Effect of herpesvirus infection on pancreatic duct cell secretion

Péter Hegyi, Balázs Ördög, Zoltán Rakonczai Jr, Tamás Takács, János Lonovics, Annamária Szabolcs, Réka Sári, András Tóth, Julius G Papp, András Varró, Mária K Kovács, Mike A Gray, Barry E Argent, Zsolt Boldogköi

Abstract

AIM: To examine the effect of acute infection caused by herpesvirus (pseudorabies virus, PRV) on pancreatic ductal secretion.

METHODS: The virulent Ba-DupGreen (BDG) and non-virulent Ka-RReP0lacgfp (KEG) genetically modified strains of PRV were used in this study and both of them contain the gene for green fluorescent protein (GFP). Small intra/interlobular ducts were infected with BDG virus (10⁷ PFU/mL) or with KEG virus (10⁷ PFU/mL for 6 h), while the ducts were then cultured for a further 18 h. All the experiments were performed in HCO₃⁻-buffered Ringer solution at 37 °C. The BDG strain of PRV, which is able to initiate a lytic viral cycle, stimulates HCO₃⁻-secretion in guinea pig pancreatic duct by about four- to fivefold, 24 h after the infection. As we found with the inhibitor stop method, no change was observed in the group KEG compared to the non-infected group.

CONCLUSION: Incubation with the BDG or KEG strains of PRV results in an effective infection of ductal epithelial cells. The BDG strain of PRV, which is able to initiate a lytic viral cycle, stimulates HCO₃⁻ secretion in guinea pig pancreatic duct by about four- to fivefold, 24 h after the infection. However, the KEG strain of PRV, which can infect, but fails to replicate, has no effect on HCO₃⁻ secretion. We suggest that this response of pancreatic ducts to virulent PRV infection may represent a defense mechanism against invasive pathogens to avoid pancreatic injury.

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Key words: Pancreas; Hypersecretion; Ductal cells; Infection; Pseudorabies virus

INTRODUCTION

Pseudorabies virus (PRV) is an α-herpesvirus closely related to the herpes simplex virus (HSV), a well-known human pathogen[4,5]. The natural host of PRV is the pig, but it has a very wide host range including several mammalian families, such as carnivores, ungulates and rodents, but humans are resistant to PRV infection[11]. PRV infection is a multistep process initiated by the receptor-mediated attachment of the virus to the cell surface. Upon entering the cells, the PRV nucleocapsid approaches the nuclear membrane and the viral DNA is released into the nuclei by a poorly understood mechanism. The lytic cycle of the virus is controlled by a transcriptional cascade mechanism[5].

Acute pancreatitis is most commonly associated with
biliary stone and alcoholism. Other causes, including infections, drugs, lipid abnormalities, congenital anomalies, trauma, tumors, and idiopathic form, account for at least 10-20% of the total number of cases[8]. The incidence of pancreatitis may even be higher since the diagnosis of acute pancreatitis may go undetected in many patients. Several viruses have been reported to cause acute pancreatitis, including coxsackievirus B3[9] and B4[10], murmuspivirus[11], varicella[12], hepatitis A[13] and B[14], cytomegalovirus[15], varicella-zoster virus[16] and HSV[17].

We have previously shown that hypersecretion can be observed during the early phase of experimental edematous and necrotizing pancreatitis[18]. However, the source and the role of this hypersecretion are unknown. Either acinar cells and/or the ductal epithelium could be responsible for hypersecretion.

Our aim in this study was to examine the effect of acute infection caused by PRV on pancreatic ductal secretion.

