ORIGINAL ARTICLE

Immunohistochemical detection of enteroviruses in pancreatic tissues of patients with type 1 diabetes using a polyclonal antibody against 2A protease of Coxsackievirus

Erika Jimbo[1], Tetsuro Kobayashi[1,2,]*(iD), Akira Takeshita[2], Keiichiro Mine[3](iD), Seiho Nagafuchi[3], Tomoyasu Fukui[4], Soroku Yagihashi[5](iD)

[1]Division of Immunology and Molecular Medicine, Okinaka Memorial Institute for Medical Research, Tokyo, Japan, [2]Department of Endocrinology and Metabolism, Toranomon Hospital, Tokyo, Japan, [3]Division of Metabolism and Endocrinology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, Japan, [4]Division of Diabetes, Metabolism and Endocrinology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan, and [5]Department of Exploratory Medicine on Nature, Life, and Man, Toho University School of Medicine, Chiba, Japan

ABSTRACT

Aims/Introduction: The need for antiserum for immunohistochemical (IHC) detection of enterovirus (EV) in formaldehyde-fixed and paraffin-embedded samples is increasing. The gold standard monoclonal antibody (clone 5D8/1) against EV-envelope protein (VP1) was proven to cross-react with other proteins. Another candidate marker of EV proteins is 2A protease ($2A^{pro}$), which is encoded by the EV gene and translated by the host cells during EV replication, and participates processing proproteins to viral capsid proteins.

Materials and Methods: We raised polyclonal antiserum by immunizing a rabbit with an 18-mer peptide of Coxsackievirus B1 (CVB1)-$2A^{pro}$, and examined the specificity and sensitivity for EV on formaldehyde-fixed and paraffin-embedded tissue samples.

Results: Enzyme-linked immunosorbent assay study showed a high titer of antibody for 18-mer peptide of CVB1-$2A^{pro}$, cross-reacting with CVB3-$2A^{pro}$ peptide. IHC showed that antiserum against $2A^{pro}$ reacted with CVB1-infected and VP1-positive Vero cells. Confocal laser scanning microscopy showed that antigen stained by the $2A^{pro}$ antibody located in the same cell with VP1 stained by 5D8/1. IHC using $2A^{pro}$ antiserum showed dense staining in the islets of EV-associated fulminant type 1 diabetes pancreas and that located in the same cell stained positive for VP1 (5D8/1). Specificity of $2A^{pro}$ antiserum by IHC staining was confirmed by negative $2A^{pro}$ in 14 VP1-negative non-diabetes control pancreases.

Conclusions: Our study provides a new polyclonal antiserum against CVB1-$2A^{pro}$, which might be useful for IHC of EV-infected human tissues stored as archive of formaldehyde-fixed and paraffin-embedded tissue samples.

INTRODUCTION

Recently, there has been a drastic increase in the practical and basic need for an antibody against enterovirus (EV)-2A protease ($2A^{pro}$) as a diagnostic means for immunohistochemical (IHC) staining of human diseases, including type 1 diabetes and chronic myocarditis[1–3]. $2A^{pro}$ is induced in EV-infected cells, and cleaves EV preproteins to produce structural proteins required for viral replication[2]. It is also implicated as an

inflammatory factor for neighboring host cell proteins that trigger autoimmune cell destruction in Coxsackievirus B (CVB)-induced chronic myocarditis[4,5] and type 1 diabetes[1]. However, no polyclonal or monoclonal antibodies against $2A^{pro}$ of EV are yet to be commercially available for IHC analysis[6]. In addition, the standard antiserum against EV-capsid protein 1 (VP1) often suffers from problematic cross-reactions with other protein epitopes[7,8]. Thus, the establishment of optimized conditions that preclude cross-reactions with non-target protein epitopes is required in IHC analysis[7–9]. For the identification of microbes, it is often difficult to obtain positive results *in situ*

hybridization, because EV-RNA, especially in pancreatic tissues, is likely degraded by many digestive enzymes over the prolonged post-mortem conditions before fixation of the samples[10]. In addition, the density of IHC by antibody (5D8/1) is less dense, which is a common characteristic of monoclonal antibodies.

EV-2A[pro] plays an important role in viral replication and can be used as an EV protein marker. We raised polyclonal antiserum against EV-2A[pro] peptide, the sequences of which show relatively low homology with other enterovirus 2A proteases. Its specificity and reactivity can be validated on the cell line or human pancreatic tissues infected with EV using IHC. In this communication, we report that the polyclonal antibody we raised was specific to EV-2A[pro] and useful as a diagnostic means for IHC of EV-infected formaldehyde-fixed paraffin-embedded (FFPE) tissues, applicable to archived tissues of type 1 diabetes patients.

