Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells

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Abstract

Aims/hypothesis. It is thought that enterovirus infections cause beta-cell damage and contribute to the development of Type 1 diabetes by replicating in the pancreatic islets. We sought evidence for this through autopsy studies and by investigating known enterovirus receptors in cultured human islets.

Methods. Autopsy pancreases from 12 newborn infants who died of fulminant coxsackievirus infections and from 65 Type 1 diabetic patients were studied for presence of enteroviral ribonucleic acid by in situ hybridisation. Forty non-diabetic control pancreases were included in the study. The expression and role of receptor candidates in cultured human islets were investigated with receptor-specific antibodies using immunocytochemistry and functional assays.

Results. Enterovirus-positive islet cells were found in some of both autopsy specimen collections, but not in control pancreases. No infected cells were seen in exocrine tissue. The cell surface molecules, poliovirus re-

ceptor and integrin $\alpha v\beta_3$, which act as enterovirus receptors in established cell lines, were expressed in beta cells. Antibodies to poliovirus receptor, human coxsackievirus and adenovirus receptor and integrin $\alpha v\beta_3$ protected islets and beta cells from adverse effects of poliovirus, coxsackie B viruses, and several of the arginine-glycine-aspartic acid motifs containing enteroviruses and human parechovirus 1 respectively. No evidence was found for expression of the decay-accelerating factor which acts as a receptor for several islet-cell-replicating echoviruses in established cell lines.

Conclusions/interpretation. The results show a definite islet-cell tropism of enteroviruses in the human pancreas. Some enteroviruses seem to use previously identified cell surface molecules as receptors in beta cells, whereas the identity of receptors used by other enteroviruses remains unknown. [Diabetologia (2004) 47:225–239]

Keywords Enterovirus · Coxsackievirus · Echovirus · Poliovirus · Human pancreas · Islets · Beta cells · Receptors

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Abbreviations: A-549, Human lung carcinoma cell line · CAV, coxsackie A virus · CBV, coxsackie B virus; DAF, decay-accelerating factor · EV, echovirus · HBSS, Hanks' balanced salt solution · FITC, fluorescein isothiocyanate · GMK, a green monkey kidney cell line · HCAR, human coxsackievirus and adenovirus receptor · HPEV-1, human parechovirus 1 · PV-1, poliovirus type 1 · PVR, poliovirus receptor · RGD, arginine-glycine-aspartic acid · RNA, ribonucleic acid

Type 1 diabetes is characterised by destruction of the beta cells in pancreatic islets. The process, which finally leads to complete beta-cell loss and onset of clinical disease, starts years before any clinical symptoms and is thought to result from several factors involving host genes, autoimmune responses and cytokines, as well as environmental factors. Results from previous cross-sectional and prospective studies on patients with Type 1 diabetes and/or prediabetic individuals have suggested that enterovirus infections are involved in the development of the disease [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15].

The *Enterovirus* genus of the *Picornaviridae* family is a large group of human pathogens traditionally divided into polioviruses, coxsackieviruses, echoviruses (EV) and the new enteroviruses. Recently, enteroviruses have been reclassified as poliovirus and human enterovirus A to D on the basis of molecular homology [16].

P. Ylipaasto and K. Klingel contributed equally to the study

The first step in viral infection is the attachment of the virus to its receptor, a cell surface molecule which viruses have adapted to gain entry into the cell. Receptors are also considered to be key determinants in tissue tropism of viruses. To date several cell surface receptors are known for enteroviruses, among them members of the integrin and immunoglobulin superfamilies normally involved in cell adhesion. Three serotypes of poliovirus use poliovirus receptor (PVR), a member of the immunoglobulin superfamily [17] and members of human enterovirus C use intercellular adhesion molecule-1 [18]. Integrin $\alpha v \beta 3$, also known as the vitronectin receptor, works as a receptor for coxsackievirus A 9 (CAV-9) [19] and other arginine-glycine-aspartic acid motifs (RGD) containing enteroviruses [20, 21], whereas another integrin, $\alpha 2 \beta 1$, works as a receptor for EV-1 [22, 23]. In addition, a complement regulatory protein, i.e. decay-accelerating factor (DAF), and a protein called human coxsackievirus and adenovirus receptor (HCAR) are used for cellular entry by coxsackieviruses and several EVs [24, 25, 26, 27, 28]. However, the function of these receptors in viral entry is not understood in detail and it is possible that additional host factors are needed for complete entry. The situation is further complicated by the fact that some viruses use more than one cellular receptor [18, 29, 30, 31, 32], sometimes even in one cell line [33, 34]. The current knowledge of receptor usage is mostly based on studies using continuous cell lines common in laboratories. Little if anything is known about enterovirus receptors of primary human cells and tissues.