MATERIALS AND METHODS

Virus strain

Ba-Dup Green (BDG), a genetically modified replicating strain of PRV, was used in this study. The Bartha virus[14], which is an attenuated live vaccine strain of PRV, was used as the parental virus for the generation of BDG. Two copies of a genetically modified green fluorescent protein (GFP) gene-containing expression cassettes were inserted into the PRV antisense promoter region located in the inverted repeat gene-containing expression cassettes were inserted into the virus[15,16]. Ka-EP0lacgfp (KEG) strain of PRV antisense promoter region located in the inverted repeat segment of the virus[15,16]. Ka-EP0lacgfp (KEG) strain of PRV was used as a non-replicating control virus for these experiments. KEG was constructed by deleting the small subunit of ribonucleotide reductase[17] and the early protein EP0 gene[18]. An expression cassette containing a GFP and a lacZ gene was inserted in place of the EP0 gene[19]. These mutations render the virus incapable of replication in non-dividing cells[20].

Isolation and infection of pancreatic ducts

Small intra/interlobular ducts were isolated from the pancreas of guinea pigs weighing 150-250 g. The guinea pig was humanely killed by cervical dislocation, the pancreas was removed and intra/interlobular ducts were isolated by enzymatic dissociation, microdissection and then cultured at 37 °C in 50 mL/L CO2[21]. Ducts were incubated in McCoy's-based culture medium containing BDG viruses at a dose of 10^7 PFU/mL for 6 h or KEG virus (10^10 PFU/mL for 6 h), while the non-infected ducts were exposed to culture media only. The ducts were then cultured for a further 18 h as described earlier[22], during which time the ducts seal to form a closed sac that swells due to accumulation of ions and water secretions within the duct lumen[23].

Inhibitor stop method

Exposing the ducts to dihydro-4, 4,diisothiocyanostilbene-2,2'-disulfonic acid (H2DIDS, 0.5 mmol/L) and amiloride (0.2 mmol/L) for 5 min caused a marked acidification of pH (Figure 2A). This acidification occurred due to inhibition of the basolateral Na+/HCO3- co-transporters and Na+/H+ exchangers, which normally

Thereafter, a rabbit polyclonal antiviral antibody (Affinity Bioreagents, Golden, USA) diluted at 1:500 (v/v) in blocking solution was applied for 24 h at 4 °C. After being washed with PBS, the ducts were incubated with an anti-rabbit antibody conjugated with a red light-emitting dye (Alexa Fluor® 633; Molecular probes, USA) diluted at 1:1 000 (v/v) in a blocking solution for 24 h at 4 °C. After further washing in PBS, the ducts were mounted on slides using Aqua-Poly/Mount (Polysciences Inc., Niles, USA). The specificity of immunohistochemical procedure was assessed by simultaneous staining of non-infected ducts. No immunohistochemical labeling was observed in control experiments.

Microscopy

For monitoring virally expressed GFP and immunofluorescence signals, fluorescent images and optical sections of the infected ducts were recorded by a confocal laser scanning microscope (Zeiss, LSM 400).

Measurement of intracellular pH

Cultured ducts were attached, using Cell-Tak, to a coverslip (24 mmol/L) forming the base of a perfusion chamber mounted on a Nikon Diaphot microscope (Nikon UK Ltd, Budapest, Hungary). The ducts were bathed in the standard Hepsolution at 37 °C and loaded with the pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5- (and-6)-carboxyfluorescein (BCEF) by exposure to 2 μmol/L BCECF-AM for 20-30 min. After loading, the ducts were continuously perfused with solutions at a rate of 4.5 mL/min. Intracellular pH (pHi) was measured using a microspectrofluorimeter system (Cairn, Kent, UK). A small area of 5-10 cells was excited with light at wavelengths of 490 and 440 nm, and the 490/440 fluorescence emission ratio was measured at 535 nm. Four pH measurements were obtained per second. In situ calibration of the fluorescence signal was performed using the high K+-nigericin technique[24,25]. During calibration, the ducts were bathed in high K+ HEPES solution and extracellular pH stepped between 5.95 and 8.46.

