Screening enteroviruses for \( \beta \)-cell tropism using foetal porcine \( \beta \)-cells

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Primary adult human insulin-producing \( \beta \)-cells are susceptible to infection by prototype strains of coxsackieviruses (CV) and infection may result in impaired \( \beta \)-cell function and/or cell death, as shown for coxsackie B virus (CVB) types 4 and 5, or have no apparent immediate adverse effects, as shown for CVA-9. Because of the limited availability of human pancreatic \( \beta \)-cells, the aim of this study was to find out if foetal porcine pancreatic islets could be used as a substitute in entero-virus (EV) screening. These cells resemble human \( \beta \)-cells in several biological properties. CVB infection resulted in a rapid progressive decline of insulin content and reponsiveness to insulin release. The amount of virus inoculum sufficient for this destruction was small, corresponding to only 55 infectious units per pancreas. In contrast to CVBs, CVA-9 replicated poorly, and sometimes not at all, in foetal porcine \( \beta \)-cells. The first signs of functional impairment and cell destruction, if present at all, were seen only after 1–3 weeks of incubation. Furthermore, CVA-16, several strains of echoroviruses and human parechovirus type 1 were unable to replicate in foetal porcine pancreatic \( \beta \)-cells. Based on these results, foetal porcine islets are somewhat more sensitive to CVB infection than adult human islets, whereas many other human EV strains do not infect porcine \( \beta \)-cells. Therefore, foetal porcine \( \beta \)-cells cannot be used for systematic screening of human EV strains and isolates for \( \beta \)-cell tropism, but they might provide a useful model for detailed studies on the interaction of CVBs with \( \beta \)-cells.

Introduction

Insulin-dependent diabetes mellitus (IDDM) is a chronic disease of insulin deficiency that results from the gradual destruction of insulin-producing \( \beta \)-cells located within the islets of Langerhans of the pancreas. The destructive process often starts years before the appearance of clinical symptoms and several lines of epidemiological evidence suggest that entero-virus (EV) infections may be involved in this process (Yoon et al., 1979; King et al., 1983; Frisk et al., 1992; Helfand et al., 1995; Hyöty et al., 1995; Hiltunen et al., 1997; Roivainen et al., 1998). On the assumption that direct infection of the \( \beta \)-cell is a relevant mechanism to explain the diabetogenic effect of EV infection, we have monitored the patterns and consequences of EV infections in primary adult human insulin-producing \( \beta \)-cells. Our previous results have shown that primary human \( \beta \)-cells are susceptible to prototype strains of several coxsackie B viruses (CVBs) and that infection may result in functional impairment or death of the \( \beta \)-cell, as shown for CVBs, or have no apparent immediate adverse effects, as shown for CVA-9 (Roivainen et al., 2000). While adult human \( \beta \)-cells are the preferred model for detection of the clinically relevant effects of EVs, they are not easily available. Rodent \( \beta \)-cells, although easily available, are not ideal for the purpose, since they are resistant to metabolic disturbances caused by the prototype strain of CVB-4 (Yoon et al., 1978; Szopa et al., 1985). Porcine \( \beta \)-cells might be a suitable substitute, since they are known to resemble human \( \beta \)-cells. For example, they are, unlike murine \( \beta \)-cells, relatively resistant to oxidative damage, toxins and cytokine-induced damage (Tuch & Bai, 1998; Eizirik et al., 1994; Welsh et al., 1995). While adult porcine islets are difficult to isolate and maintain in culture, foetal or neonatal \( \beta \)-cells can be generated in large numbers by tissue culture in nicotinamide-supplemented serum-free medium (Korbut et al., 1996; Otonkoski et al., 1999).

In the present study, we investigated the kinetics and consequences of EV replication in foetal porcine \( \beta \)-cells in order...
to see whether these cells could be used instead of primary human β-cells for screening human EV strains and isolates for β-cell tropism.

**Methods**

**Foetal porcine β-cells.** Pregnant Yorkshire sows were obtained from a breeding piggery and culled by high-voltage electrical stunning and immediate exsanguination at the Department of Food Technology, University of Helsinki, Finland. The length of pregnancy varied between 80 and 95 days, as determined by witnessed mating. Foetuses were collected immediately and placed on ice for transport to the laboratory. Pregnant Yorkshire sows were obtained from a breeding piggery and culled by high-voltage electrical stunning and immediate exsanguination at the Department of Food Technology, University of Helsinki, Finland. The length of pregnancy varied between 80 and 95 days, as determined by witnessed mating. Foetuses were collected immediately and placed on ice for transport to the laboratory.

