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Pathogenesis of Coxsackievirus-B5 acquired from intra-renal porcine islet cell xenografts in diabetic mice

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Abstract

BACKGROUND—We previously demonstrated the ability of a human isolate of Coxsackievirus B5 (CVB5) to productively infect adult porcine islet cells (PICs) *in vitro*. PICs infected with CVB5 remain viable, and upon transplantation reversed diabetes in C56BL/6 mice for up to 5 days.

METHODS—In the present work we expanded this graft-to-host xenozoonosis model by examining the long term functionality of CVB5-infected PIC xenografts in immunosuppressed mice. And, we characterized the pathogenesis of CVB5 infection in mice resulting from directional transmission of the virus from PIC xenografts to surrounding tissues in a mouse model for immunosuppressed human PIC xenograft recipients

RESULTS—Both acutely (12 hours) and chronically (72 hours) infected PIC xenografts functioned *in vivo* to reverse diabetes in mice. The efficacy of both infected and un-infected PICs was transient beyond 5 days post inoculation and the long-term functionality of the grafts was compromised by host-to-graft rejection. CVB5-infected PIC xenografts transmitted infectious virus to immunosuppressed recipient mice resulting in extensive histopathologic changes. The virus replicated in the heart, liver, spleen, kidney, pancreas, brain and skeletal muscle in higher levels in SCID mice that were directly inoculated with virus when compared to controls. In addition, infectious virus was recovered for up to 22 days after inoculation in SCID mice whereas it was only detected up to day 4 PI in non-SCID mice.

CONCLUSIONS—Immunosuppressed PIC xenograft recipients may be more susceptible to infection with CVB5 which could target the xenograft leading to disseminated infection in the host.

Keywords

Suz's paper

Introduction

Evaluation of the potential for viral xenozoonosis in pig-to-human islet-cell transplantation for the treatment of type II diabetes has been focused primarily on transmission of porcine viruses from the xenograft to recipients. Particular attention has focused on the transmission of porcine endogenous retroviruses (PERVs), exogenous herpesviruses, picornaviruses, influenza and circoviruses (1,2,3). An experimental model of pig-to-human xenozoonosis has been fairly well established in studies characterizing the transmission of porcine encephalomyocarditis virus (EMCV) across species. EMCV is a picornavirus which causes a generalized infection in pigs and persists in porcine myocardial and central nervous system cells. It has recently been shown to induce chronic myocarditis in cynomolgus macaques and to productively infect human cardiomyocytes (4,5). The virus is also transmitted to mice following intra-abdominal transplantation of myocardial or pancreatic tissues from infected pigs, or by intra-renal transplantation of *in vitro*-infected porcine islet cells (PICs) (6,7). Notably, EMCV-infected PICs produce normal levels of insulin both *in vitro* and following intra-renal transplantation into diabetic mice, although the recipient mice subsequently succumb to infection with the virus. These and other studies have heightened awareness and resulted in increased efforts to screen pig tissues for viral pathogens prior to transplantation into humans.

Another risk of pig-to-human xenotransplantation is infection of the xenograft by human pathogens, particularly persistent viruses in the recipient, which may severely compromise the function of the xenograft. Models characterizing host-to-graft xenozoonosis have not been fully developed. These models are needed to evaluate how successful cross species transplantation will be for treating human diseases especially considering the increased risk for de-novo infections in recipients undergoing immunosuppression therapy coincident with transplantation. Human viruses that can potentially infect engrafted porcine tissues and cells include retroviruses, herpesviruses, and picornaviruses. The human coxsackieviruses (CVs) are picornaviruses that are ubiquitous among humans and result in seasonal endemic and epidemic patterns of disease throughout the world (8,9,10). The group B coxsackieviruses (CVB) can persist in myocardial and pancreatic tissues and have frequently been implicated in isletitis and pancreatitis that can result in type I diabetes (11–15). Serotypes of CVB have been isolated from the pancreas of acute onset and fatal cases of type I diabetes and have been shown to be diabetogenic in mice and nonhuman primates (16–18). Because of its prevalence and ability to infect and persist in human pancreatic beta-cells, we wondered if CVB could also infect PIC xenografts in immunosuppressed transplant recipients (19,20). We hypothesized that immunosuppressed, human recipients of PIC xenografts are at increased risk for de novo infection with CVB which may infect the xenograft and compromise its function and/or result in disseminated infection which may be fatal.