MATERIALS AND METHODS

Preparation of antigen and immunization of antigen

Preparation and immunization of CVB1-2A[pro] antigen

18-mer peptide of CVB1-2A[pro] (Figure 1) was synthetized and conjugated with keyhole-limpet-hemocyanin (KLH). Two rabbits (weight 2.5 kg, age 7 months, housed for 12 h light/dark cycles, and free access to food and water) were immunized five times (1-month intervals) with CVB1-2A[pro] conjugated to KLH (500 μg), mixed with Freund's complete adjuvant (Sigma-Aldrich Japan, Tokyo, Japan). Before each injection, blood samples were obtained from the marginal vein of the rabbit ear, centrifuged at 268 g for 10 min at 4°C, and the sera were used to determine antibody titer by enzyme-linked immunosorbent assay (ELISA). Antiserum from a rabbit (ET2112) was purified by protein A Sepharose CL-4B column (Cytiva, Tokyo, Japan). Serum was incubated with KLH at 20 μg/mL overnight at 4°C and spin down at 603 g for 20 min at 4°C for evaluation of validity of antiserum.

Cell lines and viruses

Vero cells were first established from African Green Monkey kidney cells in 1962 in Japan, showing epithelial cell phenotype with contact inhibition. The Vero cells were obtained from the RIKEN CELL BANK of Japan, and maintained in Saga University, Saga, Japan. The cells grown in Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Japan, Tokyo, Japan) at 37°C and 5% CO_2 were used for viral infection. They were infected with CVB1 (strain number: 16-20289) at multiplicity of infection (MOI) of 0.1 and 3.0 for 24 h, and fixed with 5% formaldehyde phosphate-buffered saline (PBS) for 24 h and subjected to IHC examination. CVB1 (strain No. 16-20289) was isolated from a Japanese child with aseptic meningitis in 2016.

Validation of antibody

Antiserum titer determination by ELISA

Two antigens of CVB1-2A[pro] peptide (Figure 1) and CVB3-2A[pro] peptide, (`916casknkhypisfegpglv923`), were applied to 96 well plates for routine ELISA: 5 μg/mL CVB1- or CVB3-2A[pro] 18-mer peptides (Figure 1) were mixed with coating buffer (0.01 mol/L phosphate buffer, pH 7.6) at 100 μL/well and incubated at 4°C overnight. The wells were then washed five times with 0.05% Tween 20/PBS. Blocking was carried out with 0.5% gelatin/Tween 20/PBS (200 μL/well) for 1 h. After three washes with PBS, antiserum diluted at a range from ×500–×7,812,500 (concentration range) of 100 μL/well was applied and incubated for 1 h as the primary antibodies. Horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG; 1:5,000) was added at 100 μL/well and incubated for 1 h at room temperature. After washing three times, 5 mg/mL o-phenylenediamine dihydrochloride (Sigma-Aldrich Japan) was added at 100 μL/well and incubated for 20 min. The reaction was stopped by the addition of 50 μL/well 2 N H_2SO_4 and absorbance was measured at 490 nm. The antibody titer value was determined as the lowest dilution point at which the absorbance of the antiserum showed no statistically significant difference from that of the pre-immune serum.

IHC of formalin-fixed cultured cell tissues and FFPE tissues

The raised rabbit polyclonal antiserum (ET2112) was diluted at 1:800, 1:1600, 1:3200, 1:6400 and 1:12800. Vero cells grown on

coxb1 (QEV81859.1)	909	gvyf**casrgkh**y**p**vifeg**pgli**evqeseyypkrfqshvllaagfsepgdcggilrcehgv	968
coxb3 (P03313.4)	912	gvyfcasknkhypisfegpglvevqeseyyprryqshvllaagfsepgdcggilrcehgv	971
coxb4 (P08292.4)	910	gvyfcasksknhypvsfegpglvevqeseyypkryqshvllatgfsepgdcggilrcehgv	969
ec13 (BAI87840.1)	915	gvyycasrnkhypvtfegpglvevqeseyypkkyqshvllaaglsepgdcggilrcehgv	974
ec6 (SPS68038.1)	918	gvyfcasknkhypvafegpglvevqeseyypkryqshvllaagfsepgdcggilrcehgv	967
he70 (ABA43708.1)	925	gvyyckhknrsypvcfegpgiqwinetdyyparyqtntllamgpcqpgdcggllvcshgv	984
svdvu (P16604.3)	912	gvyfcasrnkhypvtfegpglvevqeseyypkkhqshvllaagfaepgdcggilrcqhgv	971
bobev (AAF05693.1)	900	gvyfcksknkhypivvtppsifkieandyypermqthillgigfaepgdcggllrcehgv	959