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**Viruses.** Prototype strains of CVBs (CVB-1 to -6), echoviruses (E-1, -7 and -11), some CVAs (CVA-9/Griggs and CVA-16), human parechovirus 1 (HPEV-1, previously known as E-22) and poliovirus type 1/Mahoney (PV-1) were obtained either from the ATCC or from the World Health Organization Enterovirus reference laboratory. The diabetes-associated CVB-4 strain E2 was kindly provided by J.-W. Yoon (Yoon et al., 1979). Viruses were passaged in continuous cell lines of monkey kidney (GMK or Vero) or human lung carcinoma (A-549) origin. The identity of all virus preparations used in this study was confirmed using a plaque neutralization assay with type-specific antisera.

**Replication of viruses.** Free-floating islets were infected with different virus preparations at an apparent high m.o.i. of between 30 and 100 (if not otherwise stated). After adsorption for 1 h at 36 °C, virus inoculum was removed. Cells were then washed twice with Hank's balanced salt solution supplemented with 20 mmol/l HEPES, pH 7.4. Incubation medium was added to all cultures, including uninfected controls, and the virus was allowed to replicate at 36 °C. Samples of suspended islets taken at different time-points were freeze–thawed three times to release virus, clarified by low-speed centrifugation and assayed for total infectivity using end-point dilutions in microwell cultures of GMK, Vero (CVA-16) or A-549 (HPEV-1) cells. TCID_{50} titres were calculated by the Kärber formula (Lennette, 1969) and expressed as the mean of two parallel series of dilutions.

**Immunocytochemistry.** Samples of infected and uninfected islets were harvested 1 day after infection on glass slides using a cytocentrifuge and fixed with cold methanol for 15 min at −20 °C. After washing three times with PBS, cells were double-stained overnight at room temperature with EV-specific polyclonal rabbit antisera (1:300, Hovi & Roivainen, 1993) and insulin-specific polyclonal sheep antisera (30 μg/ml, PC059; The Binding Site). Visualization was achieved by FITC (711-095-152; Jackson ImmunoResearch Laboratory) and lissamine rhodamine sulfonyl chloride (LRSC) (713-085-147; Jackson ImmunoResearch Laboratory) conjugated anti-species sera. Photographs were taken using a confocal microscope (Leica TCS NT) and Paint Shop Pro software.

**DNA and insulin content of cells.** Islet cells were ultrasonically homogenized in distilled water. DNA was measured from dried samples using a fluorometric method based on diaminobenzoic acid-induced fluorescence (Hinegardner, 1971). Insulin was measured with a commercial Solid-Phase Insulin RIA kit (DPC) after overnight extraction with 67 mmol/l HEPES, pH 7.4, 1% BSA. After a 60 min stabilization period in low glucose (1 mmol/l), cells were plated onto non-tissue culture dishes in incubation medium (Ham’s F-10 medium containing 25 mM HEPES, pH 7.4, 1% BSA, penicillin and streptomycin) supplemented with 0.1 mmol/l 3-isobutyl-1-methylxanthine (Sigma) and 10 mmol/l nicotinamide (Sigma). In order to enrich β-cells, islets were incubated for 10–14 days with medium changed twice a week prior to the onset of experiments. At this time, the mean β-cell content of the preparations was 23%, which increased up to 33% by day 21 of culture (Otonkoski et al., 1999).

**Cell viability.** After infection, the viability of islet cells was measured using a commercial live/dead cell assay kit (L-3224; Molecular Probes). This assay is based on the simultaneous determination of live and dead cells with two fluorescent probes. Live cells are stained green with calcein due to their esterase activity and the nuclei of dead cells are stained red with ethidium homodimer-1. Islets harvested at different time-points were incubated with the labelling solution for 30 min at room temperature in the dark, according to the manufacturer’s instructions, cytocentrifuged onto glass slides and analysed using confocal microscopy.