To address this hypothesis, our group had previously shown that a prevalent Pennsylvania isolate of CVB (CVB5/PA) can productively infect adult PICs using the coxsackie-adenovirus receptor (21). And, transplanting CVB5-infected PICs into diabetic C57BL/6 mice reversed diabetes within 48 hours and for up to 5 days post transplantation (21). These results demonstrate that PICs, though susceptible to infection by CVB5, can function *in vivo*

at least temporarily to restore glucose balance in diabetic recipient mice. In the present study we extended our analysis of the functionality of CVB5-infected PICs transplanted into immunosuppressed, diabetic mice beyond the immediate post transplantation period. Additionally we characterized the pathogenesis of disseminated CVB5 infection in both immunosuppressed recipient mice resulting from transmission of the virus from the xenograft as well as in SCID mice with de novo acquired infection. Results of these studies are presented here in the context of building a comprehensive mouse model of host-to-graft xenozoonosis in immunosuppressed human xenograft recipients and as preliminary data to further investigations which will address the question of whether un-infected PIC xenografts may be targeted for infection in recipients that acquire de novo infection with CVB5 following transplantation.

Materials and Methods

Infection of porcine islet cells with CVB5

Porcine islet cells (PICs) were obtained from the University of Minnesota's Diabetes Institute for Immunology and Transplantation. The PICs were maintained and assessed for viability and functionality before infecting with CVB5 as described previously (21). In the experiments described here 3000 PICs were inoculated with CVB5/PA (10 pfu/cell) for 1 hour at 37°C then further incubated (1000 cells/ml) for 3 hours (acutely-infected PICs) or 4 days (chronically-infected PICs) with 50% media change every other day.

Transplantation of PICs into diabetic mice

Induction of diabetes and transplantation of CVB5-infected PICs were performed as described previously (21). Briefly C57BL/6 mice were immunosuppressed the day of surgery with intraperitoneal injection of 200 μ l of rabbit anti-mouse lymphocyte serum (1mg/ml) 1 – 2 hours before surgery (Accurate Chemical and Scientific Corporation; Westbury, NY). Then 2500 infected PICs were infused under the kidney capsule. Control mice received 2500 uninfected PICs.

Infection of immunosuppressed mice with CVB5/PA

The same PA-1 strain of CVB5 used to infect PICs was inoculated into 3 – 5 week-old severe-combined immunodeficient (SCID) C57BL/6 mice purchased from Charles River Laboratory and housed at a biosafety level-2 facility in compliance with university approved animal care and use guidelines. In addition, immunocompetent C57BL/6 mice were infected to serve as controls. Six mice of each group were inoculated intraperitoneally with 1×10^4 PFU of CVB5 in a total volume of 1 ml sterile saline. One C57BL/6 and one SCID mouse were inoculated with 1 milliliter of sterile saline as an infection control. Mice were monitored daily for clinical signs and euthanized at 4 and 22 days post-inoculation (PI) with sodium pentobarbital IP.

Histopathology

Tissues (heart, brain, liver, kidney, pancreas, skeletal muscle, and spleen) from each mouse were collected for histopathologic analysis. Tissues were fixed in 10% buffered neutral formalin, sectioned at 4 μ m and stained with hematoxylin and eosin. Pathological changes were scored from 0–4 with increasing severity as follows: 0 = No pathologic changes; 1 = *Pancreas*: rare foci of degeneration or inflammatory cell infiltrate; *Liver*: mild thickening of capsule by fibrous connective tissue, multiple foci infiltrated by small numbers of lymphocytes and macrophages; *Heart*: Multiple foci of mineralization of the atrial epicardium; 2 = *Pancreas*: multifocal acute necrosis of acinar cells with infiltration by small numbers of neutrophils, macrophages and lymphocytes infiltrating the interstitium; few foci

of inflammation and necrosis in peri-pancreatic fat; *Liver*: marked thickening of capsule by fibrous connective tissue, subcapsular areas of disruption of hepatocellular architecture, multiple foci infiltrated by small numbers of lymphocytes and macrophages; 3 = *Pancreas*: extensive acute necrosis with infiltration by neutrophils, macrophages, and lymphocytes; necrosis and infiltration by neutrophils and macrophages in peri-pancreatic fat; *Heart*: multifocal areas of myocardial fiber necrosis infiltrated by neutrophils, lymphocytes, and macrophages; a few foci of small deposits of fibrin and inflammatory cells on atrial epicardium; 4 = *Pancreas*: multiple areas of atrophy and infiltration of interstitium by moderate numbers of lymphocytes and macrophages.