Figure 1 | Peptide sequences of Coxsackievirus B1 (CVB1)-2A protease (2A[pro]) immunized in this study (red bold characters). The region of the sequence chosen for immunization shows low sequence homology with cogent regions of other enteroviruses. cox, Coxsackievirus; ec, echovirus; he, human enterovirus; svdvu, swine vesicular virus; bobev, bovine enterovirus.

24-well glass plates were infected with CVB1 for 24 h. The cell tissues were fixed with 5% formaldehyde-PBS. Cells were stained with 2A^{Pro} antibodies (ET2112; 1:1600), VP1 antibodies (5D8/1; DAKO, Carpinteria, CA, USA; 1:400[11] followed by an aminomethylcoumarin acetate-conjugated donkey anti-rabbit IgG secondary antibody (1:50; 711-156-152; Jackson ImmunoResearch, West Grove, PA, USA), rhodamine (tetramethylrhodamine isothiocyanate)-conjugated donkey anti-mouse IgG secondary antibody (1:200; 715-025-151; Jackson ImmunoResearch) and horseradish peroxidase-conjugated anti-mouse/rabbit secondary antibody Envision Kit (K4007, Ready-to-use; DAKO). IHC staining was visualized by confocal laser scanning microscopy (FV 3000; Olympus, Tokyo, Japan) and conventional microscopy (DP 73; Olympus). CVB1 incubated in Dulbecco's modified Eagle medium and fixed with 5% formaldehyde-PBS were stained as a positive control. As disease controls, autopsied pancreases from three patients who died 3–5 days after onset of fulminant type 1 diabetes due to diabetic ketoacidosis[10] were stained with IHC[10–12]. The presence of CVB1-ribonucleic acid (RNA) in the pancreatic islets and exocrine pancreas of these fulminant type 1 diabetes patients was ascertained by *in situ* hybridization[13]. Nuclei were visualized with TO-PRO-3 iodide (Themo Fisher Japan, Tokyo, Japan).

Absorption test of 2A^{pro} antibody using immunizing peptide
Antiserum against 2A^{pro} (1:1600) was incubated with 2A^{pro} immunogen at 20 μg/mL for overnight at 4°C and subjected to immunostaining for 2A^{pro} in pancreatic sections of FFPE-fulminant type 1 diabetes pancreatic tissues[10–13].

Sensitivity examination on FFPE tissues and formaldehyde-fixed cultured cells using IHC
IHC analysis for the staining of 5% FFPE tissues or formaldehyde-fixed tissues and the localization of CVB1-2A^{pro} was carried out using 1:800, 1:1,600, 1:3,200, 1:6,400 and 1:12,800 diluted sera. The 1:6,400 ratio provided the best staining. Optimized IHC of EV-VP1 using antibody 5D8/1 included the use of freshly prepared sections and an antibody dilution of 1:400, as previously reported[10,12]. The stained samples were observed by conventional fluorescent microscopy (DP73; Olympus) and confocal laser scanning microscopy (FV3000; Olympus), and images stored as photos were analyzed by two investigators and judged independently in a double-blind manner.

Specificity examination on FFPE tissues using IHC
Cultured Vero cells, which were not infected by CVB1, were stained by 2A^{pro} antiserum (ET2112) and VP1 antibody (5D8/1) as negative controls. Pancreatic sections of FFPE tissue were processed as first antibody with isotype-matched control rabbit IgG (1:1000; X0903; DAKO) or pre-immune rabbit serum (1:6,400), or in the absence of primary antibody to confirm the specificity of immunostaining. FFPE tissues from nine autopsied non-diabetes controls (3 men, 6 women, age 66 ± 6 years, range 59–74 years), whose islets were negative for VP1 by ICH using antibody 5D8/1, were examined for 2A^{pro} in the pancreas, stomach, duodenum, liver, colon and thyroid gland using antiserum against CVB1-2A^{pro} (ET2112).

Ethics
Written, informed consent was obtained from the next of kin of the autopsied patients. The Toranomon Hospital research ethics committee approved all procedures, that conform to the provisions of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013).

Statistical analysis
The statistical analyses were carried out using Student's *t*-test for comparison between two group values. Values are expressed as the mean ± standard deviation. *P*-values <0.05 were considered significant.

