion of pancreas transplants (7) are going to patients who have had failure of a previous allograft, further

depleting our supply of available organs. Unfortunately, allograft biopsy, the standard for the diagnosis of pancreas rejection, is an invasive procedure that is often technically difficult and associated with risk to the patient (8,9). Because of the incidence of acute rejection and the difficulty in clinical diagnosis, a less invasive method to determine acute rejection is uniquely important in pancreas transplant recipients.

In kidney transplant recipients it has been suggested that treatment of subclinical rejection episodes can prevent deterioration of graft function (10,11) and lead to improved long-term outcomes. Simon et al. suggested that in kidney transplant recipients serial measures of peripheral blood perforin and granzyme B gene expression have been a way to recognize graft rejection in subclinical stages (12). Using immunohistochemical techniques, marked expression of major histocompatibility complex, class II, DR alpha (HLA-DRA) and granzyme B has been associated with acute rejection in pancreas allograft biopsies (9). We have used a cross-sectional design approach to demonstrate variations in expression levels of granzyme B among patients with and without biopsy-proven pancreas rejection (8). The purpose of the current study was to determine if serial measurements of rejection biomarkers could be used to detect and monitor clinical rejection in pancreas transplant recipients. To achieve this purpose we followed a group of newly transplanted patients who were enrolled in the monitoring study prior to transplantation and were followed for the first 3 months following transplantation. We also enrolled a group of patients at the time of clinically-indicated biopsy and followed for 3 months to explore changes in biomarker levels during and following any treatment modifications for those diagnosed with acute rejection. For all participants, we collected clinical follow-up data at 12 to 24 months following enrollment to determine long-term organ status. We hypothesized that the elevation in gene expression levels of perforin, granzyme B, and HLA-DRA would be predictive biomarkers for acute rejection in pancreas allograft recipients and would diminish following resolution of an acute rejection episode.

Patients and methods

Between April 2001 and April 2003 serial blood samples were collected from adults awaiting or having had a pancreas transplantation with enteric portal drainage at an urban transplant center. Appropriate institutional review board approval was obtained and all subjects were consented prior to enrollment into the study. Potential subjects were enrolled at one of two time points: prior to pancreas alone transplantation or following clinically-indicated pancreas biopsy. Subjects enrolled prior to pancreas transplantation had blood samples obtained prior to and at set time points for 3 months after transplantation. Subjects enrolled at time of pancreas biopsy for evaluations of suspected rejection were followed with serial blood samples for three months. Nine of the pancreas alone transplant recipients had received previous kidney transplantations. Because none of our subjects had a renal biopsy during the study, we confirmed that the rejection episode was pancreas (not kidney) in origin using a two-step approach. First, we monitored markers of kidney clinical rejection (i.e., creatinine, blood urea nitrogen) throughout the study period and all were determined to be within normal ranges. Furthermore, we did a chart review to determine if any subject experienced kidney rejection episodes within one year of completion of the study; none did. Thus, we categorized these individuals as undergoing pancreas only rejection. In addition, all recipients in the rejection group had a pancreas biopsy that demonstrated acute rejection.

Thirteen individuals met study inclusion criteria. Of those 13, seven had rejection episodes and six were without rejection episodes. This study was restricted to recipients who had from 4–20 (mean of 7) samples available for biomarker evaluation. Exclusion criteria for all subjects included diagnosis of chronic rejection alone on initial biopsy or at time of enrollment, pancreatitis, and kidney rejection within 2 months after pancreas biopsy. No

subject experienced any evidence of acute or chronic kidney rejection while enrolled in the study and for the 12 months after study completion. Biomarker levels were measured for gene expression of perforin, granzyme B, and HLA-DRA in 135 samples from these 13 subjects. Detailed demographic and clinical data, including induction treatment, immunosuppressants, and biopsy diagnosis and treatment can be found in Table 1 and 2. Clinical rejection diagnosis was determined by histologic diagnosis from a pancreas biopsy.

Immunosuppressant therapy

The standard immunosuppressant therapy for pancreas transplant recipients enrolled at time of transplant included induction with rabbit-derived anti-thymocyte globulin (rATG) and maintenance immunosuppression with tacrolimus, mycophenolate mofetil, and prednisone. The four subjects who received a pancreas transplant prior to 2001 received no induction (n=1), or induction with rATG (n=1) or a monoclonal antibody (n=2). During collection of longitudinal data, the immunosuppression regimens were tailored based on individual clinical presentation and response.

