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RESEARCH ARTICLE

Coxsackievirus B4 can infect human pancreas ductal cells and persist in ductal-like cell cultures which results in inhibition of Pdx1 expression and disturbed formation of islet-like cell aggregates

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Abstract The role of enteroviruses, especially Coxsackievirus B (CVB), in type 1 diabetes is suspected, but the mechanisms of the virus-induced or aggravated pathogenesis of the disease are unknown. The hypothesis of an enterovirus-induced disturbance of pancreatic β-cells regeneration has been investigated in the human system. The infection of human pancreas ductal cells and pancreatic duct cell line, PANC-1, with CVB4E2 has been studied. Primary ductal cells and PANC-1 cells were infectable with CVB4E2 and a RT-PCR assay without extraction displayed that a larger proportion of cells harbored viral RNA than predicted by the detection of the viral capsid protein VP1 by indirect immunofluorescence. The detection of intracellular positive- and negative-strands of enterovirus genomes in cellular extracts by RT-PCR and the presence of infectious particles in supernatant fluids during the 37 weeks of monitoring demonstrated that CVB4E2 could persist in the pancreatic duct cell line. A persistent infection of these cells resulted in an impaired expression of Pdx1, a transcription factor required for the formation of endocrine pancreas, and a disturbed formation of islet-like cell aggregates of which the viability was decreased. These data support the hypothesis of an impact of enteroviruses onto pancreatic ductal cells which are involved in the renewal of pancreatic β-cells.

Keywords Coxsackievirus B4 · Enterovirus · Pancreas · Ductal cells · Persistence · Type 1 diabetes

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by lymphocytic infiltration of the islets of Langerhans and destruction of the insulin-producing β cells. The selective autoimmune destruction of pancreatic islet β cells, occurring in genetically predisposed subjects, is possibly triggered or accelerated by environmental agents [1]. Enterovirus infections are among the main environmental risk factors for T1D [2, 3]. Epidemiological data have showed an increased incidence of type 1 diabetes after epidemics due to enteroviruses and enterovirus infections have been diagnosed more frequently in T1D patients than in healthy subjects [4–6]. The detection of enteroviruses in various tissues of T1D patients suggests an association between these viruses and the disease. Particularly for studies that have applied molecular methods, a significant association between enterovirus infection and T1D has been displayed and the most often involved enteroviruses were Coxsackieviruses B (CVB) [7, 8].

The physiopathological link between CVB infections and T1D has not been deciphered yet. However, experimental studies have shown that various mechanisms such as direct β-cell lysis, viral persistence, molecular mimicry, antibody-dependent enhancement, thymus function

disturbance and bystander activation could be proposed to explain CVB-induced T1D [7, 9–11]. More direct support for a viral etiology has, first, come from the demonstration that a strain of CVB4, isolated from the pancreas of a recent-onset type 1 diabetic patient, was capable of inducing diabetes in mice [12]. More recently, enteroviral RNA and viral capsid protein VP1 have been detected in pancreas of recently diagnosed T1D patients. Although enteroviral RNA has been mainly localized in endocrine cells [13, 14], it has also been detected in pancreatic ductal cells [15].

Adult pancreatic ductal cells are considered as pancreatic stem cells that have retained the ability to generate endocrine cells [16, 17]. Human pancreatic ductal cells have the potential to expand and differentiate into islet cells in vitro [18].

It has been shown in vitro in human and rodent and in vivo in rodent that there is a "turnover" of β-cells with apoptotic death and replacement by both β-cell replication and neogenesis of islets which involves the differentiation of ductal progenitor cells [19]. Although the two pathways for new β-cell formation are likely to exist, the neogenesis pathway is thought to be predominant in humans, whereas the replicative pathway is predominant in rodents [20, 21]. It has been observed that pancreatic endocrine tissue can regenerate following injury or increased insulin requirements. Indeed, substantial regeneration of both the endocrine and exocrine pancreas from precursor cells in the ductal epithelium has been observed in partial pancreatectomised young adult rats [22]. In human beings, the β-cell mass has been increased by neogenesis from the ducts in obese subjects [23]. Moreover, a differentiation of epithelial non-endocrine cells, including ductal cells, from adult human pancreas into endocrine cells was obtained in vitro [24]. Taken together, these studies reinforce the hypothesis that human pancreatic ductal cells can serve as a pool of endocrine progenitor cells.

Endocrine pancreatic cells grow and differentiate during organogenesis from ductal epithelial progenitor cells [25]. The differentiation of β-cells is not strictly limited to embryogenesis and continues, albeit to a lesser degree, throughout lifetime [22]. The pancreatic duct cell line, PANC-1, has been obtained from pancreatic duct epithelial carcinoma [26]. The PANC-1 cell line can be transformed into islet-like cell aggregates (ICAs) when exposed to a defined serum-free media [27]. Pdx1 is a transcription factor required for the formation of endocrine pancreas, which is spontaneously expressed by the PANC-1 cell line [28–31].

Based on studies in mice, it was suggested that the mechanism of CVB4-induced T1D might be related to a disturbance of islet replacement by neogenesis from pancreatic ductal cells rather than by β-cell destruction [32]. Whether such a mechanism can play a role in the human system has been addressed in the current study. The infection of human pancreas ductal cells with CVB4E2 and the results onto the differentiation of these cells have been investigated in vitro.

Materials and methods

Cells

The human duct cell line PANC-1 was purchased from ATCC and cultured in serum-containing medium (SCM), a mixture of Dulbecco's modified Eagle's medium DMEM 4.5 g/L (Invitrogen, France) supplemented with 10 % fetal calf serum (FCS), plus 100 U penicillin and 100 µg streptomycin per mL (Invitrogen, France).