Determination of base efflux

The intrinsic buffering capacity (βi) of duct cells was estimated according to the NH4+ pre-pulse technique[26,27]. β refers to the ability of intrinsic cellular components (excluding HCO3-/CO2) to buffer changes of pH. Briefly, pancreatic duct cells were exposed to various concentrations of NH4Cl, while Na+ and HCO3- were omitted from the solution in order to block the Na+-dependent pH regulatory mechanisms. βi was estimated by the Henderson-Hasselbach equation. The total buffering capacity (βtotal) was calculated from: βtotal = β[10HCO3-] = β + [HCO3-]×[HCO3-]H2O, where [HCO3-] is the buffering capacity of the HCO3-/CO2 system and [HCO3-] is the intracellular HCO3- concentration[28].

Measurement of HCO3- efflux

Inhibitor stop method

Exposing the ducts to dihydro-4, 4,diisothiocyanostilbene-2,2'-disulfonic acid (H2DIDS, 0.5 mmol/L) and amiloride (0.2 mmol/L) for 5 min caused a marked acidification of pH (Figure 2A). This acidification occurred due to inhibition of the basolateral Na+/HCO3- co-transporters and Na+/H+ exchangers, which normally
act to transport HCO₃⁻ into the duct cell from the blood[23,24]. The rate of pHₐₐcidification after the exposure to H₂DIDS and amiloride could reflect the intracellular buffering capacity and the rate at which HCO₃⁻ effluxes (i.e., secreted) across the apical membrane via Cl-/HCO₃⁻ exchangers and CFTR channels[25,26].

The initial rate of intracellular acidification (dpH/dt), over the first 60 s of exposure to amiloride and H₂DIDS, was calculated by linear regression analysis using 240 data points (four pH measurements per second)[24].

**Alkali load method** Exposing the ducts to 20 mol/L NH₄Cl caused an alkalization of pH due to the rapid influx of NH₃ into cells (Figure 3A). Recently, we demonstrated that the recovery of pHᵢ under these conditions was dependent on the presence of HCO₃⁻ in the bathing solution, suggesting that it results from HCO₃⁻ efflux (i.e., secretion) out of the duct cells[25,26]. In the present study, the initial rate of recovery from alkalosis (dpH/dt) over the first 30 s (120 pHᵢ measurements) in the continued presence of NH₄Cl was calculated as described previously[26].

The rates of pHᵢ change measured in these inhibitors stopped and alkali load experiments were converted to transmembrane base flux J(B⁻) using the equation: J(B⁻)= dpH/dtβₑₓₑₐₑ. We denoted base influx as J(B⁻) and base efflux (secretion) as -J(B⁻).

**Solutions and chemicals**

The standard Hepes-buffered solution contained (mmol/L) 130 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 D-glucose, and 10 Na-Hepes. The high K⁺ Hepes-buffered solution contained (mmol/L) 130 KCl, 5 NaCl, 1 CaCl₂, 1 MgCl₂, 10 D-glucose, and 10 Na-Hepes. Hepes-buffered solutions were gassed with 100% O₂ and their pH was set to 7.4 at 37 °C with HCl. The standard HCO₃⁻-buffered solution contained (mmol/L) 115 NaCl, 25 NaHCO₃, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 D-glucose. The ammonia pulse solution contained (mmol/L) 95 NaCl, 20 NH₄Cl, 25 NaHCO₃, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 D-glucose. HCO₃⁻-buffered solutions were gassed with 95% O₂/50 mL/L CO₂ to set pH to 7.4 at 37 °C.

Chromatographically pure collagenase was obtained from Worthington (Lakewood, NJ, USA), culture media from Sigma (Budapest, Hungary), Nigericin (Sigma, Budapest, Hungary) was dissolved in absolute ethanol, H₂DIDS (from Molecular Probes, Eugene, OR, USA) and amiloride in dimethyl sulfoxide (DMSO). CellTak was obtained from Becton Dickinson Labware (Bedford, MA, USA). BCECF-AM was obtained from Molecular Probes (Eugene, OR, USA) and was made up as a 2 mmol/L stock solution using DMSO. The 10-fold concentrated PBS stock solution contained (g/L) 80 NaCl, 2 KCl, 2.4 Na₂HPO₄, 14.4 Na₂HPO₄ (pH 7.2). Chemicals were obtained from Merck (Darmstadt, Germany). Blocking solution contained 10 g/L bovine serum albumin from Sigma (Budapest, Hungary) and 1 mL/L scintillation grade Triton X-100 (BDH Chemicals, Poole, UK).