Detection of infectious virus and viral RNA

Infectious virus was detected and quantified in tissues by plaque assay as previously described using confluent bovine green monkey (BGM) cells (22). Briefly, clarified supernatants from homogenized and sonicated samples were diluted ten-fold (10^{-1} – 10^{-6}), added to confluent BGM cells, incubated for 36–40 hours and plaques counted. Results are expressed as PFU/gm tissue. Viral nucleic acid (190 bp product) was detected using a single-step RT-PCR and primer pairs specific for the VP1 gene of CVB5/PA sequenced in our laboratory. Primers were generated using Primer3 software and shown to be specific for CVB5 by sequencing and positive identification of the 190 bp reaction amplicon using the GenBank sequence database and blast search criteria. The sequence of the 5' primer was TTA CGG CGA AAG CTT GAG AT and the 3' primer was GTG GAC GTC TGC CAA CTG TA (5' to 3' direction). The sensitivity of the RT-PCR assay was assessed by 10-fold serial dilutions of RNA obtained from purified virus stock and determined to be 10 copies. Tissues were homogenized in TRIzol® (Invitrogen, San Diego, CA) followed by chloroform extraction of total RNA. RNA was resuspended in 100 µl of purified water and 10 µl of a 1:10 dilution was reverse transcribed and cDNA amplified using the Superscript™ One-Step RT-PCR reverse transcription kit (Invitrogen). Reactions were performed using 0.2 µM primer pairs, 1.2 mM MgSO₄, and 200 µM dNTPs in a total reaction volume of 50 µl and the following reaction conditions: 50°C for 30 minutes followed by 40 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. A final extension at 72°C for 10 minutes was performed and 20 µl of amplicon was visualized by agarose gel electrophoresis.

Results

CVB5-infected PICs transiently reverse diabetes *in vivo*

We previously demonstrated the susceptibility of PICs to infection with CVB5 and the ability of intra-renally transplanted, CVB5-infected PIC xenografts to reverse STZ-induced diabetes in mice for up to 5 days post-transplantation (21). Here we determined whether CVB5-infected PICs could sustain *in vivo* function beyond this immediate post-transplantation period. Infected PICs transplanted into diabetic mice significantly decreased blood glucose levels from an average of 407 mg/dl ($\pm$ 46) on the day of surgery to an average of 158 mg/dl ($\pm$ 79) within 48 hours of transplantation ($p=0.002$, Figure 1). However, the *in vivo* efficacy of infected PICs appeared to be transient, since blood glucose in recipient mice rose to diabetic levels again by day 12 post-transplantation. Blood glucose levels in mice that received uninfected PICs were lower at this time point than in mice that received infected PICs but this difference was not significant. There was no difference in glucose levels between non-diabetic mice that received CVB5 infected PICs and no treatment control mice.

Infected PIC xenografts transmit the virus to recipients

Mice transplanted with CVB5-infected PICs were sacrificed 6 and 13 days later and virus spread and histopathologic changes in various tissues analyzed. Infectious virus or viral

RNA was detected in blood (B), heart (Ht), brain (Br), liver (L), spleen (S), pancreas (P), kidney (K), and skeletal muscle (SM) tissues harvested at day 6 PI (N = 5; Figure 2). In contrast, infectious virus was only recovered from the spleen and pancreas of mice sacrificed at day 13 PI (N = 3, Figure 2). No virus or viral RNA was detected in mice that received uninfected PIC xenografts (N = 4). The pathogenesis of CVB5 infection in infected recipient mice was similar to that observed in mice that received direct inoculation of the virus. The pancreas was the most severely affected organ characterized by necrosis of acinar cells and infiltration of inflammatory cells including neutrophils, macrophages and lymphocytes in the interstitium. These histopathologic changes were most severe in mice sacrificed at day 6 PI (average score = 3) when compared to mice sacrificed at 13 days post inoculation (average score = 1). No histopathologic changes were detected in tissues of uninfected control mice (Figure 3).