Primary human ductal cells were isolated from exocrine-enriched preparations following islet purification. Human pancreases were harvested from brain-dead adults in agreement with French law and the Ethical Committee of our institution. Briefly, pancreases were dissociated using the automated method of Ricordi [33] with collagenase solution (Liberase; Roche Diagnostics, Meylan, France). The exocrine and endocrine (islet of Langerhans) tissue was segregated by purification in a continuous euroficoll gradient with a cell separator (Cobe 2991; Cobe BCT, Lakewood, CO) as previously described [33]. The fraction containing exocrine tissue was washed in Hank's solution, 80 µL of pellet was placed in a 75 cm^2 dish in DMEM containing 3 g/L glucose and supplemented with 10 % FCS, 1 % insulin transferrin selenium (ITS, Sigma-Aldrich), and 50 µg/mL Geneticin (G418, Sigma-Aldrich) for fibroblast overgrowth limitation, as indicated by Vila et al. [34]. The cells adhered within 12 h of culture afterwards the medium was changed and the monolayer cultures were maintained for 1 week, with media changing every 3 days. The 8-day-old cell cultures were dissociated with a solution of trypsin–EDTA and the cells seeded in culture plates (Becton–Dickinson, France). The fraction containing exocrine tissue that adhered within 12 h in plastic culture plates were de-/trans- differentiated into ductal cells 3 days later. It was previously shown that in these cells there was a dramatic loss of amylase protein and a simultaneous increase of ductal cytokeratin 19 protein (up to sevenfold on day 9 compared with day 1) [35–37]. For cell proliferation and viability testing, UptiBlue (Uptima) and Trypan blue (Sigma) were used as routinely described.

Virus

The CVB4E2 diabetogenic strain was provided by Ji-Won Yoon (Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada) and grown in HEp-2 cells (BioWhittaker, Verviers, Belgium) supplemented with 10 % fetal calf serum (Invitrogen, France) and 1 % L-glutamine (Invitrogen, France). This strain has induced diabetes when first

inoculated on mice [12]. Viral particles were released from cells by three freeze–thaw cycles; afterwards the supernatants were clarified at 2,000 g for 10 min at 4 °C. Virus titers were determined on Hep-2 cells by limiting dilution assay for 50 % tissue culture infection doses by the method of Reed–Muench and aliquots of virus preparations were then stored frozen at −80 °C.

Cell infection

Human primary ductal cells and PANC-1 cells were seeded at 1.25×10^5 cells per well in 24-well plates before being infected with 0.01 MOI of diabetogenic strain CVB4E2 and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. Human primary ductal cells were infected the day after trypsinization, on day 9 post isolation. After 24 h post infection (p.i.), cells were washed three times with cold DMEM and then resuspended in fresh growth medium and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. CVB4E2-infected and mock-infected cell cultures were followed-up during 15 days for human ductal cells and up to 37 weeks for PANC-1 cells, with media changing every 3 days. Culture supernatant fluids were harvested and stored at −80 °C for infectivity tests. The monitoring of infection consisted of a microscopic evaluation of cell morphology, cell viability and the search for viral markers: the viral capsid protein VP1 and viral RNA.

Islet-like cell aggregates (ICAs) formation

Islet-like cell aggregates formation was induced in PANC-1 cells. Cells were seeded at 5×10^5 cells per well in six-well plates and grown in SCM. To induce ICA formation, SCM was removed and cells were exposed for 120 s to 0.05 % trypsin (Sigma) at 25 % to loosen but not detach the cells from their extracellular matrix, and then they were cultured in serum-free medium (SFM), as described by Hardikar et al. [27]. The SFM was DMEM/F12 supplemented with 1 % bovine serum albumin (BSA, Sigma) and 1 % insulin–transferrin–selenium (ITS, Sigma).

RNA isolation and reverse transcription–polymerase chain reaction (RT-PCR)

Cellular RNA was extracted as routinely described by using RNAgent® Total RNA Isolation System kit (Promega). Quant-it® RNA Assay kit (Invitrogen) was used for total RNA titration on 96 well-plates using the Mx3000P® system (Stratagen). The PCR were carried out in a Perkin-Elmer-Applied Biosystems GeneAmp 9700 thermocycler.

The Superscript ™ One-step RT-PCR with Platinum® Taq (Invitrogen) was used for RT-PCR. This kit allows cDNA synthesis and amplification in one step. Reverse transcription is directly followed by the amplification step. The reaction mixture, a volume of 50 μL, is undergoing an initial reverse transcription step of 30 min at 50 °C, followed by 5 min at 94 °C of denaturation phase. Amplification was performed for 35 cycles at a denaturing temperature of 94 °C for 30 s, an annealing temperature of *X* °C for 30 s, and an extension temperature of 72 °C for 1 min. The final extension has lasted 7 min at 72 °C. *X* varied with the targeted transcript of interest: 52 °C for GAPDH, 56 °C for CK19, 55 °C for MxA, 52 °C for CVB4E2, 53° for Pdx1 and 55° for IGF2. Amplification results were

Table 1 Oligonucleotide primers and probes

Target gene	Primer sequence	Size of PCR product (bp)	Size of semi-nested product (bp)
MxA	TCC AGT CCA GCT CGG CAA CA (+)		
	TGG AGC ATG AAG AAC TGG ATG A (−)	127	
CVB4E2[a]			
(EV1)	CAA GCA CTT CTG TTT CCC CGG (+)		
(EV2)	ATT GTC ACC ATA AGC AGC CA (−)	435	362
(EV3)	TCCTCCGGCCCCTGAATGCG (−)		
IGFII	ATG GGG AAG TCG ATG CTG GTG (+)		
	ACG GGG TAT CTG GGG AAG TTG (−)	316	
GAPDH	GTC TTC ACC ACC ATG GAG A (+)		
	GAT GGC ATG GAC TGT GGT CAT G (−)	206	
PDX-1	CCC ATG GAT GAA GTC TAC C (+)		
	GTC CTC CTC CTT TTT CCA C (−)	262	
CK19	CTGGAGATGCAGATCGAAGG (+)		
	CGGTTCAATTCTTCAGTCCG (−)	241	
Beta actin	TTGCCGACAGGATGCAGAA		
	GCCGATCCACACGGAGTACT	101	

[a] Primers were selected in 5′ untranslated region of the viral genome

revealed by «DNA grade» (Type 5, Euromedex®) agarose electrophoresis gel. Oligonucleotide primers, see Table 1 MxA, CK19 and IGF2 transcripts are cellular mRNA used as controls. Myxovirus resistance protein A (MxA) gene transcripts are present in many cell types, and they can be induced by viruses and interferon alpha [38, 39]. Cytokeratin 19 (CK19) is a specific marker of pancreatic ductal cells [36]. Insulin-like growth factor 2 (IGF2) is a growth factor involved the regulation and the development of pancreatic β-cell [40].