Biopsies

Surveillance biopsies were routinely performed 2, 4, 8, and 12 weeks after pancreas transplantation for pancreas recipients. Rejection severity was graded on a scale of 1–5 (5 being the most severe) according to the modification of the Maryland classification of allograft rejection for pancreas biopsies (13,14). Recipients of simultaneous pancreas-kidney transplants (SPK) did not undergo surveillance biopsies. These recipients were monitored for rejection by evaluating pancreas function. None of these recipients had evidence of rejection based on clinical markers alone and were therefore classified with the no-rejection group. Clinical pancreas biopsies were performed on 5 subjects (9–25 months after initial transplantation) for evaluation of elevated amylase and lipase.

Sample collection

Peripheral blood samples were collected from all subjects. Samples for gene expression were collected in acid citrate dextrose containing tubes from which peripheral blood mononuclear cells were separated using Histopaque (Sigma, St. Louis, MO, USA) within 24 hours of blood collection. Cells were stored in RNAlater™ (Ambion, Inc., Austin TX, USA) at –20°C until RNA extraction. Collection of chemistry and hematology samples were performed according to transplant center guidelines as well as those of the individual locations at which the phlebotomy and analysis of these samples was performed.

RNA extraction

RNA extraction was performed using RNAqueous kit (Ambion Inc). DNase I treatment [DNA-free™ (Ambion Inc)] was done following RNA extraction to remove any residual genomic DNA. RNA concentration was quantitated using the GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotechnology) and the RNA was then used in the one-step real-time PCR assay.

TaqMan® probes and primers

The primers and probes for granzyme B, perforin, and HLA-DRA were designed using the Primer Express® software (PE Applied Biosystems, California, USA) (Table 3). The primers and probes for the housekeeping gene (18S) were obtained from PE Applied Biosystems as pre-developed assay reagents. All primer pairs were designed to produce amplicons smaller than 150 bp. Furthermore, the primers for granzyme B and perforin were designed to cross exon-exon junctions to further reduce the risk of genomic DNA amplification. Because this was not feasible for the HLA-DRA, DNase treatment of

extracted RNA was conducted on all samples to reduce the possibility of genomic DNA amplification.

Real-time PCR assay

Reverse transcription and real-time quantitative PCR were performed by the TaqMan® EZ RT-PCR kit (PE Applied Biosystems) as a one-step reaction and using the RNA TaqMan® EZ RT-PCR Reagents and the protocol provided by the manufacture. The *rTth* DNA polymerase used in these assay functions as both a thermoreactive reverse transcriptase and a thermostable DNA polymerase that provides the 5'-3' nuclease activities necessary for the cleavage of the fluorogenic probe. A typical reaction contained 1ng total RNA and 5 units (2μL) of *rTth* DNA polymerase, 3mM Mn acetate in 50μL reaction volume. Primers and TaqMan probes were added at a final concentration 500 nM and 100 nM respectively. The dNTP final concentration was 0.3 mM except for dUTP, which was at 0.6 mM. Amplification of the target genes tested and the 18S RNA was performed in the same plate using the ABI-PRISM 7700 Sequence Detection System. The program consisted of heating at 50 °C for 2 min, 60 °C for 30 min, and 95 °C for 5 min, followed by 40 cycles of a two-stage temperature profile of 94 °C for 20 seconds and 62 °C for 1 min. Accumulation of the PCR products was detected by directly monitoring the increase in fluorescence of the reporter dye. Data points collected in this manner were analyzed at the end of thermal cycling. The mean of the background fluorescence emission for all the tested wells measured between cycles 3 and 15 was recorded and used to set the baseline. A threshold for the amplification of each gene of interest was then set by drawing a line that intersects the exponential phase of the logarithmic amplification curves for all samples being analyzed for expression of target gene. The cycle number at which the threshold line intersects the linear curve for each sample was used to determine the threshold cycle (C_T) value.

Analysis of patient samples and data calculation

The RNA extracted from each sample was used to amplify genes of interest as well as a chosen housekeeping gene (18S) to control for variations in amounts of RNA. The quantification of the target gene was calculated using the relative standard method. The relative standard includes known amounts of the target nucleic acid, range from 10^9 - 10^3 copies, in each run and was used to quantify the amount of a target sequence normalized to the copy number of 18S, the housekeeping gene. All results were expressed as the ratio of copy number of the target gene to the copy number of 18S.

Statistical analysis

Descriptive statistics were used to characterize the groups (rejection and no-rejection). Group differences in demographic and clinical variables were examined using chi-square for categorical data and t-test or ANOVA for interval level data.