RESULTS
Titers of antiserum against CVB1-2A^{pro} by ELISA
The interaction between antiserum against CVB1 2A^{Pro} (ET2112) and the peptide used for immunization without carrier protein KLH was confirmed by ELISA, showing significant binding with plate-coated CVB1 and CVB3 peptide (Figure 2). Antiserum against 2A^{pro} (ET2112) bound more intensely with CVB1 peptide than CVB3 peptide. The end-point titer of antiserum ET2112 using plate-coating CVB1 peptide was $15,625 \times 10^2$, whereas the end-point titer using plate-coating CVB3 peptide was $3,125 \times 10^2$, suggesting that antiserum (ET2112) against CVB1 2A^{pro} peptide shares cross-reactivity with CVB3 2A^{pro} peptide.

Validation of antiserum against CVB1-2A^{pro} by IHC
2A^{pro} in formalin-fixed CVB1 infected or uninfected cultured Vero cells by IHC
Conventional and confocal fluorescence microscopy showed antiserum against CVB1-2A^{pro} stained significant immunostaining in cytosol of CVB1-infected Vero cells for 24 h after infection and fixed by 5% formaldehyde-PBS (Figure 3a-l). The number of cultured cells stained for 2A^{pro} and VP1 were higher in 3.0 MOI of CVB1 infected cells than 0.1 MOI of CVB1 infected cells (Figure 3a,b,d,e). Mock cultured cells did not show positive staining for both 2A^{pro} and VP1 (Figure 3c,f,n,m,o,p).

Double immunostaining for 2A^{pro} and VP1 in formalin-fixed CVB1-infected Vero cells at MOI 3.0 incubated for 24 h showed that 2A^{pro} and VP1 colocalized in most cells (Figure 3g,h,i). Confocal laser scanning microscopy showed positive 2A^{pro} and VP1 in the cytosol of same cultured cells. VP1 staining was mainly in perinuclear areas and less intense in peripheral areas of cells in comparison with 2A^{pro} staining, which was mainly in the area of peripheral cytosol in the same cells with 2A^{pro} (Figure 3j–m). Mock-manipulated uninfected cells were negative for 2A^{pro} and VP1 (Figure 3n,o,p).
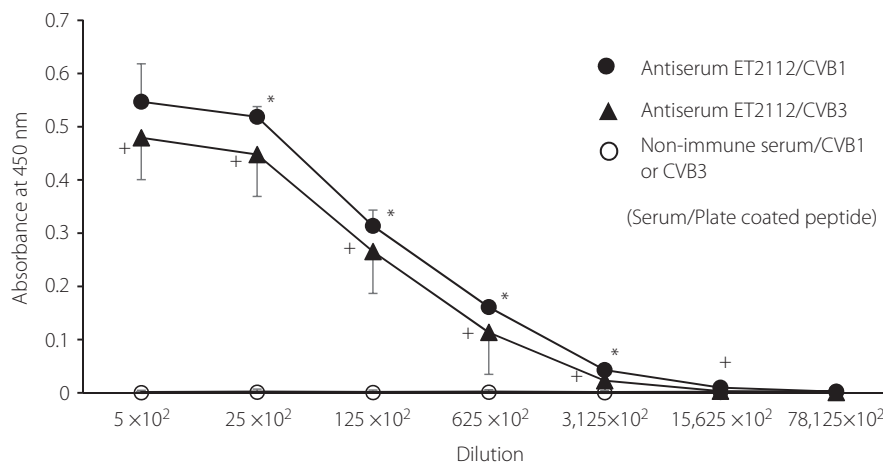
Figure 2 | Enzyme-linked immunosorbent assay analysis of interactions between antiserum against Coxsackievirus B1 2A protease (CVB1-2A^{pro}; ET2112) and non-immune serum and CVB1- or CVB3-2A^{pro} peptides (Figure 1). Mean ± standard deviation, $n = 4$, *$P < 0.05$ values between antiserum ET2112/CVB1-2A^{pro} -coated plate (●) and antiserum ET2112/CVB3-2A^{pro}- coated plate (○) data, +$P < 0.05$ versus non-immune serum.