Two-step RT-PCR for positive- and negative-strand EV RNA detection

A downstream or upstream EV primer at 0.4 mM was used as a template in synthesis of complementary DNA (cDNA) for 0.1 mg of positive- or negative strand EV RNA, respectively, in a total volume of 20 μL containing 20 U of enhanced avian reverse transcriptase, 0.5 mM each dNTP, 20 U of RNase inhibitor, 50 mM Tris–HCl (pH 8), 40 mM KCl, 8 mM $MgCl_2$, and 1 mM dithiothreitol by using the enhanced avian RT-PCR kit according to the manufacturer's instructions. The RT reaction was performed at 50 °C for 50 min and was stopped by heating the samples for 5 min at 95 °C. The PCR was carried out in a Perkin-Elmer Applied Biosystems GeneAmp 9700 thermocycler, with 5 μL of cDNA samples and 0.4 mM each primer, in a total volume of 50 μL containing 2.5 U of Accu *Taq* LA DNA polymerase, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 50 mM Tris–HCl, and 15 mM ammonium sulfate (pH 9.3). The PCR mixture was subjected to 35 cycles of amplification, consisting of denaturation for 30 s at 94 °C, annealing for 45 s at 55 °C, and extension for 45 s at 68 °C. RNA extracted from HEp-2 cells infected with CVB4 for 4 h was retrotranscribed and then amplified in the same way for the detection of positive- and negative-strand EV RNA. For all specimens, β-actin mRNA was amplified by using specific primers.

Sn-RT-PCR applied directly on a few cells

The detection of mRNA and viral RNA in a few cells by RT-PCR without extraction has been performed as previously described with modifications [41–43]. Briefly, mock- and CVB4E2-chronically-infected PANC-1 cell cultures, maintained in six-well plates, were washed eight times with cold PBS. The last washing fluid was harvested and tested for detection of viral RNA by RT-PCR (see RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Cells were then detached with trypsin–EDTA 5 min at 37 °C, recovered in 2 ml per well of DMEM culture medium in 15 mL centrifugation tube. After 5 min of centrifugation at 400 g at 4 °C, supernatants were removed and pellets dissociated in 1 mL PBS. Cell suspensions were then counted with Trypan blue and diluted to give approximately 10^5 cells/mL. Samples were then serially two-fold diluted in PBS on 96-well microtiter plates, to obtain approximately one to ten cells in the last wells. Plates were then centrifuged at 1,600 g for 10 min, incubated at 65 °C for 20 min, and immediately frozen at −80 °C. To avoid viral RNA destruction by cell enzymes, an RNase inhibitor (Roche) was added immediately after thawing and samples were directly used for RT-PCR without prior extraction. cDNA synthesis and amplification were performed in a tube by using the SuperScript™ One-Step RT-PCR with Platinum® Taq kit (Invitrogen) according to manufacturer's instructions. The reaction was performed in a total volume of 50 μL containing 25 μL of 2× Buffer Reaction Mix, 1 μM of each primer (EV1 and EV2), 2.5 U of RT enzyme/Platinum Taq polymerase Mix. GAPDH primers were also included in the mix to ensure the presence of cells in each sample. Samples were subjected to a first step of reverse transcription for 30 min at 50 °C, followed by 5 min of denaturation at 94 °C, followed by 35 cycles consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and extension for 1 min at 72 °C, followed by a final extension step for 7 min at 72 °C. RT-PCR products were then directly submitted to a semi-nested (Sn)-PCR by using the JumpStart AccuTaq LA DNA Polymerase Mix (Sigma) according to manufacturer's instructions. The reaction was carried out with 5 μL of amplified DNA samples and 1 μM each of primers (EV1 and EV3), in a total volume of 50 μL containing 1 U of JumpStart Accu Taq LA DNA polymerase, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 5 mM Tris–HCl, 15 mM ammonium sulfate (pH 9.3), and 1 % tween 20. Samples were subjected to 3 min of denaturation at 94 °C, followed by 35 cycles consisting of denaturation for 30 s at 94 °C, annealing for 30 s at 52 °C, and extension for 30 s at 72 °C, followed by a final extension step for 7 min at 72 °C. All reactions were performed by using a preheated Perkin Elmer Applied GeneAmp PCR System 2400.

Immunofluorescence

PANC-1 cells

After washing with PBS, PANC-1 cells were fixed with fresh 4 % paraformaldehyde and permeabilized with chilled methanol/acetone. The blocking of nonspecific and avidin–biotin sites was respectively carried out with rabbit serum/anti-Fc receptor solution (Miltenyibiotec®) and avidin/biotin solution (Vector®). Cells were first labeled with primary antibodies, mouse anti-enterovirus VP1 antibody (clone 5D8/1) or mouse anti-CK19 antibody (clone BA17) (Dako®), and then after washing, they were labeled with goat anti-mouse TRITC antibody (Sigma®); for VP1, and with rabbit anti-mouse Alexa Fluor 488 (Molecular

Probes®) for CK19. Cell nuclei were revealed by Hoesch dye solution (Sigma®). Fluoroprobes were visualized by using a Zeiss LSM 710 confocal laser-scanning microscope equipped with argon and helium–neon lasers.

Ductal cells

Mock-infected and CVB4E2-infected ductal cells were carefully washed in PBS. The slides were air dried and fixed in a solution of 4 % paraformaldehyde (Sigma) for 20 min at 4 °C. The slides were washed twice in PBS and permeabilized with cold acetone–methanol (1 vol/2 vol) for 10 min at 220 °C. Free aldehyde groups were reduced by immersing the slides in solution containing 50 mM NH_4Cl (Merck) and 20 mM glycine (Sigma) for 30 min at room temperature. Unspecific protein binding sites were blocked by incubating the slides for 30 min at room temperature in PBS supplemented with 10 % mouse serum, 10 % rabbit serum (Sigma) and 10 % FCS. After rinsing in PBS, cells were stained with primary antibodies, mouse anti-enterovirus VP1 antibody (clone 5D8/1) or mouse anti-CK19 antibody (clone BA17) (Dako®) for 1 h at room temperature. Following three washes in PBS, incubation with goat anti-mouse Alexa Fluor 546 antibody (VP1) (Invitrogen, France) or Alexa Fluor 488-conjugated rabbit anti-mouse IgG (CK19) (Invitrogen, France) second antibody was performed for 1 h at RT. For double staining, mouse anti-Enterovirus VP1 antibody (clone 5D8/1) and goat anti-mouse Alexa Fluor 546 were used; and polyclonal rabbit anti-CK19 (Invitrogen, France) and goat anti-rabbit Alexa Fluor 488 antibody. The respective antibodies were applied sequentially. The slides were then mounted with Permafluor (Coulter Immunotech), and positive cells were visualized with specific detector filters using a fluorescence microscope (Leitz Diaplan, Wetzlar, Germany).