Enterovirus serotypes associated with Type 1 diabetes have been tentatively identified in several ways [1, 35]. Assuming that infection of pancreatic beta cells is relevant to enterovirus-induced damage, we have studied patterns and consequences of enterovirus infections in human beta cells isolated from the organs of deceased persons. According to previous results, human beta cells are highly susceptible to enterovirus infection [36, 37, 38, 39, 40]. The effect of virus replication on beta-cell survival and function was, however, dependent on serotype and/or strain, ranging from rapid cytolysis with simultaneous severe functional damage of surviving cells to subtle morphological changes only [38]. These observations are of interest as there are very few published data on enterovirus infections in human pancreata *in vivo*. Moreover, in most experimental models of murine coxsackie B virus (CBV) infection, the exocrine pancreas is destroyed and the islets often remain intact [41, 42, 43, 44, 45, 46, 47].

For this study we had access to two sets of autopsy materials suitable for assessing potential islet tropism of enterovirus infections *in vivo*. This enabled us to study *in situ* hybridisation in pancreata from newborn infants who died of fulminant coxsackievirus infections, as well as in pancreata from deceased adoles-

cent and adult Type 1 diabetic patients. In another line of studies, we examined the background for this tropism and studied the presence and function of the selected enterovirus receptors in cultured primary human islets.

Subjects and methods

Autopsy pancreases. Autopsy pancreases from 12 newborn infants who died of fulminant coxsackievirus infections at the age of 3 days to 10 months [48] were studied for presence of enteroviral ribonucleic acid (RNA) by *in situ* hybridisation. Most of these patients had coxsackievirus myocarditis and six out of seven enterovirus-positive pancreases showed evidence of insulinitis. We also studied 65 autopsy pancreases from adolescent and adult Type 1 diabetic patients. The age span of the Type 1 diabetic patients was 18 to 52 years, the ratio of men to women was 2:3 and the duration of diabetes ranged from a few weeks up to 19 years. The enterovirus status of the patients was not known but their autopsy hearts were negative for coxsackievirus myocarditis. In addition, 40 non-diabetic pancreases from subjects matched for age and sex were included in the study as controls. Autopsy specimens obtained from [48] and the University of Tübingen were treated as anonymous tissue, and studies carried out in accordance with the Declaration of Helsinki of the World Medical Assembly. According to the statement of the ethics committee of the University of Tübingen this kind of historic study can be done without specific permissions.

***In situ* hybridisation for detection of enteroviral RNA.** Formalin-fixed, paraffin-embedded pancreatic tissue was obtained from newborn infants who died of fulminant coxsackievirus infection [48] and from autopsies of patients suffering from Type 1 diabetes. Control tissue included normal pancreases from newborn infants and adults and pancreas from patients with chronic pancreatitis. Enteroviral plus-strand RNA in pancreatic tissue was detected using single-stranded ^{35}S -labelled RNA probes, which were synthesised from the dual-promoter plasmid pCVB3-R1 by using SP6 RNA polymerase [49]. Control RNA probes were obtained from non-recombinant transcription vector pSPT18 (Amersham Pharmacia Biotech, Piscataway, N.J., USA). Pre-treatment, hybridisation, and washing of de-waxed paraffin tissue sections (6 μm thick) were carried out as described previously. Briefly, tissue sections were incubated for 18 h at 42°C in hybridisation buffer containing ^{35}S -labelled strand-specific CVB3 RNA probes (500 ng/ml) in 10 mmol/l Tris-HCl, pH 7.4, 50% (volume by volume) deionised formamide, 600 mmol/l NaCl, 1 mmol/l EDTA with 0.02% polyvinylpyrrolidone, 0.02% dithiothreitol, denatured sonicated salmon sperm DNA at 200 $\mu\text{g}/\text{ml}$, and rabbit liver transfer RNA (Boehringer Mannheim, Mannheim, Germany) at 100 $\mu\text{g}/\text{ml}$. Washing steps, including digestion for 30 min at 37°C of non-hybridised single-stranded RNA probes by RNase A (20 $\mu\text{g}/\text{ml}$) (Boehringer Mannheim) in 10 mmol/l Tris-HCl, pH 8.0, 0.5 mol/l NaCl, were done as described previously [49]. Slide preparations were subjected to autoradiography (NTB2 film, Kodak, Rochester, N.Y., USA), exposed for 3 weeks at 4°C and counterstained with haematoxylin-eosin.

