cirrhosis (LC), known as hepatogenous diabetes, may differ from T2DM in important ways.

The liver plays a central role in regulating postabsorptive and postprandial glucose levels by means of glycogenolysis and gluconeogenesis [2–4]. Glucose is absorbed by the intestinal tract and reaches the liver via the portal vein. Approximately 60 % of glucose is converted to glycogen in the liver, and the remaining glucose is subsequently carried by the bloodstream to peripheral tissues such as adipose tissue and skeletal muscle, resulting in decreased postprandial glucose levels. On the other hand, in the postabsorptive state, the plasma glucose level is maintained by hepatic glycogenolysis and by release of glucose synthesized from amino acids or glycerol in the liver [4, 5]. In LC, postprandial hyperglycemia is observed, reflecting the depletion of hepatic parenchymal cells, which results in impaired glucose uptake and glycogen synthesis. The fasting plasma glucose level does not rise, reflecting impaired gluconeogenesis and glycogen depletion in LC [4, 6, 7]. Correspondingly, β cell function and insulin resistance are also important factors in glucose metabolism in LC [8]. Notably, hyperinsulinemia has been reported in hepatogenous diabetes [9]. However, the pathophysiological process responsible for hyperinsulinemia in LC is still not precisely known.

Hepatic parenchymal damage is thought to be an important factor in peripheral hyperinsulinism by decreasing the hepatic insulin degradation rate, and portosystemic shunts may promote postprandial hyperglycemia [10–12]. In fact, hyperinsulinemia is not always explained by compensatory hypersecretion of insulin from β cells, even in hepatitis C virus (HCV)-related liver diseases [10, 13]. However, Greco et al. [14] reported that hyperinsulinemia might be caused by increased pancreatic β cell sensitivity to glucose, even in patients with Child B grade LC. Furthermore, in HCV-related hepatitis in particular, without progression of LC, insulin resistance and hyperinsulinemia are frequently seen due to both direct and indirect involvement of the virus [13, 15–18]. Hyperinsulinemia, frequently accompanied by insulin resistance, is clearly associated with hepatocarcinogenesis, growth of hepatocellular carcinoma, and poor prognosis in liver disease [19–22]. The effect of antidiabetic therapy on hepatoma is still a matter of debate [23–25]. In order to design appropriate treatments for hepatogenous diabetes, etiologic and environmental factors in addition to pathological changes in β cells must be considered just as they are in the treatment of T2DM [26].

The mechanism of pathogenesis in T2DM has been widely discussed [1, 27, 28]. Recent work has focused on pancreatic duodenal homeobox-1 (PDX-1), a transcription factor that plays a pivotal role in differentiation and maintaining the function of pancreatic endocrine cells [29, 30]. In particular, PDX-1 is associated with compensatory β cell expansion and insulin secretory function [29]. Although hypertrophy of islets has been reported in LC and in HCV-core transgenic mice, no evidence exists of pathological changes in pancreatic endocrine cells in these conditions [16, 31–33].

The aim of this study was to investigate the morphological and endocrinological changes in islets of Langerhans in cirrhotic and diabetic patients, with a focus on PDX-1 expression in islet cells in both groups.

Materials and methods

Case selection and subjects

Human autopsy tissue samples were collected at Kurume University School of Medicine, Japan, and the study design was approved by the Institutional Review Board (IRB) of Kurume University School of Medicine. A total of 35 adult Japanese subjects who were autopsied in the Department of Diagnostic Pathology of Kurume University between the years 2000 and 2010 were retrospectively included, and tissue samples of the pancreatic tail were taken from each subject. Eighteen patients who had died of non-hepatic causes with no history of diabetes were selected for controls. Eleven patients with hepatitis C virus-related cirrhosis who died of hepatic diseases with no history of diabetic care were selected for the LC group. Six patients with T2DM who died of non-hepatic causes were selected for the T2DM group. Diabetes care in the T2DM group was as follows: no medication ($n = 3$) and sulfonylurea with metformin ($n = 3$). Subjects were excluded if they had exogenous insulin treatment or exhibited obesity [body mass index (BMI) more than 25], as determined on weighing after removal of the body cavity effusion at autopsy. The following clinical and laboratory data were assessed based on medical charts and preserved serum (Table 1). All of the blood samples were taken at fasting state in the morning during hospitalization: sex, age, BMI, levels of hemoglobin A1c (HbA1c) in the T2DM group, plasma glucose level, insulin level, and levels of C-peptide and free fatty acids in the LC group.