Statistical analysis

Data are summarized as mean ± standard deviations. The significance of differences was determined by Mann–Whitney *U* test.

Results

CVB4E2 can infect ductal cells in vitro

Human pancreas-derived cell cultures encompassed up to 85 % of ductal cells as shown by IF labelling with a CK19 antibody (see Fig. 1). Human primary ductal cell cultures were inoculated with 0.01 MOI of CVB4E2. The Upti-Blue test enabled the calculation of a viability index which increased for 6 days in mock-infected cultures (4–5 days in virus-infected cultures) ($n = 3$) and afterwards decreased dramatically suggesting that an optimal viability of CVB4-infected primary ductal cells in culture can be maintained for 4–5 days of culture (see Fig. 1A). These cultures consistently allowed replication of CVB4E2. As shown in Fig. 1B, infectivity assays have revealed a steady increase in viral titers, peaking at 6.10^4 TCID50/mL at day 3 p.i. followed by a dramatic decrease, most likely related to the significant cytopathic effect with cell death revealed by Fig. 1A. The viability of CVB4E2-infected ductal cell cultures was lower than the one of mock-infected cells but the difference was not significant ($P > 0.5$, see Fig. 1A).

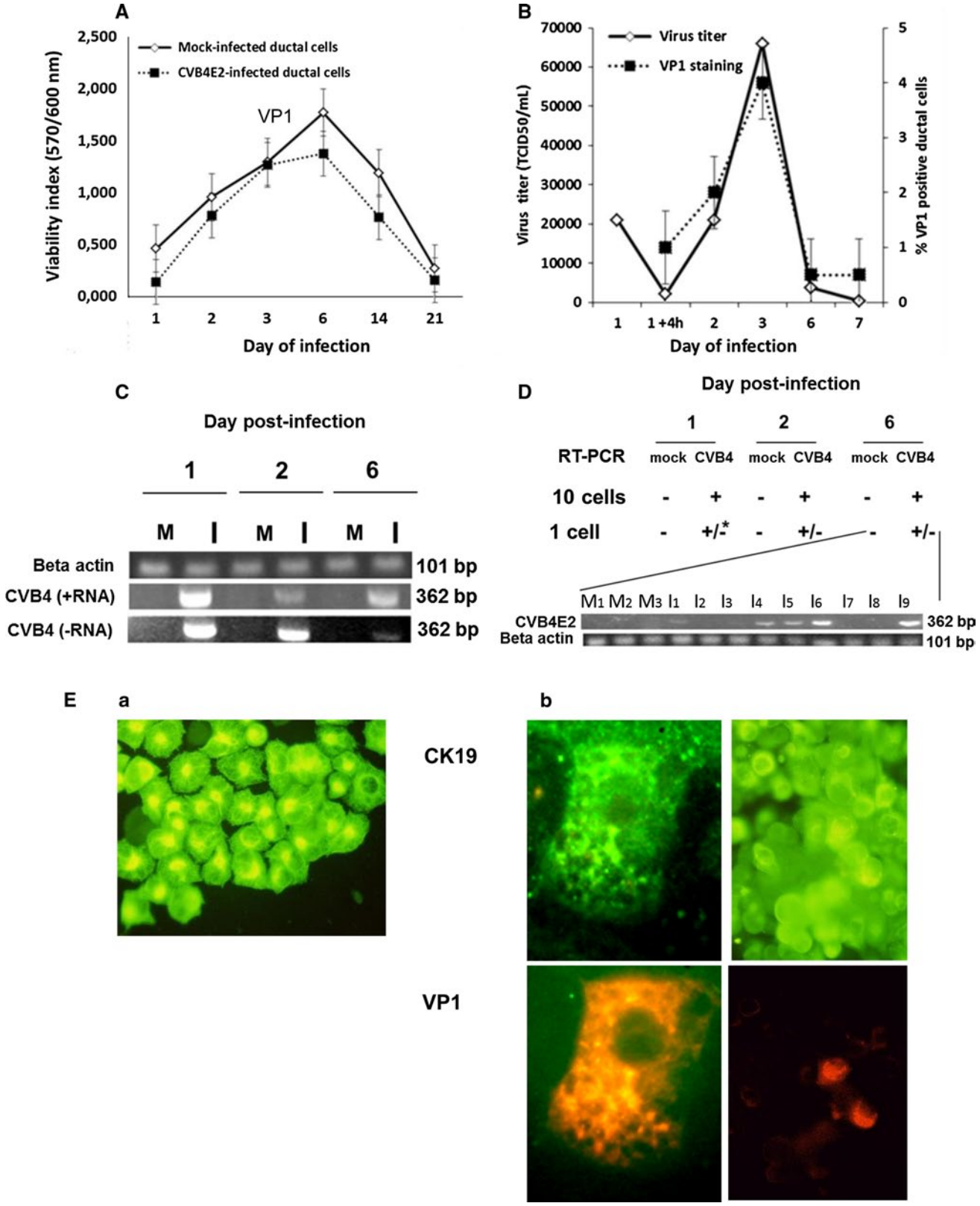
The viral protein VP1 was detected in infected cultures by IFI but the rate of stained cells was low (1.14 ± 0.7 %, $n = 3$), peaking at 4 % on day 3 p.i. (Fig. 1B). Intracellular positive-strand and negative-strand viral RNA was displayed by RT-PCR. (see Fig. 1C).

To investigate the extent of infection in ductal cell cultures in our experiments, cells at day 6 p.i. were thoroughly washed and distributed to obtain around one cell and ten cells per tube. Then the presence of enteroviral RNA in these cells was studied by RT-PCR without RNA extraction as described in the "Materials and methods" section. Thus, viral RNA was detected in all tubes containing around ten cells and in five out of nine of tubes containing around one cell (see Fig. 1D), whereas the supernatant fluid of the final washing was negative for the detection of viral RNA by RT-PCR. To affirm that intracellular viral RNA, but not viral RNA from membrane bound particles, was detected by this method, cells were incubated in presence of virus at +4 °C which allows binding but not infection. Mock- and CVB4E2-incubated ductal-like cells were washed eight times with cold PBS. In this condition, no viral RNA was detected by RT-PCR without extraction (data not shown).