Human beta cells. Human pancreatic islets were isolated and purified either in Brussels, Belgium, at the Central Unit of the β -Cell Transplant or in Uppsala, Sweden, at Uppsala University Hospital. They were then sent to Helsinki, Finland, as free-floating islets after 1–15 days of culture in Ham's F10 medium

supplemented with 10 mmol/l HEPES, pH 7.35, and 2% calf serum. Before experiments, the suspended islets were maintained in sterile non-adherent culture plates in serum-free medium (Ham's F10 medium containing 2 mmol/l HEPES, pH 7.4, 1% BSA, penicillin and streptomycin).

Viruses. Laboratory strains of CBV (CBV-2/Ohio-1, 4/J.V.B, 5/Faulkner), EV (6/D'Amori) and 9 (Hill and Barty), CAV-9 (CAV-9/Griggs), human parechovirus 1 (HPEV-1/Harris, previously known as echovirus 22) and poliovirus type 1 (PV-1/Sabin) were obtained either from the American Type Culture Collection (Manassas, Va., USA) or from the World Health Organization Enterovirus Reference Laboratory (Copenhagen, Denmark). Viruses were passaged in continuous cell lines of monkey kidney origin (GMK) or human lung carcinomas (A-549) (American Type Culture Collection).

A clinical strain isolated from a diabetic patient was included in the study. The EV 9 DM strain was isolated from a stool sample of a 6-week-old baby suffering from acute Type 1 diabetes. The strain was isolated in tertiary monkey kidney cells [50] and passaged twice in primary monkey kidney cells before experiments.

The serotype of all virus preparations used was confirmed by a neutralisation assay with type-specific antisera.

Enterovirus receptor-specific antibodies. Polyclonal antibodies to PVR (NAETZ8) and integrin $\alpha\beta 3$ were produced in-house by immunising rabbits with three to four doses of purified proteins together with Freund's complete (the first dose) or incomplete adjuvant (with boosters). The highly purified form of integrin $\alpha\beta 3$ from human placenta was purchased from Chemicon (cat no. CC1021, Temecula, Calif., USA), and purified soluble His-tagged fusion protein of PVR was provided by E. Wimmer from the Department of Molecular Genetics and Microbiology, State University of New York, New York, USA. Monoclonal antibodies to DAF [51] used in immunostaining were provided by T. Kinoshita, Department of Immunoregulation, Research Institute of Microbial Diseases, Osaka University, Japan. The monoclonal antibody against HCAR, RmcB [52] was kindly provided by L. Philipson, Karolinska Institute, Stockholm, Sweden.

Replication of viruses. Free-floating islets pretreated with Hanks' balanced salt solution (HBSS) supplemented with 20 mmol/l HEPES, pH 7.4, h-HBSS, penicillin and streptomycin or receptor-specific antibodies (30–60 min at room temperature) were infected with apparent high multiplicity of infection (30 to 100) by different virus preparations in the absence or presence of the same receptor antibodies (anti-HCAR, 1:50; anti-PVR and anti- $\alpha\beta 3$, 1:50 to 1:300). After adsorption for 1 h at 36°C, the inoculum virus was removed and the cells were washed twice with h-HBSS. Then serum-free incubation medium (Ham's F10 medium plus 1% BSA, 25 mmol/l HEPES, pH 7.4, penicillin and streptomycin), supplemented or not with the receptor antibodies (dilution 1:50 to 1:300), was added to all cultures including uninfected controls. Infected and uninfected islets were divided into aliquots on 96-well non-tissue culture plates (for virus titration, immunofluorescence and viability stainings) or 24-well non-tissue culture plates (for perfusion, insulin and DNA), and incubated at 36°C. During the experiment, the incubation medium was changed at two- or three-day intervals.

For virus titration, samples of suspended islets harvested at 0, 1 and 2 days after infection were frozen and thawed three times to release the virus, clarified by low-speed centrifugation, and assayed for total infectivity using end-point dilutions in microwell cultures of GMK cells (HPEV-1 in A-549 cells).

Cytopathic effects were assessed by microscopy after one week and tissue culture infectious dose titres were calculated as described earlier [37].

Immunocytochemistry of infected islets. Samples of infected and uninfected islets were harvested one 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), they were double-stained for 1 h at 36°C with enterovirus-specific polyclonal rabbit antiserum (1:400; KTL-910, ref. [53]) and insulin-specific polyclonal sheep antiserum (1:200; The Binding Site, Birmingham, England). Visualisation was achieved using fluorescein isothiocyanate (FITC) and Red-X conjugated anti-species sera (711-095-152 and 713-295-147 respectively; Jackson ImmunoResearch Laboratory, West Grove, Pa. USA), and analysed using a confocal microscope (Leica TCS NT, Wetzlar, Germany).