IHC staining for 2A^{pro} and VP1 in FFPE tissues of EV-induced fulminant type 1 diabetes

Pancreatic islets were positively stained for CVB1-2A^{pro} in EV-induced fulminant type 1 diabetes (Figure 4a). Pre-absorption with 2A^{pro} peptide at 10 μg/mL abolished positive immunostaining (Figure 4b). Immunostaining for VP1 using antibody (5D8/1) on the islet of serial sections of (a) concordantly stained positive in the islet (Figure 4c). Staining for 2A^{pro}-positive fulminant type 1 diabetes samples processed as omission of first antibody, usage of isotype-matched control (rabbit IgG) or pre-immune rabbit serum showed negative staining (data not shown). Double immunostaining of FFPE-fulminant type 1 diabetes pancreas for 2A^{pro} and VP1 showed concordant location of 2A^{pro} and VP1 in the islet cell, in which VP1 mainly located peri-nuclear areas and 2A^{pro} mainly located peripheral cytosolic areas (Figure 5a–h), according with the findings of immune-stained CVB1 infected Vero cells, as shown in Figure 3g–m.

Specificity of IHC staining using 2A^{pro} antibody on control FFPE tissues

FFPE pancreases from three fulminant type 1 diabetes cases showed positive for 2A^{pro} (Figure 6a). FFPE pancreases from nine non-diabetes controls, whose islets were negative for VP1 by IHC, showed negative staining for 2A^{pro} (Figure 6b). The non-diabetes human FFPE tissues including stomach, duodenum, liver, colon and thyroid glands were negative for 2A^{pro} (Figure 6c–g).