To investigate further whether CVB4E2 can infect ductal cells in primary cultures, indirect immunofluorescent staining for the enterovirus capsid protein VP1 and CK19 were performed. As shown in Fig. 1E, the VP1 protein was localized in CK19 positive cells, indicating that CVB4E2 can replicate in primary ductal cells. There was no VP1 staining in CK19 negative cells. These data indicate that CVB4 can infect ductal cells and that around 50 % of cells can harbor CVB4E2 RNA in human pancreas-derived cell cultures encompassing a majority of ductal cells (80–85 %). Insofar as the viability of virus-infected human pancreas-derived cell cultures decreased dramatically as soon as day 4–5 p.i, we decided to use a ductal-like cell line, PANC-1 as a model, in the rest of our studies.

Persistent infection of PANC-1 cells with CVB4E2

PANC-1 cell cultures were inoculated with CVB4E2 (0.01 MOI). Culture medium was renewed every 3 days, and the



culture was followed-up for 15 days. There was no difference between mock-infected and virus-infected cultures up to 6 days p.i when they were observed under an inverted microscope. The pattern of results was similar during the first 6 days when the viability of mock-infected and virus-infected cultures was assessed by UptiBlue. Beyond day

Fig. 1 Infection of primary human ductal cells with CVB4E2. **A** Effect of CVB4E2 on primary human ductal cells viability assessed by using Uptiblue® viable cell reagent. The results are expressed as a viability index (ratio between the absorbance at a wavelength of 570 and 600 nm) measured 4 h after reagent application at 37 °C to CVB4E2- and mock-infected cultures (results are expressed as mean ± SD. $n = 3$). **B** Release of infectious particles by ductal cell cultures and proportion of infected cells. The viral titers in culture supernatants at various times p.i. were determined by the TCID50 assay with Hep-2 cell cultures. Results from one representative experiment out of three are shown. The percentage of CVB4E2-infected cells was determined by detection of viral protein VP1 by indirect immunofluorescent staining (results are expressed as mean ± SD). **C** Representative agarose gel electrophoresis of amplicons specific to positive and negative strands of enterovirus (EV) genome, and amplicons specific to beta actin mRNA. RT-PCR was carried out on total RNA extracted from mock- (M) ($n = 3$) and CVB4-infected (I) ($n = 9$) cells at day 1, day 2 and day 6 p.i.. One representative experiment out of three is shown. **D** RT-PCR without RNA extraction. Mock- and CVB4E2-infected human primary ductal cells were harvested and extensively washed at 1, 2 and 6 days post-infection and distributed to obtain around 10 (8–11) and 1 (1–3) cells per tube. Specific RT-PCR for the positive strand of EV genome, and for beta actin, was applied without RNA extraction. The electrophoresis in agarose gel of specific amplicons from 12 samples, 3 from mock (M) and 9 from infected cultures (I) containing around 1 cell per tube harvested on day 6 p.i. is presented. Results from one representative experiment out of three are shown. **E** Indirect immunofluorescent staining. (a) Ductal cell culture stained for CK19 with mouse anti-CK19 antibody and goat anti-mouse Alexa Fluor 546 antibody (original magnification, ×40). (b) CVB4-E2 infected ductal cell culture stained for VP1 and CK19. For VP1 with goat anti-mouse Alexa Fluor 546-conjugated antibody and for CK19 with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody. The staining was performed on day 4 post-infection with CVB4-E2. The respective antibodies were applied sequentially for double staining and positive cells were visualized with specific detector filters using a fluorescence microscope (Leitz Diaplan microscope, original magnification, ×100 and ×20)

6 p.i., cell viability was lower in CVB4E2-infected cells than in mock-infected cells. Thereafter, infected cell layers began to pull away, with many dead cells in culture supernatant from day 6 up to day 9 p.i. At day 14 p.i., adherent infected cells were rare and Trypan blue exclusion assay has revealed that only 3 % of cells were still alive in cultures inoculated with CVB4E2 whereas 68 % of cells were alive in mock-infected cultures (data not shown).

In order to study further the infection of PANC-1 cell cultures, the cells that remained alive after the acute phase of infection on day 7 were scraped and harvested and the culture was continued. Infectious particles were released in culture supernatant fluids of CVB4E2-infected PANC-1 cells, as evidenced by titration on Hep-2 cells (Fig. 2a). High titers of virus were found in the supernatant fluids, peaking between day 5 and 10 p.i. Then, viral titers were slightly lower but remained at relatively constant levels with moderate fluctuations until the end of the follow up. A viral progeny up to 2.5×10^5 TCID50/ml was still detectable on day 260 p.i. Virus titration of supernatant fluids of mock-infected PANC-1 cell cultures was also carried out and no viral particle was detected in these samples (data not shown).

Every week during the first 8 weeks, and thereafter occasionally up to the end of the follow-up (260 days), supernatant fluid and cells were harvested and RNA was extracted to investigate the presence of enteroviral RNA by RT-PCR. CVB4E2 RNA was detected in each sample of supernatant fluid and positive- and negative-strand CVB4E2 RNA was detected in each sample of RNA extracted from cells, starting from day 1 p.i. up to the end of the follow-up (day 260 p.i.). CVB4E2 positive- or negative-strand RNA was not detected in mock-infected cells (data not shown).

When cultures inoculated with CVB4E2 were scraped and tested for RT-PCR without extraction, the proportion of cells harboring intracellular viral RNA appeared to be high. Indeed, for example, on day 30 p.i. the detection of viral RNA in 7 out of 13 tubes containing around 1 cell per tube (actual number 1–3 cells) was positive (Fig. 2b). In contrast the proportion of CVB4E2-infected cells with a positive detection of VP1 by IFI was low, ranging from 1 to 5 % (Fig. 2c).