Immunocytochemistry of cell surface expression of enterovirus receptors. Expression of enterovirus receptors on primary human islets was carried out by staining samples of live unfixed islets with receptor-specific antibodies (polyclonal antibodies, dilution 1:500 for 30 min at 22°C; monoclonal antibodies, dilution 10 $\mu\text{g/ml}$ for 2 h at 4°C). After staining with polyclonal receptor antibodies, the islets were fixed and made impermeable with cold methanol (15 min at -20°C), and stained for 1 h at 36°C with insulin-specific antibody (1:200; The Binding Site), followed by a 30-min incubation with FITC and Red X-labelled anti-species conjugate (Jackson ImmunoResearch). The anti-DAF staining was followed by a 30-min incubation with Red X-conjugated anti-mouse antibody (Jackson ImmunoResearch) and then fixed with cold methanol. The samples were analysed using a confocal microscope.

Co-localisation of enterovirus receptors with virus particles. Samples of live islets were infected with an apparently high multiplicity of PV-1 or coxsackievirus A9. After 60 min incubation at 16 to 18°C the infected islets were stained with anti-receptor antibodies (anti-PVR or anti- $\alpha\beta 3$, dilution 1:500) and enterovirus group-specific anti-peptide antibody (produced in house by immunising sheep four times with the immunodominant highly conservative VP1-derived peptide; dilution 1:600). Visualisation was achieved by FITC- and Red X-labelled anti-species conjugates (Jackson ImmunoResearch). Before analysis the islets were cytocentrifuged (Thermo Shandon, Runcorn, UK) on glass slides and fixed with cold methanol (15 min at -20°C).

DNA and insulin content of cells. For the measurement of cellular DNA and insulin content, islet cells were homogenised ultrasonically in distilled water. DNA was measured from dried samples fluorometrically based on diaminobenzoic acid-induced fluorescence [54]. Insulin was measured with a commercial solid-phase insulin RIA kit (DPC, Los Angeles, Calif., USA) after overnight extraction with acid ethanol [55].

Cell viability. The viability of islet cells after infection was measured using a commercial kit (the live/dead-cell assay, L-3224, Molecular Probes, Leiden, The Netherlands). According to the manufacturer's instructions for staining, islets harvested at different time points were incubated with the labelling solution for 30 min at RT in the dark, and analysed with a confocal microscope (Leica).

Beta-cell function. Insulin secretion in response to glucose and glucose plus theophylline was studied by perfusion as described previously [37, 56]. Briefly, after taking samples for

Fig. 1a-e. Islet-cell tropism of enteroviruses in human pancreas. Human pancreases obtained by autopsies were analysed for enteroviral RNA by in situ hybridisation. The first three panels show pancreases from one uninfected control baby (a) and from two newborn infants who died of coxsackievirus infections (b, c). Viral RNA (black silver grains) was found in several islets and some pancreatic duct cells but not in exocrine tissue. Other panels (d, e) show pancreases from two Type 1 diabetic patients. Note the black silver grains in the islets, reflecting cells which contain virus RNA

insulin content and DNA measurement, islets were loaded in perfusion chambers in Krebs-Ringer bicarbonate buffer, supplemented with 20 mmol/l HEPES (pH 7.35) and 0.2% BSA. After a 60-min stabilising period in low glucose (1.67 mmol/l), the cells were stimulated first with a 16.7 mmol/l concentration of glucose and then with a mixture of 16.7 mmol/l glucose and 10 mmol/l theophylline (Sigma, St. Louis, Mo., USA). Fractions of 1 ml were collected every 4 min and analysed for insulin by RIA. After stimulations the basal buffer (1.67 mmol/l glucose) was used during the final five fractions. Up to six perfusion lines were run in parallel using a multi-channel perfusion apparatus (Brandel, Gaithersburg, Md., USA). Stimulation indices were calculated separately for the first and second glucose response and glucose plus theophylline.

Results

Compartments of human pancreas infected by enteroviruses in vivo. In our in situ hybridisation studies on 12 pancreases from autopsies of coxsackievirus-infected children, 7 out of the 12 pancreases were positive for enteroviral RNA, which was found in numerous pancreatic islets and in some duct cells but not in exocrine tissue (Fig. 1b,c).