**Statistical analysis**

Results were expressed as mean±SE (n = 5 ducts). Statistical analyses were performed using ANOVA. P<0.05 was accepted as statistically significant.

**RESULTS**

**Efficacy of viral infection**

Two GFP-expressing cassettes were inserted into the PRV genome[28] enabling virus infection to be visualized in both living and fixed cells. Monitoring of GFP expression of BDG was utilized to assess the extent of viral infection of ductal epithelial cells. We observed that BDG virus practically infected all accessible cells of the duct when used at a titer of 10⁷ PFU/mL after 24 h (Figure 1A). We used a higher concentration (10⁸ PFU/mL) of KEG virus and found a similarly high efficiency of infection (Figure 1C).

**Cytotoxic effect of PRV infection**

BDG is a virulent strain; therefore, virus uptake by cells results in the immediate initiation of the lytic viral cycle and the unavoidable death of the infected cells. Productive virus infection was detected in the BDG group by labeling the structural viral proteins by means of immunohistochemistry. The GFP gene was placed under the control of the constitutive human cytomegalovirus immediate early promoter, which conferred a very early expression of the reporter gene. In contrast, viral structural proteins, which are recognized by the anti-PRV antibodies, appeared at a later stage of viral infection[15]; therefore, a shift between the appearance of GFP and immunofluorescence could be observed (Figure 1B). In contrast, in the KEG group, we did not observe any cytopathic effects and could not detect viral antigens. However, GFP expression indicated the presence of the virus within the infected cells (Figure 1C).

**Pancreatic ductal bicarbonate secretion**

Exposing the ducts to 0.5 mmol/L H₂DIDS and 0.2 mmol/L amiloride caused an acidification of pH (Figure 2A), due to inhibition of the basolateral Na⁺/HCO₃⁻ co-transporters and Na⁺/H⁺ exchangers. The effect of H₂DIDS and amiloride was reversible, removal of the inhibitors caused pHᵢ to return to the control value. In these series of experiments, we tested whether virus infection affected the net HCO₃⁻ secretion of pancreatic duct cells. Base efflux was significantly elevated...
in the BDG group compared to the non-infected ducts, 24 h after the infection (7.84±1.24 mmol/L B-/min vs 2.2±0.18 mmol/L B-/min, respectively; n = 5). However, no change was observed in the KEG group (2.4±0.32 mmol/L B-/min; n = 5) compared to the non-infected ducts. These data are summarized in Figure 2B.

Figure 2 Inhibitor stop method for determining bicarbonate secretion. A: Representative pHi traces; B: summary of the results obtained using the inhibitory stop method. *P<0.001 vs the control (ANOVA).

Exposure of duct cells to 20 mmol/L NH₄Cl induced an immediate rise in pH due to the rapid entry of NH₃ into the duct cells (Figure 3A). In this series of experiments, base efflux was significantly elevated in the BDG group compared to the control group 24 h after the infection (160.04±19.16 mmol/L B-/min vs 36.37±1.08 mmol/L B-/min, respectively; n = 5). However, as we found with the inhibitor stop method, no change was observed in the KEG group (39.34±3.49 mmol/L B-/min, n = 5) compared to the non-infected ducts (Figure 3B).

Figure 3 Alkali load method for determining bicarbonate secretion. A: Representative pH traces; B: summary of the results obtained using the alkali load method. *P<0.001 vs the control (ANOVA).

Using the ammonium pulse technique, we also tested whether PRV affected the ability of duct cells to recover from an acid load following removal of NH₄Cl from the superfusate. The transporters most likely to be involved in this process are the Na⁺/HCO₃⁻ co-transporter, the Na⁺/H⁺ exchanger and the H⁺ pump located on the basolateral membrane of the duct cells. No significant change was observed between the PRV infected and non-infected groups (Figure 3A, n = 5).