The pattern of virus persistence has been investigated. Chronically infected cells (day 33 p.i.) were scraped, subcultured in fresh medium and treated with rabbit anti-CVB4E2 neutralizing anti-serum. After 2 days of monitoring, the culture medium was renewed and complemented by rabbit anti-CVB4E2 antiserum. The above procedure was repeated after 1 week of monitoring and so on. At each scraping, cells and culture supernatant fluids were collected for viral RNA detection by RT-PCR. After 4 months (16 passages), viral RNA was still detected in infected cells treated with control rabbit anti-serum, but not in infected cells treated with anti-CVB4E2 rabbit anti-serum (data not shown). Together these data are in favor of a carrier state persistent infection characterized by a productive infection of a part of cells in the culture.

CVB4E2 can disturb the expression of Pdx1 mRNA by PANC-1 cells and their differentiation into islet-like cell aggregate (ICA)

Pdx1 and CK19 mRNA were detected in primary ductal cells and in the PANC-1 cell line as well. Insofar as a persistent infection was obtained in ductal-like cells, it was decided to investigate the result of the infection with CVB4E2 onto the expression of cellular factors in this model. Messenger RNAs for IGF2, MxA and CK19 were found in cells by RT-PCR throughout the follow-up of mock- and CVB4-infected cells (data not shown). In contrast, Pdx1 mRNA was no more detected after day 36 p.i. (see Fig. 3a).

Mock-infected PANC-1 cell cultures were exposed to trypsin and cultured in serum-free medium (SFM) which

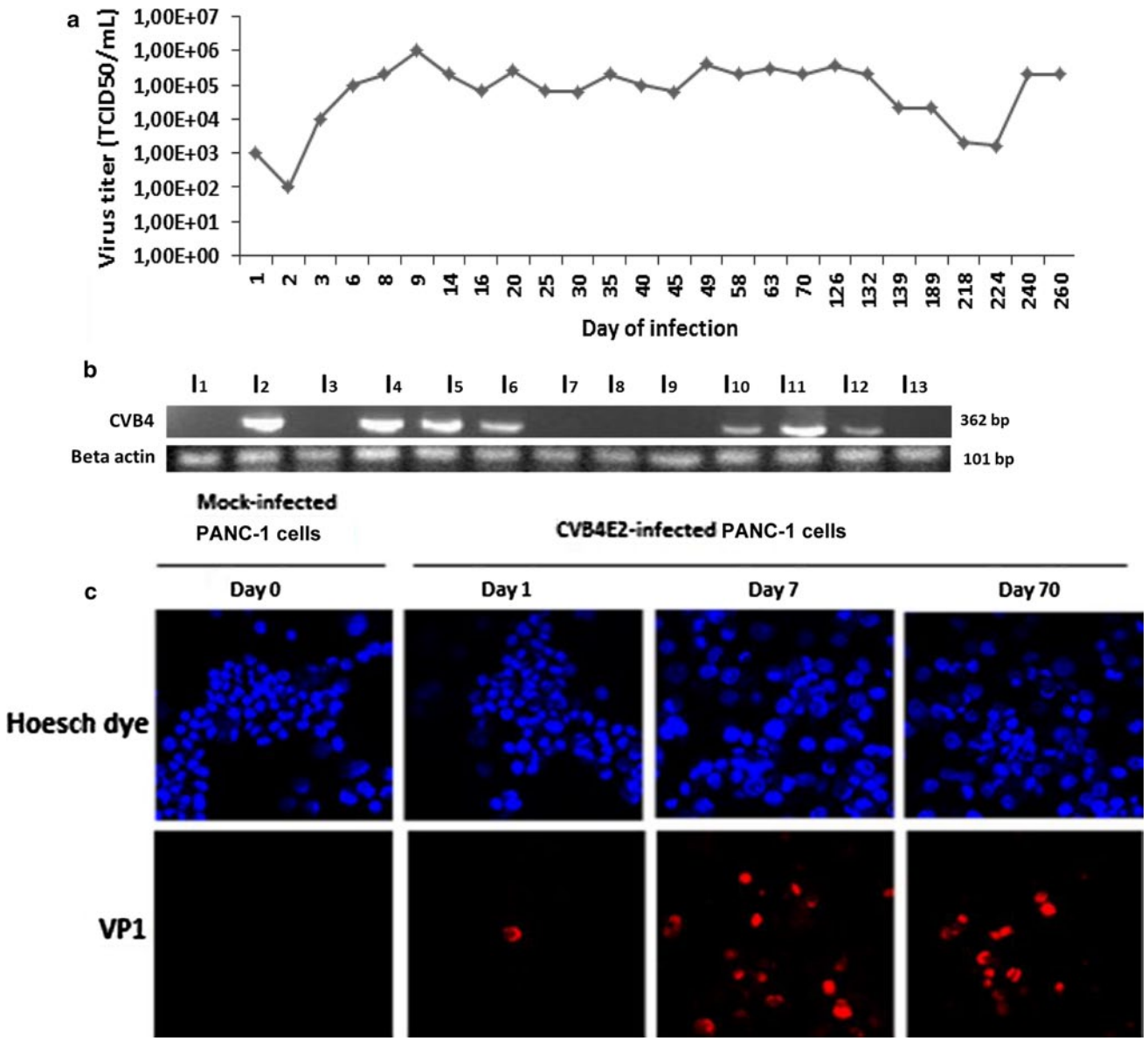


Fig. 2 Persistent infection of human ductal-like cells with CVB4E2 human ductal-like cell cultures were inoculated with CVB4E2, after the acute phase of infection, on day 7, cells were scraped and subcultured in fresh growth medium once a week and the culture was continued. **a** Titration of viral progenies. Supernatant fluids of CVB4E2-infected ductal-like cell cultures were harvested every 3 days of subculture. The results expressed as TCID50/ml are representative of one experiment out of three. **b** RT-PCR without RNA extraction. CVB4E2- infected human ductal-like cells were harvested on day 30 and extensively washed and distributed to obtain around 1 cell (1–3) per tube. Specific RT-PCR for the positive strand of EV genome, and for beta actin, was applied without RNA extraction. The electrophoresis in agarose gel of specific amplicons from 13 samples is presented. The results of one representative experiment out of three are shown. **c** Detection of enterovirus capsid protein VP1 by IF staining. Mock- and CVB4E2-infected ductal-like cells were tested for the presence of VP1 by IF staining at various times p.i. (TRITC-conjugated antibodies). Cell nucleuses were stained with Hoechst 33342. Fluoroprobes were visualized by using a Zeiss LSM 710 confocal laser-scanning microscope (×40). The results of one representative experiment out of three are shown

resulted, between day 2 and day 4, in reorganization of cell monolayers in ICAs formed with 3D clusters of rounded cells (see Fig. 3b). In CVB4E2-infected-PANC-1 cell cultures treated on day 40 p.i. to induce a reorganization of cell monolayers, a few structures looking like ICAs were obtained. They appeared as a disturbed flat structure with every cell positive for trypan blue dye on day 6. In contrast, larger three-dimensional structures appeared and only a few peripheral trypan blue-positive cells were observed in mock-infected PANC-1 cell cultures on day 6 (see Fig. 3b).