Fig. 2a, b. Expression of enterovirus receptors in beta cells. Samples of cultured islets (**a**) were stained with polyclonal anti-receptor antibodies, dilution 1:500 (poliovirus receptor PVR; integrin $\alpha v \beta 3$). Reactions were made visible with fluorescein isothiocyanate-conjugated anti-rabbit conjugate. After receptor staining the islets were fixed with methanol and stained with insulin-specific antibody followed by Red X-labelled anti-sheep conjugate. Colour codes: green, receptor; red, insulin; yellow-orange, receptors in beta cells. Samples of cultured islets and human rhabdomyosarcoma cells (**b**) were stained with two different monoclonal antibodies to decay-accelerating factor (dilution: 10 $\mu\text{g/ml}$). Visualisation was achieved by Red X-labelled anti-mouse conjugate

Insulinitis was evident in six out of seven enterovirus-positive pancreases, showing that the morphological characteristic of insulinitis matched well with the presence of viral RNA. Uninfected neonatal control pancreases were negative, as shown in one newborn infant (Fig. 1a). When pancreases from Type 1 diabetic patients (18–52 years of age) were studied for enteroviral RNA using the same in situ hybridisation technique, positive results were obtained in 4 out of 65 patients. In all four of these diabetic pancreases the enterovirus-positive cells were detected exclusively in islets, as shown in two patients (Fig. 1d,e). In contrast, pancreatic control tissues from 40 non-diabetic patients were consistently negative for enteroviral RNA.

Fig. 3. Co-localisation of enterovirus receptors with virus particles in human islet cells. Samples of live islets were infected, with apparent high multiplicity, with poliovirus type 1 (PV-1) or coxsackievirus A9 (CAV-9). After 60 min incubation at 16–18°C the infected islets were stained with anti-receptor antibody (anti-poliovirus receptor PVR or anti- $\alpha\text{v}\beta 3$, dilution 1:500) and virus-recognising polyclonal antibody (1:600, produced in a sheep). Visualisation was achieved by fluorescein isothiocyanate- and Red X-labelled anti-species conjugates (green: receptor; red: virus; yellow-orange: co-localisation). At the end of the assay the islets were cytocentrifuged on glass slides and fixed with cold methanol

Expression of different enterovirus receptors in primary human islets. Cell surface expression of PVR and integrin $\alpha\text{v}\beta 3$, both known to act as enterovirus receptors in established cell lines, was clearly evident (Fig. 2a). Double immunofluorescence staining for insulin and virus receptors verified expression of these cell surface molecules in beta cells (Fig. 2a). In contrast, islets stained with monoclonal antibodies to DAF were completely negative (Fig. 2b). As a positive control for DAF, cells from human rhabdomyosarcoma cell line were stained simultaneously using the same preparations of DAF-monoclonal antibodies, and a strong cell surface expression of DAF was seen in these cells when stained with two different antibodies.

Confocal microscopy of live human islets exposed to infectious viruses revealed a bright co-localisation of virus particles with studied cell surface molecules, suggesting that the studied cell surface molecules are capable of binding the virus (Fig. 3).

PVR as a receptor for PV-1/Sabin. In primary human islets infected, in the absence or presence of PVR-specific antibody, with the Sabin strain of PV-1 we found that PV-1/Sabin replicated well, and that the replication took place in beta cells as shown by double immunofluorescence staining with virus- and insulin-specific antibodies (green and red respectively) (Fig. 4a, where the yellow-orange label corresponds to infected beta cells). The replication of PV-1/Sabin in primary human islets was blocked completely by the PVR-specific polyclonal antibody, as shown by double immunofluorescence staining (Fig. 4a), live-dead cell staining (Fig. 5d) and infectivity titration (Fig. 5a). The observed blocking effect was specific for this antibody, as poliovirus replication was not affected by the polyclonal antibody to the vitronectin receptor, $\alpha\text{v}\beta 3$ (Fig. 5a).

Effects of the polyclonal antibodies to PVR and integrin $\alpha\text{v}\beta 3$ on virus-induced beta-cell-specific function and destruction, as well as on stimulated insulin release and intracellular insulin content, are shown in Fig. 5b,c. Poliovirus-induced disturbance in beta cells was completely prevented by the PVR-specific antibody.

Fig. 4a, b. Anti-receptor antibodies protect human beta cells from infections caused by poliovirus type 1 (PV-1) and coxsackievirus B4 and 5 (CBV-4, CBV-5) as shown by double immunofluorescence staining with insulin and virus-specific antibodies. Samples of islets infected in the presence or absence of anti-poliovirus receptor PVR, dilution 1:100 (**a**) and anti-human coxsackievirus and adenovirus receptor (HCAR) at dilution 1:50 (**b**) were harvested one day after infection, cytocentrifuged on glass slides, fixed with cold methanol and double-stained with enterovirus-specific rabbit antiserum and insulin-specific sheep antiserum. Visualisation was achieved by staining with fluorescein isothiocyanate anti-rabbit conjugate (green: virus antigen) and Red-X-anti-sheep conjugate (red: insulin). Infected beta cells are yellow-orange

HCAR as a receptor for coxsackievirus B4 and 5. In control human islets not pretreated with the HCAR antibody the infections of both coxsackieviruses progressed well. Virus-induced beta-cell-specific destruction was clearly evident in experiments where stimulated insulin release or intracellular insulin content per cellular DNA were assayed (Fig. 6b,c). In the case of both viruses, the HCAR-specific monoclonal antibody

protected islets from virus-induced cellular death and beta-cell-specific destruction (Fig. 6b,c). Even 14 days after infection the islets were still alive when RmcB was present (Fig. 6d). Likewise, immunofluorescence staining for infected islets did not show any virus-positive cells in cultures inoculated in the presence of HCAR antibody (Fig. 4b). The HCAR antibody inhibited virus replication in islets infected with CBV-4 (Fig. 6a). In contrast, a clear progeny virus production was seen in cultures infected with CBV-5 even in the presence of the HCAR antibody (Fig. 6a).