**DISCUSSION**

Though the broad spectrum of etiological factors is involved in acute pancreatitis, the pathophysiology of the disease is less understood. Most investigators believe that acute pancreatitis results from an early intra-acinar cell activation of zymogens. Following this early activation, a trypsin cascade occurs in the gland leading to the auto-digestion of acinar cells. However, a possible pathophysiological role of the ductal epithelium has not been investigated. The permeability of the pancreatic ductal epithelium to HCO₃⁻ and Cl⁻ is increased by exposure to various bile salts at concentrations within the range normally found in the duodenum. Moreover, *E. coli*-infected bile causes further increases of the permeability of the ductal epithelium to HCO₃⁻ and Cl⁻. Ethanol could induce fluid hypersecretion from guinea pig pancreatic duct cells. Low concentrations of ethanol directly augment pancreatic ductal fluid secretion stimulated by physiological and pharmacological concentrations of secretin (cAMP pathway) and via Ca²⁺ mobilization. The effects of other etiologic factors for acute pancreatitis on pancreatic ductal HCO₃⁻ secretion have not been characterized as yet.

Viruses can alter ion transport by epithelial cells. Kunzelmann et al., reported that parainfluenza virus I (Sendai virus) produces rapid changes in ion transport across tracheal epithelium. The Sendai virus, at concentrations observed during respiratory infections, activates Cl⁻ secretion and inhibits Na⁺ absorption by triggering the release of ATP, which then acts on apical P2Y receptors to produce changes in ion transport. Bacterial infection (e.g., *Pseudomonas aeruginosa* or *Staphylococcus aureus*) triggers mucus and interleukin production. Mucus clearance is a primary innate defense mechanism for mammalian airways.

In this study, we developed a model to investigate the effect of acute infection with a herpes virus (PRV) on the pancreatic ductal epithelium. Incubating BDG at a dose of 10⁷ PFU/mL for 6 h resulted in the infection of the majority
of accessible epithelial cells within the duct. As expected, the virulent PRV strain resulted in a productive infection of epithelial cells, indicated by the appearance of viral antigens. We used two different measures of HCO\textsubscript{3}- secretion (inhibitor stop and alkali load methods) to study the effect of acute PRV infection on pancreatic ducts. Both methods showed that BDG infection stimulated HCO\textsubscript{3} secretion in guinea pig pancreatic duct by about four or fivefold. However, BDG had no effect on the pH\textsubscript{i} recovery after an acid load, suggesting that neither the basolateral Na+/HCO\textsubscript{3} co-transporter nor the basolateral Na\textsuperscript{+}/H\textsuperscript{+} exchanger is involved in the hypersecretory effect. We have previously reported that pancreatic hypersecretion is observed during the early phase of acute necrotizing pancreatitis\textsuperscript{[33,34]}. Furthermore, the early phase hypersecretion is accompanied with a simultaneous decrease in protein output\textsuperscript{[34]}, suggesting that the pancreatic ducts are at least in part involved in the change of secretory pattern. In our study, the genetically engineered control virus (KEG) did not evoke hypersecretion in the ductal cells, indicating that the presence of the virus in the cell is not enough to trigger hypersecretion. Hypersecretion was only induced by the BDG virus, which is able to initiate a lytic viral cycle.

Our finding that BDG stimulates HCO\textsubscript{3} secretion from pancreatic ducts may represent a defense mechanism against invasive pathogens in order to avoid pancreatic injury. We speculate that the stimulated secretion can wash out activated enzymes, viruses and other toxic factors from the pancreas. Moreover, the high efficiency of PRV infection in the pancreatic ductal epithelium may open the possibility for gene transfer and gene therapy of the duct cells using non-replicating or conditionally replicating PRV variants (KEG)\textsuperscript{[2,19]}.

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