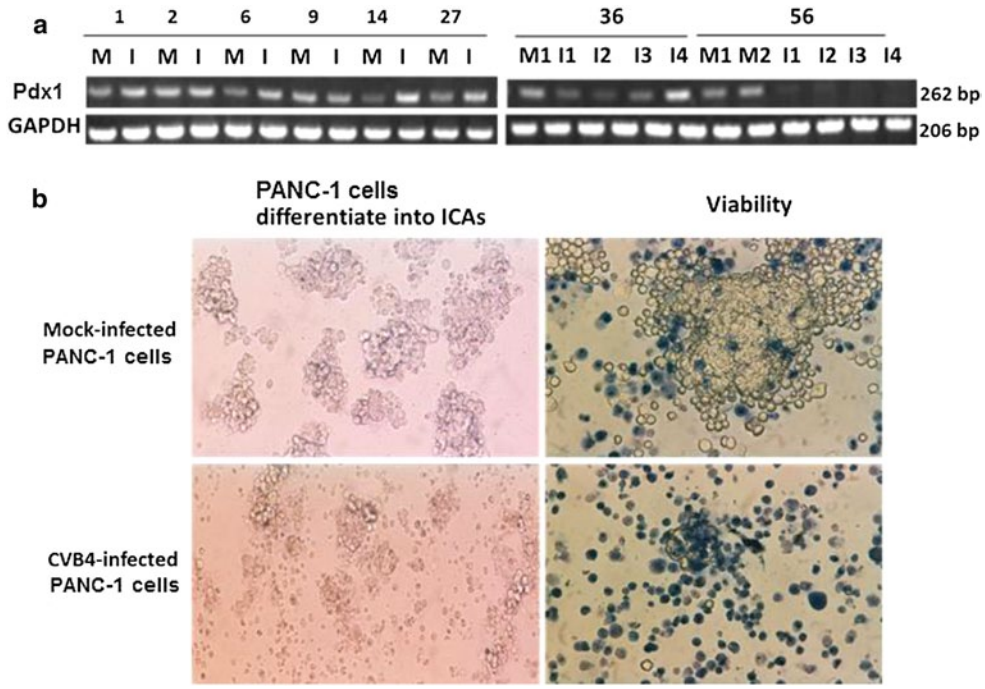


Fig. 3 Effects of CVB4E2 on ductal-like cell cultures. **a** Expression of Pdx1 mRNA. Agarose gel electrophoresis of amplicons specific to the Pdx1 (*top*) and beta actin (*bottom*) transcripts. RT-PCR carried out on total RNA taken from mock- (M) and CVB4E2-(I) infected cultures from 1 through 56 days p.i. are presented. One representative experiment out of three is shown. **b** Differentiation into islet-like cell aggregates (ICAs) mock-and CVB4E2-infected ductal like cell cultures were tested to differentiate into structures like ICAs. At day 40 p.i., cells were briefly exposed to trypsin and then cultured in serum-free medium (SFM) at 37 °C. Structures like ICAs observed under an inverted microscope were obtained between day 2 and day 4 after exposure to SFM (×10). The viability of these structures was evaluated by using Trypan blue dye exclusion test. (×40). One representative experiment out of three is shown

In further experiments, mock-infected PANC-1 cell cultures were exposed to serum free medium to induce a transformation into ICAs and then, 4 days later, they were infected with CVB4E2. In these conditions, dissociated structures were obtained on day 2 p.i. and trypan blue exclusion test displayed that almost all the cells were dead 2 days later (data not shown).

Discussion

Several studies have previously revealed that adult pancreatic ductal cells could differentiate into endocrine cells [18, 22, 24]. Pancreatic ductal cells could therefore be considered as a pool of precursor cells involved in pancreatic β-cell turnover [19, 20]. The aim of this study was to investigate the infection of human ductal cells with CVB4E2 in vitro and its consequences in the differentiation process. It is noteworthy that primary cultures of human ductal cells can be productively infected by the diabetogenic strain CVB4E2. However, assessing in primary cells the persistence of the virus and the impact onto the cells, especially onto the differentiation process, was hindered by a technical limitation. Indeed, the viability of CVB4E2-infected primary ductal cells cultures dramatically decreased as soon as day 4–day 5 p.i., that is a period shorter than the optimal duration (6 days) needed to obtain clusters of differentiated cells. This technical limitation prompted us to use a ductal-like cell line as a model for investigating the persistence of CVB4E2 and its effects.

Like primary cultures of ductal cells, human PANC-1 cell cultures were productively infected with CVB4E2. The infection was characterized by an acute phase during the first week of monitoring. Thereafter, the detection of viral capsid protein VP1 and intracellular positive- and negative-strand CVB4E2 RNA in cellular extracts and CVB4E2 RNA in supernatant fluids during the 37 weeks of monitoring have shown that CVB4E2 could persistently infect ductal-like cells.

Following the initial production of CVB4E2, virus synthesis and liberation into the cell culture medium remained moderate and stable during the follow-up. Persistent infection with CVB4 in vitro in human systems has been previously reported: pancreatic islets [44], thymic epithelial cells [45] and K1 cell line derived from a thyroid carcinoma [46]. In these studies and in the present one a carrier state persistent infection with CVB4E2 was characterized. A carrier state-like infection has been proposed in other

studies to explain the persistence of CVB in human lymphoid cells and in myocardial fibroblasts [47–49].