Integrin α V β 3 as a receptor for CAV-9 but also for EV-9 and HPEV-1. In primary human islets infected with CAV-9 and HPEV-1 the polyclonal antibody to integrin α V β 3 prevented the destruction of beta cells at 2 weeks after infection (Fig. 7). The protective effect of the antibody was also seen in live/dead cell staining of islets, as shown below for CAV-9.

Studies on the protective effect of the polyclonal integrin α V β 3 antibody were extended to other RGD-containing strains of viruses, including the laboratory strain of EV-9/Barty and a clinical isolate of EV-9 / DM. PV-1/Sabin was used as a negative control. No

Fig. 5a–d. Poliovirus receptor-specific antibody inhibits replication of poliovirus type 1 (PV-1) and virus-induced beta cell consequences in primary human islets. Parallel aliquots of islets were incubated with the antibody (anti-poliovirus receptor PVR, anti-αVβ3 was used as a negative control; dilution of 1:100) or the diluent and then infected with PV-1 at an apparent high multiplicity. Samples taken at 0, 1 and 2 days after infection were assayed for total infectivity (**a**), at one week after infection for intracellular insulin content expressed per cellular DNA (**b**), for stimulated insulin release (**c**) and for cell viability (**d**). Results (**b**, **c**) are expressed as the relative change from the uninfected control. Live cells (**d**) are stained green by calcein due to their esterase activity, whereas red fluorescence was induced in the nuclei of dead cells by ethidium homodimer-1. Results obtained by several different methods from one complete experiment are shown. The blocking effect of the anti-receptor antibody was also evident in other islet cell preparations as shown by double-immunofluorescence staining with insulin and virus-specific antisera and live/dead-cell stainings

signs of progeny virus were found in the presence of the antibody in cultures infected with EV-9/Barty, EV-9/DM or the prototype strain of CAV-9/Griggs (Fig. 8). In contrast, progeny virus production of PV-1 / Sabin was not affected by the antibody (Fig. 8).

The protective effect of the antibody on beta-cell-specific destruction was shown by determining intracellular insulin content/DNA. All islets infected with CAV-9 or EV-9 were protected by the antibody. Likewise, results from live/dead-cell staining of cultures infected with tested non-polio enteroviruses showed the clear protective effect of the polyclonal antibody (Fig. 8).

The prototype strain of EV-9 Hill was also included in the study because according to our previous results this virus, although not having the RGD motif, is capable of using integrin αVβ3 in its entry process at

Fig. 6a–d. Human coxsackievirus (HCV)-specific antibody inhibits replication of coxsackievirus B4 and 5 (CBV-4 and -5) and virus-induced beta cell-specific consequences in primary human islets. Parallel aliquots of islets were incubated with HCV-specific antibody (dilution 1:100) or the diluent and then infected with CBV-4 or CBV-5 at an apparent high multiplicity. Samples taken at 0, 1 and 2 days after infection were assayed for total infectivity (**a**), at one week after infection for stimulated insulin release (**b**) and for intracellular insulin content per cellular DNA (**c**), and at two weeks after infection for

cell viability (**d**). Results (**b**, **c**) are expressed as the relative change from the uninfected control. Live cells (**d**) are stained green by calcein due to their esterase activity, whereas red fluorescence was induced in the nuclei of dead cells by ethidium homodimer-1. Results obtained by several different methods from one complete experiment are shown. The blocking effect of the anti-receptor antibody was also evident in other islet cell preparations as shown by double-immunofluorescence staining with insulin and virus-specific antisera and live/dead-cell stainings

Fig. 7. Integrin α V β 3-specific antibody protects islets from infections caused by coxsackievirus A9 (CAV-9) and human parechovirus 1 (HPEV-1). Parallel aliquots of islets were incubated with α V β 3-specific antibody (dilution 1:300) or the diluent and then infected with CAV-9 or HPEV-1 at an apparent high multiplicity. Samples taken at 2 weeks after infection were assayed for intracellular insulin per cellular DNA. The results are expressed as the relative change from the uninfected control

least in an established cell line of green monkey kidney [21]. However, replication of this virus in human islets, as well as virus-induced cellular death, was prevented by the polyclonal antibody to integrin α V β 3 (Fig. 9).