Results

Gene expression levels of granzyme B, perforin, and HLA-DRA were serially measured in the peripheral blood of patients with (n=7) and without (n=6) biopsy proven rejection of pancreas allografts. There was a great deal of inter-individual variance for the biomarker gene expression levels. Therefore, the reported gene expression levels, which were calculated based on gene copy numbers/18s copy number, varies between individuals.

Elevation of biomarkers prior to biopsy proven rejection

Three out of the seven recipients with acute rejection episodes had blood collected at the pre-transplantation time point and were followed for up to 44 weeks post transplantation.

Our data demonstrate that three recipients showed a significant increase in granzyme B, HLA-DR, as well as perforin gene expression as early as 5 weeks, and possible as early as 27 weeks, before the traditional diagnosis as shown in the Figure 1a, b, c. It should be noted that there was a gap of 27 weeks without data for one subject. Specifically, at week 13 (Figure 1c), there was an increase in biomarkers; yet, there was no change in clinical management to indicate a diagnosis of clinical rejection at that time and samples for gene expression determination were not collected again until week 40. At week 40, there were clinical indications of rejection resulting in a clinically indicated biopsy and reentry into the study. Biopsy-proven acute rejection was identified. It is possible, therefore, that the elevation in biomarkers at week 13 heralded a future acute rejection episode despite the lack of clinical evidence at that time.

Decrease in biomarker levels following effective treatment with immunosuppressants

The effect of immunosuppressant therapy on gene expression was investigated in all of the seven patients with biopsy proven rejection (three from pre-transplant and four from time of clinically indicated biopsy). All seven patients with biopsy proven rejection demonstrated a decrease in the levels of granzyme B and perforin within days following the adjustment of their immunosuppression therapy. For the two patients' data presented in Figure 1A and 1B, the peaks that appear after the episode of acute rejection and the immunosuppression therapy did not correlate with any known events. Interestingly, two patients who had biopsy proven rejection at entry into the study did not have resolution of their rejection episode following initial treatment with immunosuppressants. As can be seen (Figure 2a, b) each patient continued to have elevated biomarkers until a clinically indicated second biopsy confirmed ongoing rejection and further immunosuppression therapy was given resulting in a decrease in biomarker levels and resolution of acute rejection episodes.

Gene expression of biomarkers in recipients without biopsy-proven rejection

Six recipients did not experience an acute rejection episode. Immediately after transplantation, in those without rejection episodes, biomarkers either diminished or remained stable during the study period. Throughout the follow-up time period, we found that individuals without acute rejection episodes had minimal variations in their biomarker expression levels (Figure 3a, b, c). Prior to onset of acute rejection, there is no similar pattern demonstrating elevations in levels of amylase and lipase such as that seen with the biomarkers. However, at time of biopsy-proven rejection, mean serum amylase and lipase values were increased by at least 2 fold (data not shown). Correlation between elevated amylase, lipase, and biomarkers at time of acute rejection has been demonstrated (8). These values all diminished during treatment with immunosuppressants.

Discussion

Pancreas rejection is diagnosed on the basis of clinical observation and histological examination of a biopsy. The diagnosis may be uncertain if biopsy cannot be obtained or there was not an adequate representative sample. Earlier we have reported that the mean values for granzyme B in patients with biopsy-proven pancreas rejection had a significantly higher level than control subjects with type 1 diabetes ($p \leq 0.03$). Our findings suggested that, with additional evaluation of a larger number of patients as well as a longitudinal approach, analyses of granzyme B expression levels in peripheral blood may be shown to be a noninvasive method of monitoring pancreas allograft rejection (8). The mean level of perforin and HLA-DRA expression was also higher in patients with acute rejection.

The relevance of perforin and granzyme B as a molecular signature that correlates with rejection is supported by clinical (12,15–20) as well as animal studies (21,22). Perforin-

deficient mice heart allografts survived longer (mean 87.8 days) than in control mice (mean 31.5 days) (21). Heusel et al. showed in an elegant study that granzyme B serves a critical and non redundant role for the rapid induction of target cell DNA fragmentation and apoptosis by alloreactive cytotoxic T lymphocytes and that granzyme deficient mice have reduced cytotoxic activity (22).