**Insulin secretion.** Insulin release in response to glucose and glucose plus theophylline was studied separately by perfusion, as described previously (Otonkoski & Hayek, 1995; Roivainen et al., 2000). Briefly, after taking samples for insulin and DNA content measurement, islets were loaded into perfusion chambers in Krebs–Ringer bicarbonate buffer supplemented with 20 mmol/l HEPES (pH 7.35) and 0.2% BSA. After a 60 min stabilization period in low glucose (1 mmol/l), cells were stimulated first with high glucose (16.7 mmol/l) and then with a mixture of 16.7 mmol/l glucose and 10 mmol/l theophylline (Sigma). Fractions of 1 ml were collected every 4 min. After stimulation, the basal
buffer (1.67 mmol/l glucose) was used for the final five fractions. Five or six perifusion lines were run in parallel using a multi-channel perifusion apparatus (Brandel).

**Statistical methods.** Differences between groups were tested with Microsoft Excel 97 software using one-way analysis of variance and taking 95% as the limit of significance.

**Results**

**Replication of EVs in foetal porcine pancreatic cells**

The replication of different EVs was studied in foetal porcine β-cells and cumulative data obtained with different viruses are shown in Fig. 1. All CVBs replicated well and the maximum titres of progeny virus were detected 1 or 2 days after infection. Double immunofluorescence staining for insulin and viral antigens verified infection of insulin-producing β-cells as shown for CVB-1 and -6 in Fig. 2. CVA-9 replicated poorly and sometimes not at all. In experiments where marginal progeny virus production (0.5–1 log TCID_{50}/75 µl) was seen it was completed already at one day after infection (data not shown). All tested echoviruses (E-1, -7 and -11), CVA-16, PV-1 and HPEV-1 did not replicate in foetal porcine pancreatic β-cells.

**Consequences of EV infection in foetal porcine β-cells**

The viability of islets was studied using a commercial live/dead cell assay kit. Throughout all of our experiments, uninfected control islets remained viable and only a few solitary dead cells were seen (Fig. 3). After 3 days of infection, islets infected with prototype strains of CVB-1 to -6 or the diabetogenic strain E2 of CVB-4 were almost completely destroyed (Fig. 3). In contrast, islets inoculated with CVA-9, E-1, -7 and -11, PV-1 or HPEV-1 were intact (Fig. 3). However, in two out of three experiments where the viability of CVA-9-
infected islets was followed for 3 to 6 weeks, signs of islet cell destruction became apparent 39–40 days after infection.

Due to their functional immaturity, the response of uninfected porcine foetal islets to high glucose alone was minimal (Fig. 4 A), as shown previously (Otonkoski et al., 1999), while the response to a mixture of theophylline and high glucose was, on average, 9-fold higher (Fig. 4 A). Therefore, in subsequent experiments, the effect of EV infection on responsiveness of insulin release was studied only after stimulation with a mixture of glucose and theophylline. The cumulative results of EVs capable of replicating in foetal porcine islets are summarized in Fig. 4 (B). PV-1, which cannot replicate in non-primate cells, was used as a control. As expected, PV-1 was unable to cause any disturbance in stimulated insulin release (Fig. 4).

All strains of CVBs had very deleterious effects on foetal porcine islets. Insulin release responses of CVB-infected cells had already begun to deteriorate 1 day after infection and the destructive process progressed rapidly (data not shown). After 1 week of infection, islets infected with CVB-1 and -6 did not secrete any measurable insulin (Fig. 4 B), while slightly more variation was seen in the insulin-release responses of islets infected with other CVBs.

The insulin response of islets infected with a genetically related virus, CVA-9, was usually enhanced rather than decreased 1 week after infection (Fig. 4 B). Not surprisingly, a large variation in the time scale and degree of CVA-9-induced disturbance was seen in different islet cell preparations (Fig. 6). At 3 weeks after infection, a clear CVA-9-induced disturbance in stimulated insulin release was seen in two of four experiments [stimulation index (SI) 33% or less of uninfected controls]. For all four experiments, the mean stimulated insulin release (SI ± SD) of uninfected and CVA-9-infected islets at this time-point was 6·14 ± 2·95 and 3·80 ± 2·69, respectively.

In islets that supported virus replication, intracellular immunoreactive insulin began to decrease early after infection and by 1 week, insulin was barely detectable in islets infected with CVBs (Fig. 4 C). In contrast, the insulin content of CVA-9-infected cells remained comparable to that of the uninfected control cells throughout the 1 week observation period (Fig. 4 C). Likewise, the insulin content of PV-1-infected cells was practically unchanged (Fig. 4 C). In general, the decrease of insulin content correlated well with the secretory responses in porcine cells.