Discussion

We have identified the cellular compartments infected by enteroviruses in the human pancreas. Our *in situ* hybridisation studies on pancreases of Type 1 diabetic patients showed enteroviral-positive cells exclusively in islets. This observation was very specific as no reactivity was seen in exocrine cells. Likewise, the islet-cell tropism of enteroviruses was clearly evident in pancreases of newborn infants dying of fulminant coxsackievirus infections as shown here by *in situ* hybridisation and previously by means of immunohistochemistry [48]. In our study enteroviral RNA was found in numerous pancreatic islets and in some duct cells but not in exocrine cells. This finding is in direct contrast with experimental infections in mice where enteroviruses had a propensity to the exocrine compartment. Thus localisation studies on mouse pancreases have identified virus in acinar cells but not in islets [41, 42, 43, 44, 45, 46, 47, 57]. In spite of almost complete destruction of the infected exocrine pancreas, the islets, ducts and connective tissue were selectively spared in mice infected with coxsackievirus B [42, 44, 46, 47, 58]. Our observations demonstrate an important species difference in the

cell type specificity of pancreatic enterovirus infection.

Human diabetic pancreases from adults have been studied for viral infections, but no evidence of enterovirus infections was found [48, 59, 60]. In our study, too, most diabetic pancreases remained negative. Enteroviral RNA was found in only 4 of 65 pancreases obtained at autopsies. The duration of diabetes in these deceased persons was not known, and evidence of concurrent or historical enterovirus infections was not available. However, in heart specimens no signs of enterovirus myocarditis were seen. In contrast, several studies based on RT-PCR have shown the presence of enteroviral RNA in sera, blood cells or stools of people with recent onset Type 1 diabetes [9, 13, 14, 15].

Enteroviruses initiate infections in pancreatic as well as in all other target cells by attaching to cell surface receptor molecules, which are considered to play a key role in tissue tropism and hence in the generation of the spectrum of clinical symptoms. We showed that two cell surface proteins (PVR and integrin α V β 3), both of which act as enterovirus receptors in established cell lines, are also expressed in primary human islets and especially in beta cells. Recently, expression of integrin subunits including α v and β 3 in purified human beta cells was shown by semi-quantitative RT-PCR [61]. In contrast, no evidence was found for expression of DAF, which acts as a receptor for several of the islet cell replicating EVs in established cell lines, although different highly reactive DAF-specific monoclonal antibodies [51] were used. The absence of DAF was a surprise, as this glycosyl phosphatidylinositol-anchored protein, which is normally involved in regulating complement activity, is widely expressed in different tissues. It dissociates and prevents the assembly of C3/C5 convertases in the classic and alternate pathways of the complement system [62].

Antibodies to PVR, HCAR and integrin α V β 3 protected islets and beta cells from virus-induced adverse effects. In most cases the antibody also inhibited virus replication. Poliovirus infection and virus-induced effects on beta cells were completely blocked by PVR-specific polyclonal antiserum. Likewise, monoclonal antibody to HCAR prevented infection of CBV-4 and its consequences. Altogether, these results suggest that PVR and HCAR play key roles in infections caused in primary human islets by PV-1 and CBV-4 respectively. One previous study has suggested that HCAR plays a role in CBV infections of human islets [40]. However, the expression of HCAR in human beta cells is intriguing since practically no evidence of expression of the corresponding mouse protein was found in islets of murine pancreas [63]. PVR, a member of the immunoglobulin superfamily also thought to work as a receptor for vitronectin [64], is the only receptor known for three serotypes of poliovirus [17].

Fig. 8a–c. Integrin $\alpha\text{V}\beta 3$ -specific antibody protects human islets from infections of enteroviruses containing arginine-glycine-aspartic acid. Parallel aliquots of islets were incubated with $\alpha\text{V}\beta 3$ -specific antibody (dilution 1:50) or the diluent and then infected, with apparent high multiplicity, with echovirus 9/Barty (EV-9 Barty), echovirus 9/DM (EV-9 DM) or coxsackie A virus (CAV-9). Poliovirus 1 (PV-1) was used as a negative control. Samples taken 0 and 36 h after infection were assayed

for total infectivity (a) and at one week after infection for intracellular insulin content per cellular DNA (b) and cell viability (c). Results obtained by several different methods from one complete experiment are shown. The blocking effect of the anti-receptor antibody was also evident in other islet cell preparations as shown by double-immunofluorescence staining with insulin and virus-specific antisera and live/dead-cell stainings