In the present study, we performed the first serial measurements of gene expression of cytotoxic lymphocytes (granzyme B and perforin) and HLA-DRA, measured by real-time PCR on peripheral blood mononuclear cells, as biomarkers of acute rejection in recipients of pancreas allograft. Thirteen recipients were followed for up to 44 weeks with scheduled blood draws to explore the relationship between gene expression levels and episodes of biopsy-proven rejection.

Our findings demonstrate that in a subset of recipients there is an increase in the level of biomarkers as early as 5 weeks prior to clinical rejection diagnosis and in one subject possibly as early as 27 weeks. In addition, in those recipients experiencing acute rejection, biomarker levels diminish once immunosuppressant therapy is begun. Our results indicate that these biomarkers may be useful as predictive biomarkers of recipients who may be at higher risk of acute rejection. In addition, biomarkers diminished during periods of treatment with immunosuppressants following episodes of acute rejection. Therefore, use of these biomarkers may be important in determining efficacy of immunosuppressant treatment. Potentially, these biomarkers could be part of a panel of biomarkers that monitored for persistent episodes of rejection that were not responding to traditional therapy with immunosuppressants. However, further studies with larger sample sizes are needed to more accurately determine sensitivity and specificity.

With the advent of the genomic era of medicine, conducting a similar study using a panel of genes would allow for the identification of the most sensitive and specific genes allowing us to determine an index (score) that would include numerous biomarkers. Currently, AlloMap is a similar product that is FDA approved to detect acute rejection in recipients of cardiac transplantation (23–25).

Interestingly, we noted in a small subset of subjects (n=2) not included in this data analysis that individuals whose biopsy indicated pancreatitis had similar biomarker patterns as those individuals experiencing acute pancreas rejection. Limitations of our study include the preliminary nature of our data due to limited sample size. We were also unable to correlate grade of rejection with degree of biomarker elevation due to limited sample size. There were a limited number of patients who developed acute rejection after pancreas allograft transplantation, thus limiting our ability to determine the sensitivity and specificity of the biomarkers to predict acute rejection. Unfortunately, normal reference values for these biomarkers could not be determined as the subject variability was so great. This variability between subjects could be a result of the differences of expression of these genes among naïve and activated T-cells between individuals whether they are healthy or diabetic (8,12,15). Thus, these biomarkers may be most clinically useful when individuals establish their own reference points and clinicians use that reference to monitor variations.

In conclusion, we have demonstrated the feasibility of early noninvasive identification of those at increased risk of acute rejection by performing serial analysis of granzyme B, perforin and HLA-DR expression in peripheral blood thus making them possible candidates for biopsy or preemptive anti-rejection therapy. These same biomarkers have the potential to be a useful tool in measuring the efficacy of immunotherapy treatment after an episode of rejection. In addition, we showed that recipients without acute rejection had stable or decreasing biomarker levels throughout the follow-up time period.

Acknowledgments

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Figure 1.

Figure 2.

Figure 3.

Table 1

Demographic and clinical characteristics of sample who experienced acute rejection (n=7)

Subject	Arm	Transplant organ	Age (in years)	Body Mass Index	Sex	Race	Yrs since diagnosis of Type 1 DM	Induction	Immuno	Bx (type, grade)	Rx
1	Pre/Bx	PAK ^a	29	21.2	Female	C	17	rATG	Yes	1. AR5	1. rATG
2	Bx	SPK	32	30.5	Male	C	23	Unknown	Yes	1. AR3, R2; 2. CR2	1. rATG; pred taper
3	Pre/Bx	PA	51	37.8	Male	C	46	rATG	No	1. N; 2. AR1; 3. AR1; 4. IUS; 5. N	2. Pred taper
4	Bx	PA	36	24.8	Male	AA	14	Unknown	Yes	1. AR5, CR3; 2. AR, CR ^b	1. rATG, Increase sirolimus ^d , add Cellcept
5	Bx	SPK	35	27.5	Female	C	19	Unknown	Yes	1. AR, CR ^c 2. AR, CR ^c	1. increase tacrolimus, rATG; 2. Solumedrol, pred taper
6	Bx	SPK	34	26.5	Female	C	25	rATG	Yes	1. AR3, CR1; 2. IN	1. rATG; pred taper, change Neoral to tacrolimus and sirolimus
7	Pre/Bx	PAK	44	22.7	Female	H	18	rATG	Yes	1. IUS; 2. AR4	2. Increase sirolimus, pred; add Cellcept

Mean±SD or %: age = 37.3±7.6; BMI = 27.3±5.9; Sex = 50% M; Race = 66.7% C; DM = 22.7±10.8

Study Arm: Pre: enrolled prior to transplant, Bx: enrolled at time of clinically indicated biopsy, Pre/Bx: enrolled prior to transplant and re-enrolled at time of clinically indicated biopsy after completion of initial enrollment.