**Titre of cytolytic virus needed for β-cell damage**

Some of the differences found between the various viruses might be based on the different virus titres used for islet cell infections. To determine how strongly the infection-induced consequences are dependent on the kinetics of virus replication, virus-induced cell damage was studied in detail after infecting foetal porcine islets with decreasing concentrations of CBV-1. As seen in Fig. 7 (A), the infectivity of the virus inocula used correlated well with the eclipse phase titres found in the islets after virus adsorption. In spite of this, practically no differences were seen in virus replication in islets infected with our ‘normal amount’ (1 × 10⁹ TCID₅₀) of virus or 4-, 10- or even 40-fold less (corresponding to 2·6 × 10⁷, 8·3 × 10⁶ or 2·6 × 10⁶ TCID₅₀, respectively) (Fig. 7 A). By using lower titres of virus...
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Fig. 4. Stimulated release of insulin in foetal porcine islets in perifusion experiments 7 days after infection. (A) Response of uninfected porcine foetal islets to high glucose (Glu1 and Glu2) and to a mixture of high glucose and theophylline (G + T). Stimulated insulin release is shown in µIU/ml. One representative experiment is shown. (B) Cumulative effect of EV infection on the stimulated release of insulin. Insulin release is expressed as the SI (stimulated/basal level) in response to high glucose and theophylline and is shown as the relative change from the uninfected control. The number of observations is indicated at the bottom of each column. The results are shown as the mean ± 95% confidence intervals (the mean ± range if carried out only twice). (C) Insulin content relative to cellular DNA of the foetal porcine islets harvested 1 week after infection expressed as the relative change from the uninfected control. P < 0.05 (*), P < 0.01 (**) and P < 0.005 (***) as compared to the uninfected control cells.

A

B

C

(1.2 × 10⁶–55 TCID₅₀), progeny virus production was gradually delayed, but even in this case, the maximal titre of progeny virus was produced within 2 days (Fig. 7B). Islets infected with less than 55 TCID₅₀ of CBV-1 did not produce measurable amounts of infectious virus within 3 days (data not shown).

After 1 week of infection, severely impaired insulin release was clearly evident in all islets infected with the amount of virus capable of producing progeny (1.2 × 10⁶–55 TCID₅₀).

Fig. 5. Cumulative data of CV infection in primary human β-cells [data summarized from Roivainen et al. (2000)]. (A) Replication of CVBs in β-cells infected with virus at an apparent high m.o.i of between 30 and 100. The results are shown as the mean ± 95% confidence intervals of three experiments. (B) Cumulative data of stimulated insulin release from nine islet cell preparations 7 days after infection. Insulin release is expressed as the SI (stimulated/basal level) in response to 16.7 mM glucose, first (G1) and second phases (G2) separately, and to glucose and theophylline (G + T). (C) Insulin content relative to cellular DNA of the human islets harvested 7 days after infection expressed as the relative change from the uninfected control. The number of observations is indicated at the bottom of each column. P < 0.05 (*) and P < 0.01 (**) as compared to uninfected control. For experimental details see Roivainen et al. (2000).

A

B

C

(Fig. 7C). Virus-induced β-cell damage was also evident if intracellular insulin levels were studied and expressed relative to cellular DNA (Fig. 7D). Virus titre slightly affected the time scale of β-cell damage. After 2 days of infection, functional β-cell damage was evident in islets infected with 1560 TCID₅₀ of CVB-1 or more, while β-cells infected with a lower titre (127 TCID₅₀) showed enhanced insulin responses to secretagogues (Fig. 7E) before cell death, which was seen 1 week after infection (Fig. 7F). Interestingly, at this time-point, even islets
Discussion

In the present study, we have investigated the patterns and consequences of EV infection in foetal porcine pancreatic islets. We were prompted in this study to find out whether primary human β-cells could be replaced by foetal porcine β-cells in studies where β-cell tropism of human EV strains and isolates are screened systematically. According to our present results, primary foetal porcine β-cells were highly susceptible to CVBs. Soon after infection, insulin release in response to secretagogues was severely impaired and a few days later, all endocrine cells were dead. In contrast to CVBs, no immediate adverse effect was seen after CVA-9 infection. In principle, this conclusion is very similar to the one we made earlier with the same viruses in human primary β-cells. However, CVA-9 replicated well in the human β-cells, but in spite of that, no immediate adverse effect was seen (Fig. 5) (Roivainen et al., 2000), while in porcine islets, this virus replicated poorly and sometimes not at all. Furthermore, in those porcine islet experiments where we were capable of documenting CVA-9 replication, a clear destructive effect in stimulated insulin release was seen after extended follow-up. However, large variation was evident and islets infected with CVA-9 usually showed enhanced stimulation 1 week after infection, a phenomenon that is also seen regularly for CVBs before final functional destruction. Previously, the same phenomenon has been described for CVA-4 (Szopa et al., 1985).