The RT-PCR without extraction is a method that enables the detection of cellular mRNA and viral RNA in a single cell as previously described [41–43]. This method applied to a few cells has shown that the proportion of cells harboring viral RNA was higher than predicted by the level of VP1-positive cells in our systems. Thanks to this method it was displayed that viral RNA was present in primary ductal cells in our experiments since around 50 % of cells were viral RNA positive in culture encompassing 80–85 % ductal cells. Furthermore, immunofluorescence staining for both VP1 and CK19 displayed that CVB4 could replicate in ductal cells.

Our in vitro studies confirm that pancreatic ductal cells can harbor enteroviral RNA, which is in agreement with the previously reported detection of enteroviral RNA by in situ hybridization in pancreatic ductal cells from patients with type 1 diabetes [15]. The persistence of an enterovirus in PANC-1 cells in our experiments suggest that a persistency/latency of enteroviral RNA in such a cell type in vivo can not be ruled out, which may contribute to the pathogenesis of virus-induced or aggravated T1D.

Our study raises the issue of the persistence of enteroviral RNA in pancreas ductal cells in vivo. The persistence of enteroviral RNA in various tissues or organs in vivo has been already reported [50, 51]. The presence of enteroviral RNA associated with an inflammatory process in successive intestine samples from patients with T1D has been recently reported which is in favor of a prolonged or persistent enterovirus infection in this organ in such individuals [4]. The model described here may be used to investigate whether dsRNA, deletion of 5′ terminal genomic sequences during replication, or cumulative mutations in 5′NTR and 2A regions of the enterovirus genome can play a role in the persistence of CVB4 in pancreas ductal-like cells, as described in other models [52–54].

At the morphological level, in our experiments, mock-infected cells-derived ICAs were similar to those obtained by Hardikar et al. [27]. ICAs from PANC-1 cells persistently infected with CVB4 were, less compact, with fewer viable cells than those of mock-infected cells. Thus a persistent infection resulted in a deterioration of formation, morphological stability and viability of ICAs. Furthermore, when ICAs from mock-infected cells were infected with CVB4E2, structures with high rate of dead cells were observed as soon as day 2 p.i. These results suggest that the differentiation process of ductal cells in endocrine cells, mimicked in the present study by the process leading to ICAs formation can be a critical event, resulting in disorganized structures and cell death in case of CVB4E2 infection.

In ductal-like cells that have been inoculated with CVB4E2, Pdx1 mRNA was not detected by RT-PCR after 5 weeks of culture and up to the end of the follow-up (32 weeks), in contrast with other cellular factors (IGF2, MxA and CK19). The negative detection of Pdx1 mRNA in our system was not the result of the selection of Pdx1 negative cells during the acute phase of the infection, otherwise Pdx1 mRNA would have been undetectable following the acute phase as soon as 9 days post-infection.

The evaluation of the level of Pdx1 protein in CVB4E2-infected and in mock-infected PANC-1 cell cultures was hampered because it was under the limit of detection of western-blot analysis in our experiments (data not shown), which was in agreement with the low proportion of PANC-1 cells (<1 %) expressing that protein, as previously described [55].

This is the first report of a virus-induced modulation of Pdx1 expression in PANC-1 cell culture. The mechanism of the impaired expression of Pdx1 in CVB4E2 infected ductal-like cell cultures remains to be investigated. Whether the down-regulation of Pdx1 can play a role in the disturbed process leading to ICAs formation in CVB4E2-infected PANC-1 cell cultures deserves further studies.

Pdx1 characterizes the tissue of which the differentiation leads to the development of the pancreas during organogenesis [56, 57]. Endocrine pancreatic cells can be obtained from murine and human ductal cell (primary cells and continuous cell line) [18, 27, 58].

Our data reveal that CVB4E2 can infect human primary ductal cells and can persistently infect ductal-like cells, which results in a modified phenotype of cultures as shown by a disturbance of ICAs clusters formation on one hand and by inhibition of Pdx1 expression on the other hand. Therefore, we assume that a persistent CVB4E2 infection of pancreatic ductal cells may disturb their differentiation process into endocrine cells. Such an effect of CVB4E2 can be involved in the pathophysiology of the infection, since it was previously shown that a lack or a profound disturbance of islets neogenesis played a key role in β-cell depletion in mice infected by CVB4E2 [32]. A coxsackievirus B–induced disturbance of cell differentiation has been recently reported in another system; indeed, the infection of mice neural progenitors and stem cells with CVB3 in vitro resulted in an altered differentiation of these cells [59].

RT-PCR applied to a small number of cells (around 1 and 10 cells) has shown that the proportion of ductal-like cells harboring CVB4E2 RNA was higher than it had been predicted by the relatively low number of VP1-positive cells. The high rate of viral RNA containing cells is consistent with the extent of the morphological and molecular consequences of the persistent infection onto the PANC-1 cell culture. The proportion of cells with replicative RNA remains an open question. Given the time to eliminate intracellular viral RNA from ductal-like cell cultures with neutralizing antibodies (4 month, i.e., 16 passages), it is

likely that a large proportion of cells harbor latent viral RNA which can be activated in some of them to produce virions resulting in cytolysis. Further experiments are needed to investigate this hypothesis.

Type 1 diabetes is supposed to be the result of the destruction or the functional impairment of pancreatic β-cells [60]. Several studies have revealed that enteroviral infections, especially those due to CVB, are associated with the disease [8]. Whether enteroviruses can initiate or accelerate the disease is a subject of controversy. Various mechanisms can play a role in the enterovirus-induced or enterovirus-accelerated development of type 1 diabetes [7, 9, 61]. The current study demonstrates that human pancreas primary ductal cells can be infected with CVB4 in vitro, which is in agreement with the presence of enteroviral RNA in human ductal cells in vivo observed by Ylipaasto et al. [15] and suggest that a persistent infection of these cells resulting in an impaired differentiation into endocrine cells can not be excluded. Moreover it can not be discarded that the pool of endocrine cells can be reduced when ductal cells that are in the process of differentiation are exposed to the cytolytic effect of enteroviruses. Furthermore ductal cells could be sites of persistence for enteroviruses, which would enable these viruses to spread to surrounding β-cells.

Our data support the hypothesis of an impact of an enterovirus onto pancreatic ductal cells which are involved in the renewal of pancreatic β-cells. Whether the infection of human pancreas ductal cells with enteroviruses and the resulting damaging effects could play a role in vivo in the pathogenesis of type 1 diabetes deserves further investigations.

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