DISCUSSION

Type 1 diabetes is a complex chronic disease caused by environmental factors (i.e., viruses and gut microbiota) and many genetic factors (i.e., *HLA* and *IFIH1* genes)[14–17]. EVs, especially the group B Coxsackievirus 1–6 (CVB1–6), have been reported to be associated with the development of type 1 diabetes involving autoimmune mechanisms with a broad-spectrum of cellular immunity, humoral immunity and cytokine/chemokine mediated pathways[18]. Under these circumstances, studies on seeking EV-encoded proteins and EV-RNA in the affected pancreas samples will provide new insights on the pathogenetic mechanisms of β-cell failure in type 1 diabetes[1,19]. However, discordant results have been obtained between IHC and reverse transcription polymerase chain reaction in acute-onset type 1 diabetic organs[19]. This phenomenon might be related with the fact that pancreatic EV-RNA is sometimes degraded by pancreatic digestive enzymes in autopsied pancreases, making it difficult to determine the exact location of EV-RNA[12]. Busse et al.[20] reported a new method to detect EV-RNA using short fluorescently labeled oligonucleotide. In addition, it has been reported that the monoclonal antibody against EV-VP1 protein (5D8/1) cross-reacts with other proteins, including a component of the mitochondrial adenosine triphosphate synthase (ATP5B) and an isoform of creatine kinase in human organs[7] and requires optimized IHC conditions[8,9]. We demonstrated that a polyclonal antibody against CVB1-2A^{pro} showed positive staining in formalin-fixed Vero cells infected by CVB1 and the FFPE pancreatic tissues of EV-induced fulminant type 1 diabetes in the present study. This EV-2A^{pro} antibody represents an important diagnostic tool for IHC staining of FFPE tissues, because there is no antiserum against EV-2A^{pro} for IHC of FFPE tissues[6]. In general, polyclonal antibodies remain stable in various environments, including under conditions varying pH, salt concentrations and dilution ranges that are associated with reduced non-specific staining[21]. Our serum against CVB1-2A^{pro} (ET2112) is polyclonal and is likely to utilize multiple epitopes that might be associated with other EV subtypes, which are potentially associated with type 1 diabetes. Polyclonal antibodies require more rigorous validation than monoclonal antibodies, because
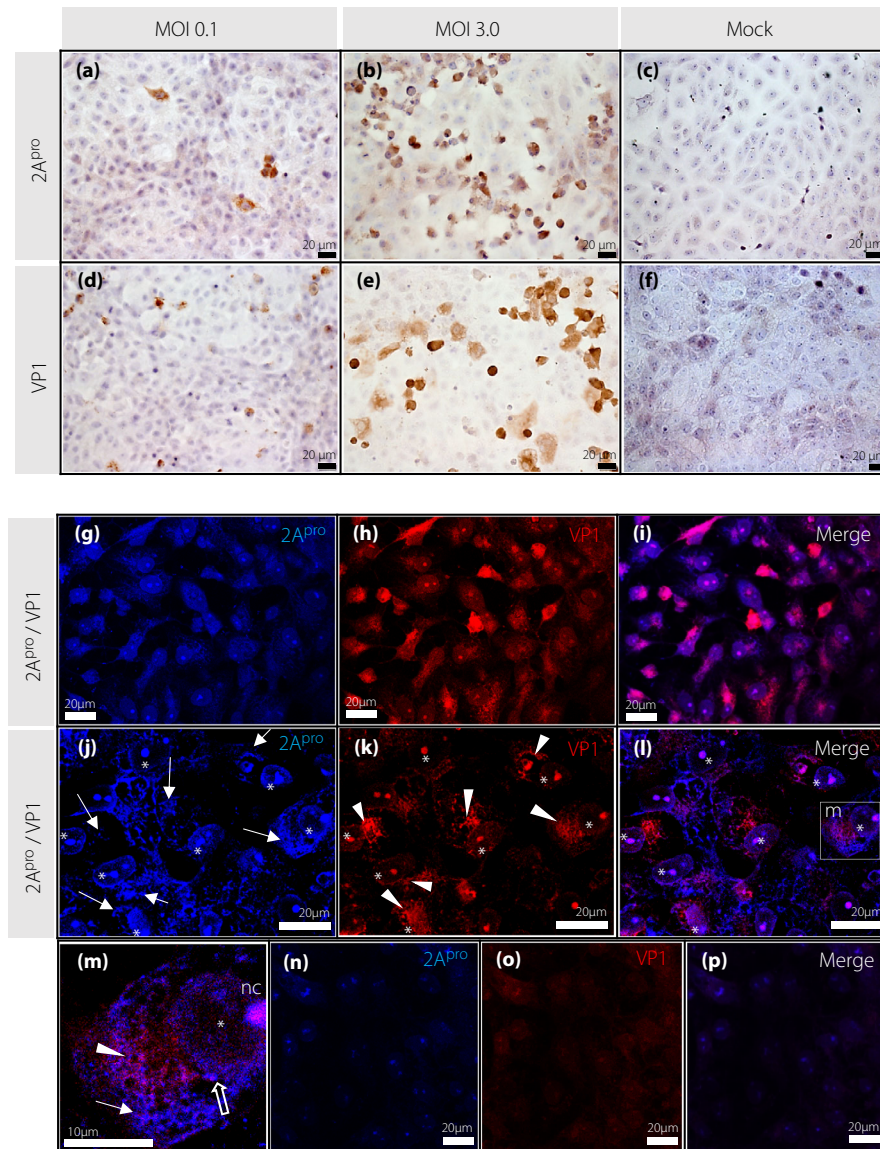
Figure 3 | Polyclonal antibody ET2112 recognizes Coxsackievirus B1 2A protease (CVB1-2A^{pro}) expressed in Vero cells. 2A^{pro} is stained positive in scattered Vero cells infected by CVB1 at (a) multiplicity of infection (MOI) 0.1 and (b) 3.0 incubated for 24 h. (c) No positive Vero cells for 2A^{pro} in mock manipulated for 24 h. (d) Enterovirus capsid protein 1 (VP1) was stained positive in Vero cells infected by CVB1 at (d) 0.1 MOI and (e) 3.0 MOI and incubated for 24 h. (f) No positive Vero cells for VP1 in mock manipulated cells for 24 h. Scale bar, 20 μm. (g–p) Double immunostaining for 2A^{pro} (blue) and VP1(red) in Vero cells incubated with or without CVB1 at 3.0 MOI for 24 h. Cytosol of Vero cells are stained positive for (g) 2A^{pro} (blue) and for (h) VP1 (red). (i) Merged image of (g) and (h) shows that 2A^{pro} and VP1 is located in the same cell stained magenta. (j–l) Magnified view of double immunostaining for 2A^{pro} and VP1 in Vero cells show that (j) 2A^{pro} (blue, arrows) and for (k) VP1 (red, arrowheads) locates in the same cell. Nucleus is shown by asterisk. (m) Magnified image of white box [m] in figure l shows that VP1 (red, arrowhead) is localized around/near the nucleus (asterisk) and cytosol (arrowhead), whereas 2A^{pro} (blue) localized in mainly the peripheral part (arrow) in addition to the perinuclear part of the cell (blank arrow). Nucleolus is marked as nc. (n–p) Vero cells uninfected by CVB1 incubated for 24 h. No (n) 2A^{pro} and (o) VP1 are stained. Scale bar, 20 μm unless otherwise mentioned.