Pathogenesis of CVB5 infection in immunosuppressed SCID mice

To better characterize the pathogenesis of de novo acquired CVB5 infection under typical immunosuppression maintained in mammalian xenograft recipients, SCID mice were intraperitoneally inoculated with 1×10^5 PFU of CVB5 and monitored daily for 3 weeks. Apart from the appearance of ruffled fur and hunched posture none of the SCID (n=6) or immunocompetent C57BL/6 (n=6) mice infected with CVB5 exhibited any detectable clinical signs. Infectious virus or viral RNA was detected in heart, brain, liver, kidney, pancreas, skeletal muscle and spleen of both SCID (N = 3) and control C57BL/6 (N = 3) mice at day 4 PI although infectious virus was detected more frequently and in higher titers from SCID than control mice (Figure 4, A). The highest viral titers were observed in the pancreas of both groups of mice, followed by the liver, heart, kidney and skeletal muscle, respectively. At day 22 PI, the frequency of virus isolation from tissues in SCID mice (N = 3) remained high whereas virus could not be isolated from any of the organs of control mice (N = 3) (Figure 4, A). Viral RNA was detected in 3/3 tissues of SCID mice that were negative for virus isolation at this time point but only in the spleens of 2 control mice (Figure 4.B). Un-infected SCID and control C57BL/6 mice were negative for infectious virus and viral RNA in all organs tested (N = 2).

There were no gross pathologic findings at necropsy in either SCID or control mice sacrificed at day 4 PI. At 22 days PI, all of the SCID mice sacrificed had abnormal spleens (atrophy or hypertrophy), whereas control mice were normal. Histopathologically, both SCID and control mice had acute necrosis of acinar cells in the pancreas accompanied by multiple foci of inflammation at 4 days PI (Figure 5, A and B). At 22 days PI, SCID mice had atrophy of the exocrine pancreas and replacement of acinar tissue with adipose cells, multifocal myocardial necrosis and inflammation and inflammatory foci in the liver with marked thickening of the hepatic capsule (Figure 5, C to E). Un-infected SCID and control C57BL/6 mice (N=12) were clinically normal throughout the study. No significant microscopic changes were detected in sections of kidney, spleen, brain or skeletal muscle from any of the mice.

Discussion

The risk of graft-to-host xenozoonosis has been greatly minimized by intensive efforts to screen xenografts for pathogens prior to transplantation. Yet the potential for xenografts to become infected with human pathogens following transplantation into humans remains a concern especially when recipients are at increased risk of infection under immunosuppressive transplant conditions. Appropriate models are needed to more fully characterize host-to-graft xenozoonosis and the impact of viral infection on the function of the graft and the overall health of xenograft recipients.

We have previously demonstrated that porcine islet cells considered for use in the treatment of diabetes in humans are susceptible to productive infection by human CVB5 *in vitro* (21). We have also demonstrated that PICs infected with CVB5 produced insulin *in vitro* in response to glucose stimulation and functioned *in vivo* for up to 5 days by restoring blood glucose levels in diabetic mice to normoglycemic levels (21). In the current study, we extended our *in vivo* analysis of CVB5-infected PICs beyond the immediate transplantation period to show that they retain their ability to restore blood glucose levels in diabetic, immunosuppressed mice within 48 hours post-transplantation but for less than 2 weeks. The *in vivo* function of uninfected PICs was also impaired over a similar time-course, suggesting that factors interfering with the *in vivo* functionality of PIC xenografts may be more related to host rejection, rather than to virus infection of the xenograft. This was supported by histopathologic analysis of the renal tissues adjacent to the xenograft that showed comparable inflammatory reaction suggestive of acute graft rejection (Figure 3). These results suggest that uninfected xenografts may also be susceptible to infection *in vivo* post transplantation in a recipient that harbors persistent CVB5 virus, or later becomes infected with the virus due to increased susceptibility to infection under immunosuppression therapy but may still be able to function and prolong the life of the recipient as long as immunologic rejection episodes are minimized.