In this study, virus replication was documented by measuring the infectivity of progeny virus in samples taken at different time-points. However, this might not be the most sensitive and accurate method to use when only marginal titres of progeny are produced. This is especially true for viruses that are sensitive to heat-induced inactivation. For example, infectivity of CVA-9, unlike that of PV, is clearly decreased after overnight incubation at 37 °C (Piirainen et al., 1998).

In addition to CVBs and CVA-9, several other strains of EVs were included in this study. PV-1, which is known to replicate only in cells of primate origin, was used as a negative control. No replication was seen with CVA-16, E-1, -7 and -11, or HPEV-1 in any porcine islet preparation, and, thus, they were not included in further functional analysis. Of course, we cannot exclude the possibility of marginal progeny production in the case of some viruses because no method other than regular progeny titration was used. In theory, replication of echoviruses could have been inhibited by the BSA (1%) used in our incubation medium after virus infection (Ward et al., 1999). However, this explanation is not plausible because the same echoviruses replicated well in primary human islets in the presence of 1% BSA (unpublished data). In conclusion, there seem to be species-specific differences in EV permissibility between human and porcine pancreatic islets.

Through our previous studies, we have used high multiplicity infections in order to confirm that all susceptible cells are infected at the beginning of the experiment. In this way, the virus-induced consequences of infection could be followed reliably, but it is not possible to study the kinetics of these effects. After extending our studies to several different serotypes, some of them more difficult to propagate at high titres than others, such a high multiplicity was not always achieved. Some of the observed differences in virus-induced consequences might have been based on differences in the infectivity of the virus inoculum used. To exclude this possibility, the kinetics of virus replication and virus-induced cell damage were studied in detail. We found that the amount of virus needed for β-cell destruction was small. Even 55 infectious virus particles were enough to destroy all β-cells of one pancreas in vitro.

Assuming that EVs are indeed involved in the pathogenesis of IDDM, one of the most obvious questions would be whether all EVs are diabetogenic or whether certain serotypes or strains are particularly diabetogenic. It is possible that in a genetically susceptible individual, several different serotypes
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Fig. 7. Lowest titre of CVB-1 needed for β-cell damage. The number of infectious virus particles used in infection (per one pancreas, as described in Methods) is shown in TCID$_{50}$. (A, B) Replication of CVB-1 in islets infected with decreasing titres of CVB-1. (C, D) Effects of infection (55–1.2 x 10$^6$ TCID$_{50}$) on stimulated insulin release and on insulin relative to DNA 1 week after infection. (E, F) Stimulated insulin release of islets infected with 15–90,000 TCID$_{50}$ units of CVB-1 2 days after infection (E) and 1 week after infection (F).

The large number of different EV serotypes makes the identification of the most pathogenic serotypes and/or strains both important and tedious. The screening process could be simplified significantly by standardized experiments with primary human islet cultures. Unfortunately, human islets are not easily available. Previous studies with isolated pancreatic islets have revealed that porcine β-cells share important properties with human β-cells (Tuch & Bai, 1998).

The aim of this study was to evaluate whether foetal porcine β-cells could be used instead of the scarcely available human β-cells for monitoring the diabetogenic properties of human EVs. The responses to CV infection were quite similar in the two species, both differing clearly from previously reported rodent β-cells. The function of porcine β-cells was affected by CVBs even more severely than human β-cells. CVA-9 did not replicate in porcine islets as readily as in human islets, but when it did replicate, a clear functional destruction of β-cells was evident after prolonged incubation, suggesting that foetal porcine islets are highly sensitive to this virus-induced damage as well. Furthermore, porcine foetal β-cells, unlike human β-cells (unpublished data), were unable to support the replication of other tested EVs. Thus, species-specific differences do exist and systematic screening of various EVs for β-cell tropism should be carried out in primary human β-cells. Foetal porcine β-cells could be used in studies that address details of the interaction between CVs and β-cells.

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