polyclonal antisera are raised against native proteins or fragments of proteins, and polyclonal antibodies potentially recognize multiple epitopes[21]. Some of these risks can partly be eliminated in immunizations with short peptide that was used as an antigen (Figure 1). Concordant location of 2A^{pro} and VP1

was confirmed by double immunostaining in CVB1-infected cultured cells and fulminant type 1 diabetes pancreas using polyclonal 2A^{pro} antibody (ET2112) and VP1 antibody (5D8/1), suggesting that the two antibodies have comparable diagnostic value of the two antibodies. Furthermore, we excluded possible
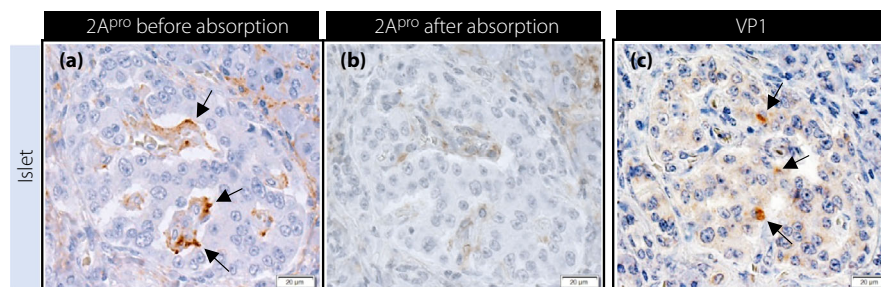
Figure 4 | Absorption test of Coxsackievirus 2A proteinase (2A^pro) antibody by immunized peptide. (a) Immunohistochemical staining for enterovirus (EV) 2A**pro** in the islet of fulminant type 1 diabetes (arrows). (b) After absorption of antiserum, immunostaining of enterovirus was completely abolished. (c) Immunohistochemical staining of the islet on serial section of (a) for enterovirus capsid protein 1 (VP1; arrows) using 5D8/1 is less dense compared with (a) 2A^pro staining. Scale bar, 20 μm.
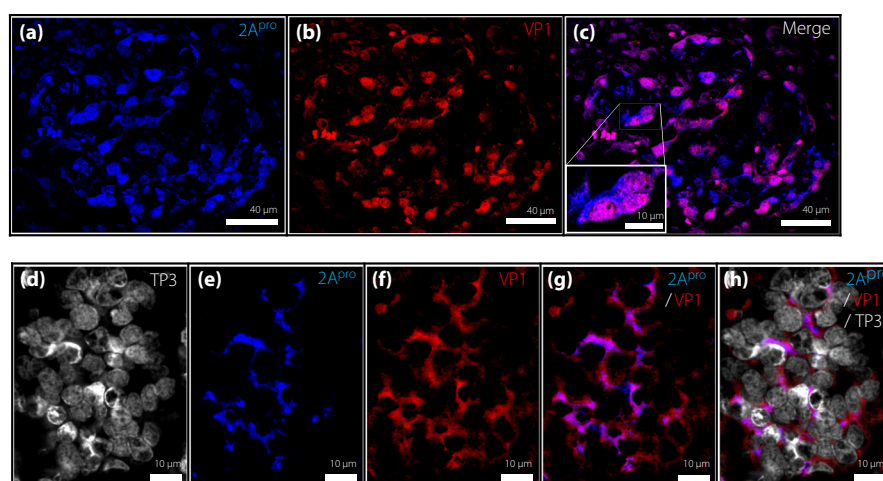

Figure 5 | Double immunostaining of fulminant type 1 diabetes pancreas for 2A proteinase (2A^pro; antiserum ET2112), enterovirus capsid protein 1 (VP1; antibody: 5D8/1) and nucleus (TO-PRO-3 iodide; TP3). (a) Immunostaining of the pancreatic islet of fulminant type 1 diabetes for 2A^pro using antiserum ET2112 (blue). Scale bar, 40 μm. (b) Immunostaining of the pancreatic islet of fulminant type 1 diabetes for VP1 using antiserum 5D8/1 (red). Scale bar, 40 μm. (c) Merged image of (a) and (b) shows that most VP1-positive islet cells are also positive for 2A^pro (magenta). Magnified photo (inset) shows that VP1 and 2A^pro are colocalized in the central part of islet cells (magenta), in addition to 2A^pro (blue) being stained in the peripheral cytosolic compartment of islet cells. Scale bar, 40 μm unless otherwise mentioned. (d) Staining of the pancreatic islet cell nuclei of fulminant type 1 diabetes with TP3. (e) Staining of the pancreatic pseudo-atrophied islet of fulminant type 1 diabetes for 2A^pro using antiserum ET2112. (f) Immunostaining of the pancreatic pseudo-atrophied islet of fulminant type 1 diabetes for VP1 using antiserum 5D8/1. (g) Merged image of (e) and (f) shows that 2A^pro and VP1 are located in the same cell stained magenta. (h) Merged image of (d), (e) and (f). Scale bar, 10 μm.