Transplant organ: PA: Pancreas alone; SPK: simultaneous pancreas-kidney; PAK: pancreas after kidney; Induction: rATG: rabbit derived antithymocyte globulin, MAb: monoclonal antibody, none: no induction immunosuppression

Race: C: Caucasian, AA: African American, H: Hispanic; Immuno: Indicates if patient is on immunosuppression at time of pancreas transplant; Bx: Each line represents an individual biopsy sample: AR-acute rejection grade, CR chronic rejection grade, IUS inflammation of undetermined significance, N-normal pathologic sample, IN-inadequate pathologic sample; Rx: Treatment for rejection episode (the numbers in front of each treatment regimen correspond to the same numbered biopsy in the previous column); N/A=not applicable.

^aSubject received PAK one week after kidney transplant and was clinically treated the same as an SPK.

^bA follow up biopsy revealed ongoing AR and progressive CR, severe grade; no numerical grade was assigned.

^cNo rejection grade was reported for this sample.

^dThis subject had not been taking immunosuppressant medications prior to rejection episode.

Table 2

Demographic and clinical characteristics of sample who did not experience acute rejection (n=6)

Subject	Arm	Transplant organ	Age (in years)	Body Mass Index	Sex	Race	Yrs since diagnosis of Type 1 DM	Induction	Immuno	Bx (type, grade)	Rx
8	Pre	PA	37	20.4	Female	C	30	rATG	No	1. IUS	N/A
9	Bx	SPK	29	23.1	Female	C	23	Unknown	Yes	1. IN	N/A
10	Pre	PAK	49	22.0	Female	C	44	rATG	Yes	1. N	N/A
11	Pre	PAK	40	19.4	Male	C	22	rATG	Yes	1. N 2. N	N/A
12	Pre	PAK	40	25.0	Female	C	35	rATG	Yes	1. IN	N/A
13	Pre	PA	48	20.8	Male	C	33	rATG	No	1. N 2. N 3. N	N/A

Mean±SD or %: age = 40.5±7.4; BMI = 21.8±2.0; Sex = 33.3% M; Race = 100% C; DM = 31.2±8.2

Study Arm: Pre: enrolled prior to transplant, Bx: enrolled at time of clinically indicated biopsy, Pre/Bx: enrolled prior to transplant and re-enrolled at time of clinically indicated biopsy after completion of initial enrollment.

Transplant organ: PA: Pancreas alone; SPK: simultaneous pancreas-kidney; PAK: pancreas after kidney; Induction: rATG: rabbit derived antithymocyte globulin, MAb: monoclonal antibody, none: no induction immunosuppression

Race: C: Caucasian, AA: African American, H: Hispanic; Immuno: Indicates if patient is on immunosuppression at time of pancreas transplant; Bx: Each line represents an individual biopsy sample: AR-acute rejection grade, CR chronic rejection grade, IUS inflammation of undetermined significance, N-normal pathologic sample, IN-inadequate pathologic sample; Rx: Treatment for rejection episode (the numbers in front of each treatment regimen correspond to the same numbered biopsy in the previous column); N/A=not applicable.

Table 3

TaqMan probe and primers

Gene	Oligonucleotide	Sequence
Granzyme B	Forward primer	5'-TGC AAC CAA TCC TGC TTC TG
	Reverse primer	5'-CCG ATG ATC TCC CCT GCA T
	Probe	(FAM) 5'-TGG CCT TCC TCC TGC TGC CCA
Perforin	Forward primer	5'-TGG AGT GCC GCT TCT ACA GTT
	Reverse primer	5'-GTG GGT GCC GTA GTT GGA GAT
	Probe	(TET) 5'-TTC AAC GCC TCC ACC CAG CCC
HLA-DRA	Forward primer	5'-AGC CCA ACG TCC TCA TCT GT
	Reverse primer	5'-TCG AAAG CCA CGT GAC ATT GA
	Probe	(FAM) 5'-TCA TCG ACA AGT TCA CCC CAC CAG TG
18S	Forward primer	5'-TTC GGA ACT GAG GCC ATG AT
	Reverse primer	5'-TTT CGC TCT GGT CCG TCT TG
	Probe	(VIC) ATT CGT ATT GCG CCG CTA

Note: HLA-DRA = major histocompatibility complex, class II, DR alpha