In PIC recipients CVB5 was transmitted directionally from the xenograft to surrounding tissues resulting in severe disseminated infection. Virus was recovered with increased frequency and from multiple organs in mice that were transplanted with chronically infected PICs (96hr) early post transplantation but only in the spleen and pancreas of mice that received acutely infected PICs (4hr) at a later time point. Histopathologic changes were also more severe in chronically infected PIC recipients. Assuming the mice were adequately immunosuppressed and the marked decreased virus isolation observed later post transplantation was not due to active antiviral immunity, this data suggests that the chronicity of infection and perhaps the level of virus replication within the xenograft may influence the level of virus transmission from the xenograft to surrounding organs. This could have important implications for recipients that harbor persistent and perhaps pancreovirulent strains of group B coxsackieviruses emphasizing the need to screen and treat xenograft recipients prior to transplantation to avoid virus infection and replication within the xenograft which could result in widespread infection in the recipient especially under immunosuppressive conditions.

In our model system immunosuppression clearly influenced susceptibility to infection with CVB5. In IP inoculated mice, infectious virus was recovered with increased frequency and in higher titers from SCID mice than from non-immunosuppressed control mice at 4 days post inoculation and was recovered only from SCID mice at 22 days post inoculation. Infected SCID mice also had a higher frequency of and more severe histopathologic lesions than did control mice.

CVB5 was isolated from the pancreas of both IP inoculated mice and CVB5-infected PIC graft recipients with higher frequency and in higher titers than from other organs and lesion scores were also highest for the pancreas in both groups of mice. These results indicate that CVB5/PA is primarily pancreovirulent in mice and similar to some non-diabetogenic strains of CVB4 in their ability to induce severe pancreatitis in mice (23). Histopathologic changes were similar in CVB5-infected PIC recipients and IP inoculated mice and were characterized by acute and severe necrotizing pancreatitis with multiple inflammatory foci. CVB5-inoculated mice also had lesions in the heart and liver while infected PIC recipients did not despite active viral replication in these organs. Experimental models of diabetes in mice have described pancreovirulent and diabetogenic strains of group B Cocksackievirus serotypes as well as persistent infection with this virus in the pancreas (24). Others have

demonstrated that CVB3-induced histopathologic changes in mice typically involve both the pancreas and heart and consist of necrotizing acute acinar pancreatitis and the subsequent development of focal myocarditis following primary virus replication in the pancreas (25–27). Together these data suggest that pancreovirulent group B coxsackieviruses present in recipients could target PIC xenografts which may serve as a site of primary virus replication in the host leading to disseminated infection.

In summary, CVB5/PA-infected PIC xenografts function *in vivo* temporarily to restore normal glucose levels in immunosuppressed diabetic mice post-transplantation but the virus is transmitted from the graft to surrounding host tissue resulting in disseminated infection that is more severe in immunosuppressed individuals. This strain of CVB5 is pathogenic for mice and is primarily pancreovirulent causing severe pancreatitis and moderate myocarditis through active replication in these target organs. Whether PICs can be infected *in-vivo* following transplantation into a recipient with disseminated CVB5 infection remains to be determined with this model system. The current data suggests that recipients of PIC xenografts should be screened for CVB5 and if possible treated prior to transplantation to avoid risk of infection and compromised function of the graft. However, at best CVB5 infected PIC xenografts may function short term to prolong the life of the recipient. Future studies aimed at investigating CVB5 infection in mice followed by xenotransplantation of un-infected PICs may offer a clinically relevant model to study host-to-graft xenozoonosis as well as prophylaxis and antiviral therapies for use in xenotransplantation.

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Figure 1.

Transplantation of CVB5-infected PICs reverses diabetes in immunosuppressed, C57BL/6 mice. Following diabetes induction in mice by intraperitoneal injection of 220 mg/kg of streptozotocin (STZ), blood glucose levels increased to 500 mg/dl (diabetic) within 3–4 days. Transplantation of 2500 CVB5-infected PICs under the kidney capsule of diabetic mice (n=4) resulted in a decrease in glucose concentration (below 158 mg/dl) within 48 hours, whereas the levels remained high (≥ 350 mg/dl) in a diabetic mouse that did not receive a PIC xenograft. The rate and levels of glucose restoration by infected PIC xenografts were similar to those observed in diabetic mice that received uninfected PICs (N=2). Glucose levels in non-diabetic mice (no STZ injection) that received CVB5-infected PICs (N=2) remained normal throughout and were similar to the control non-diabetic mouse (N=1).