non-specific reactions with non-diabetic organ tissues and ascertained that antibody ET2112 does not react with other human organ tissues. In addition, cross-reactivity was examined using synthetized CVB3-2A^pro peptide using ELISA assay. Antiserum raised by immunizing CVB1-2A^pro peptide cross-reacted with CVB3-2A^pro peptide with the ELISA assay, but the titer of antiserum against CVB3-2A^pro peptide was lower than against CVB1-2A^pro peptide (Figure 2). The data might suggest that the polyclonal antiserum ET2112 potentially cross-reacted with other enteroviral 2A^pro peptides, which have homologous amino acid sequences, as shown in Figure 1.

Further studies on the pathogenetic roles of EV-2A^pro, which potentially cleaves islet cell proteins and causes β-cell/exocrine cell damage, and subsequent autoimmunity similar to chronic myocarditis[4,5], need to be clarified in the future. Finally, the exact relationship between persistent EV infection and the immune-modulatory function of EV-2A^pro in type 1 diabetes should be clarified. This is because EV-2A^pro cleaves innate immune sensor melanoma differentiation-associated gene 5, mitochondrial antiviral signaling and eukaryotic translation initiation factor, thereby potentially allowing persistent EV infection[6,22–25].
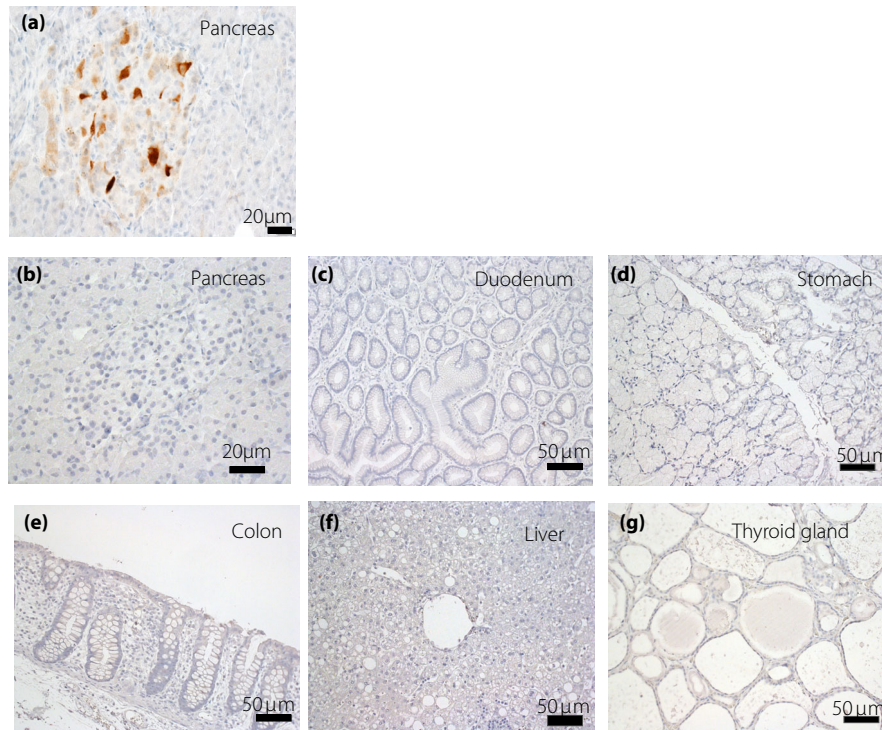
Figure 6 | (a) Immunostaining for 2A proteinase (2A^{pro}) using antiserum ET2112 in the formaldehyde-fixed paraffin-embedded pancreas obtained from a fulminant type 1 diabetes patient shows dense immunostaining of islets cells. No positive staining for 2A^{pro} using antiserum ET2112 was observed in (b) formaldehyde-fixed paraffin-embedded pancreas, (c) duodenum, (d) stomach, (e) colon, (f) liver and (g) thyroid gland of non-diabetes autopsied individuals.

The present study provides a new polyclonal antiserum against EV-2A^{pro} that is useful for the detection of EV-2A^{pro} in human tissues stored as archive of FFPE tissue samples.

DISCLOSURE

The authors declare no conflict of interest.

Approval of the research protocol: The protocol of this study was approved by the Toranomon Hospital research ethics committee (No. 948, Date: 2017, 5, 23).

Informed consent: Written, informed consent was obtained from the next of kin of the autopsied patients.

Approval date of registry and the registration no. of the study/trial: N/A.

Animal studies: N/A.

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