Fig. 9a, b. Integrin $\alpha\text{V}\beta 3$ -specific antibody protects human islets from infection of echovirus 9/Hill (EV-9 Hill). Parallel aliquots of islets were incubated with $\alpha\text{V}\beta 3$ -specific antibody (dilution 1:50) or the diluent and then infected, with apparent high multiplicity, with prototype strains of EV-9 Hill and echovirus 9 Barty (EV-9 Barty). Samples taken at different intervals were assayed for total infectivity (**a**), and at one week after infection for cell viability (**b**) by using a live/dead-cell assay kit. Results obtained by several different methods from one complete experiment are shown. The blocking effect of the anti-receptor antibody was also evident in other islet-cell preparations as shown by double-immunofluorescence staining with insulin and virus-specific antisera and live/dead-cell stainings

On the other hand, islets infected with CBV-5 showed virus replication in the presence of HCAR antibody. This was a surprise because in the same experiment the antibody protected infected islets from virus-induced cellular death and beta-cell-specific defects. It is possible that even in the presence of the antibody,

other non-insulin-producing islet cells are infected with CBV-5 in a less cytolytic way. For coxsackie B viruses, two different receptors, HCAR and DAF, have been identified in established cell lines, and especially serotypes 1, 3, 5 are known to use either or both as receptors [24, 25, 27, 52, 65]. An important role of HCAR in the development of coxsackievirus diseases has been suggested in recent studies on clinical virus strains characterised after only one passage through primary green monkey kidney cells [27, 65].

In contrast to HCAR, DAF was not expressed in primary human islets and therefore this receptor cannot serve as an alternate receptor for CBVs in islets. In our studies no attempts were made to identify cell surface molecules used by other EVs in primary human islets and beta cells. Recently, a DAF-independent entry process through heparan sulfate has been characterised for some EVs in established cell lines [30, 66]. This interaction could be of relevance in vivo as viruses showing this phenotype have been found among clinical low-passage isolates of EVs [67].

Integrin $\alpha\beta 3$, also known as the vitronectin receptor, is used as a receptor for CAV-9 in several established cell lines [19, 68] as well as in primary human islets as shown here. Virus replication and virus-induced beta-cell-specific destruction were prevented by the anti-receptor polyclonal antibody. Moreover, in primary human beta cells this receptor was used by two other RGD-containing viruses, a laboratory strain of EV-9/Barty and HPEV-1, as beta-cell-specific destruction was inhibited by the same antibody. Interestingly, EV-9 strain Hill, the prototype that does not have the RGD-motif, was found to use the same vitronectin receptor in primary human islets. The receptor footprint on the EV-9 virion is not known. The vitronectin receptor, however, is well known for RGD-independent interactions with the natural ligand, vitronectin, and with some microbes [69]. Altogether, the results obtained with the vitronectin receptor-specific antibody show that this receptor works as a receptor for enteroviruses and HPEV-1 in primary human islets. Similar results have been reported in established cell lines [19, 20, 21, 70, 71].

Receptor usage by clinical strains is not strictly determined by the serotype. Variants with altered receptor specificity can arise during infection in humans. We studied the receptor specificity in primary human islets of a clinical strain, EV-9/DM, which had been isolated from a diabetic patient. This virus strain was more destructive in human islets than the corresponding prototype strains, as has been shown in comparative studies of different EV-9 strains and their tropism for pancreatic cells [21]. In the case of EV-9/DM the vitronectin receptor was responsible for beta-cell-specific destruction and virus replication in islets. Under some conditions, a virus can change receptor specificity and use another molecule as a receptor to mediate entry into the host cell. Differences in receptor specificity are found even among prototype strains passaged in various established cell lines [72]. The adaptation of a virus, allowing it to replicate in secondary target tissues, could extend the cell tropism of that virus, also affecting organ-specific symptoms of enterovirus infections [31]. The adaptation of CBV-2 to primary human islets did not change receptor specificity.

In conclusion, we have shown islet-cell tropism of enteroviruses in human pancreases in vivo. Enteroviral genomes were found in islets, but not in exocrine cells of the deceased newborn infants studied, as well as in Type 1 diabetic patients. These findings suggest that enteroviruses play a part in the development of Type 1 diabetes and that this part is based on direct action by enteroviruses on beta cells. We showed that the cell surface molecules, PVR, HCAR and integrin $\alpha\beta 3$, which act as enterovirus receptors in established cell lines, are expressed in primary human islets and specifically in beta cells, and that these receptors are responsible for virus infections resulting in destruction of beta cells. It is possible, however, that

other cell surface molecules contribute to infections in non-beta cells. In contrast, the cell surface molecule DAF was not expressed on islet cells, suggesting that another molecule acts as a receptor for several serotypes of EVs.

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