Figure 2.

Isolation of infectious CVB5 from tissues of diabetic, immunosuppressed mice that received either chronically (96-hr) or acutely (4-hr) infected intra-renal PIC xenografts and harvested 6 and 13 days post-transplantation respectively. Infectious virus was detected in the heart, brain, liver, spleen, pancreas, kidney and skeletal muscle of mice receiving chronically infected PIC xenografts but only in the spleen and pancreas of mice that received acutely infected PICs. Virus was not recovered from any of the tissues of control mice that received un-infected xenografts.

Figure 3.

Histopathologic changes in renal tissues of mice that received CVB5-infected PIC xenografts. Tissues receiving both CVB5-infected (A and B) and un-infected (C and D) PIC xenografts initiated a similar host inflammatory response consisting of infiltration of small numbers of lymphocytes and macrophages surrounding the implants (closed arrows, all panels). There was marked fibrosis of the renal capsule next to the implants, with moderate numbers of lymphocytes and macrophages infiltrating the fibrous connective tissue (A and C, open arrows). Minimal fibrosis was observed between the islet cell implants and the renal cortex (B and D, arrow heads).

Figure 4.

A.) Isolation of infectious CVB5 from tissues of SCID (n=6) and immunocompetent control C57BL/6 mice (n=6) following intraperitoneal inoculation of the virus. Infectious virus was detected in the heart, brain, liver, kidney, pancreas, skeletal muscle, and spleen of both groups of mice at 4 days PI but only from tissues of SCID mice at 22 days PI. Infectious virus was detected more frequently and in higher titers from SCID than control mice and the highest titers were observed in the pancreas of both groups. B.) Viral RNA was detected in all organs of C57BL/6 mice sacrificed at day 4 PI (left panel) but only in the spleens of 2/3 of these mice harvested at day 22 PI (right panel). Infectious virus and viral RNA was not detected in any of the tissues of mock-infected mice (n=2).

J		Average Lesion Score			
Treatment Group	Mouse	Days PI	Pancreas	Heart	Liver
CVB5 Inoculated	SCID (n=3)	4	3	0	0.3
	SCID (n=3)	22	4	1.3	1.7
	C57BL/6 (n=3)	4	1.7	0	0.3
	C57BL/6 (=3)	22	1.7	0	0.7
Un-Inoculated	SCID	22	0	0	0
	C57BL/6	22	2	0	1

Figure 5.

CVB5-induced pathological changes in tissues of SCID and control mice. Mice were inoculated with 1×10^4 PFU of CVB5/PA and histopathologic changes in the heart, brain, liver, kidney, skeletal muscle, pancreas and spleen were analyzed at 4 (A and B) and 22 (C, D, E, F, G, H, I) days post-inoculation (PI). Pancreas from control (A and D) and SCID (B and E) mice demonstrating severe necrosis and saponification (B, arrows) of peri-pancreatic fat accompanied by dense inflammatory infiltrates (A, arrow) containing neutrophils and macrophages. At 22 days PI there was pancreatic interstitial inflammatory foci (E, arrows), acinar cell necrosis and atrophy of acinar tissue (D and E) that was replaced by adipose tissue. CVB5 infection in SCID mice resulted in multifocal areas of myocardial fiber

necrosis and inflammation in two out of three mice (I, arrows). In the liver of SCID mice there was marked thickening of the capsule by fibrous connective tissue (H, open arrow) and subcapsular dilation of the hepatic sinusoids (H, closed arrows) that was not observed in control mice (G). No significant microscopic changes were detected in sections of kidney, spleen, brain or skeletal muscle from any of the mice. Normal pancreas (C) with intact acinar cells and islets of Langerhans (C, arrows) is shown for comparison. Magnification = 40X. (J.) Average lesions scores for SCID vs control mice at 4 and 22 days PI.