Immunohistochemistry (IHC)

Paraffin-embedded tissue samples were cut at 4 μm and mounted on coated glass slides and immunostained using one of the following automated systems: BenchMark XT (Ventana Automated Systems, Inc., Tucson, AZ, USA), DAKO autostainer (DakoCytomation, Glostrup, Denmark), or Bond-Max autostainer (Leica Microsystems, Newcastle, UK). Primary antibodies (with dilutions) were as follows:

Table 1 Characteristics, clinical and fasting laboratory data of subjects

	Reference value (fasting)	Control	LC	T2DM	*P*
Number		18	11	6	
Sex (F/M)		7/11	8/3	3/3	NS
Age (years)		65.2 ± 3.2	70.5 ± 1.4	67.7 ± 4.3	NS
BMI	22–25	20.2 ± 0.7	20.8 ± 0.7	19.2 ± 1.3	NS
HbA1c (%)	4.3–5.8			6.5 ± 0.5	N/A
Plasma glucose (mg/dL)	80–109		111.5 ± 15.0		N/A
Insulin (μIU/mL)	1.84–12.2		19.1 ± 4.2		N/A
C-peptide (ng/mL)	0.61–2.09		2.6 ± 0.6		N/A
Free fatty acid (μEq/L)	140–850		534.3 ± 117.4		N/A

Values are given as number or mean ± SD

BMI body mass index, *HbA1c* hemoglobin A1c, *NS* not significant, *N/A* not applicable

Ki67 (1:200, DakoCytomation, Glostrup, Denmark), insulin (1:400, Leica Microsystems, Newcastle, UK), glucagon (1:10,000, Leica Microsystems, Newcastle, UK), somatostatin (ready to use, Nichirei, Japan), PDX-1 (1:12,000, OriGene Technologies, MD, USA), and proliferating cell nuclear antigen (PCNA) (ready to use, Thermo Scientific, CA, USA).

Ki67 immunostaining was performed on the BenchMark XT system. Briefly, slides were heat-treated in CC1 retrieval solution (Ventana) for 60 min, and incubated with the Ki67 antibody for 30 min. The system used the streptavidin–biotin complex method with 3,3′-diaminobenzidine (DAB) as the chromogen (Ventana iVIEW DAB detection kit). Immunostaining for insulin, glucagon, and somatostatin was performed on a DAKO autostainer using the ChemMate ENVISION method (DakoCytomation, Glostrup, Denmark). Briefly, samples were incubated with primary antibody at room temperature for 30 min. After slides were washed in Tris-buffered saline (TBS), they were incubated with labeled polymer-HRP secondary antibody for 30 min at room temperature. After TBS washing, the staining was visualized using DAB for glucagon and somatostatin, andwith VIP (purple) for insulin (VIP substrate Kit, Vector Laboratories, Burlingame, CA, USA). Immunostaining with PDX-1 and PCNA was performed on the same Bond-Max system using onboard heat-induced antigen retrieval with ER2 for 10 min and a Refine polymer detection system (Leica Microsystems, Newcastle, UK). DAB was used as the chromogen.

We also used double staining to reveal insulin- and glucagon-positive areas in order to assess and compare expression areas accurately. Since somatostatin-positive cells are distinct from insulin/glucagon cells [34] and account for only 5–10 % of the islets, we used somatostatin staining alone to evaluate the somatostatin-positive area.

IHC expression area analysis

Ten fields in islet size order were selected from each tissue sample at 200×. Digital images were captured using a CCD digital camera (DS-Fil, Nikon, Tokyo). Expression analysis was performed by measuring the expression area using WinROOF software (version 5.7; Mitani Co., Osaka, Japan) [35, 36]. Expression areas were measured and averaged.

Statistical analysis

All data are expressed as mean ± SD. Comparisons among groups were performed by analysis of variance followed by post hoc tests (Fisher's PLSD).

Results

Characteristics of subjects

Clinical and fasting laboratory data of subjects are summarized in Table 1. There were no significant differences in sex, age, and BMI among the three groups.

Changes of islet area

The average area of each islet was significantly enlarged in the LC group and significantly reduced in the T2DM group compared to the control group (Figs. 1a, 2).

Changes of insulin/glucagon- and somatostatin-positive area

Insulin-positive area and glucagon-positive area in islets were significantly reduced in the LC group compared with the control group (Figs. 1c, d, 2). Insulin-positive area and glucagon-positive area of the T2DM group were also significantly decreased compared with the control group (Figs. 1c, d, 2). These changes were also observed by double staining of insulin and glucagon. Somatostatin-positive area was significantly increased in LC group and trend toward increasing in T2DM groups compared with the control group (Fig. 1e, 2). Moreover,

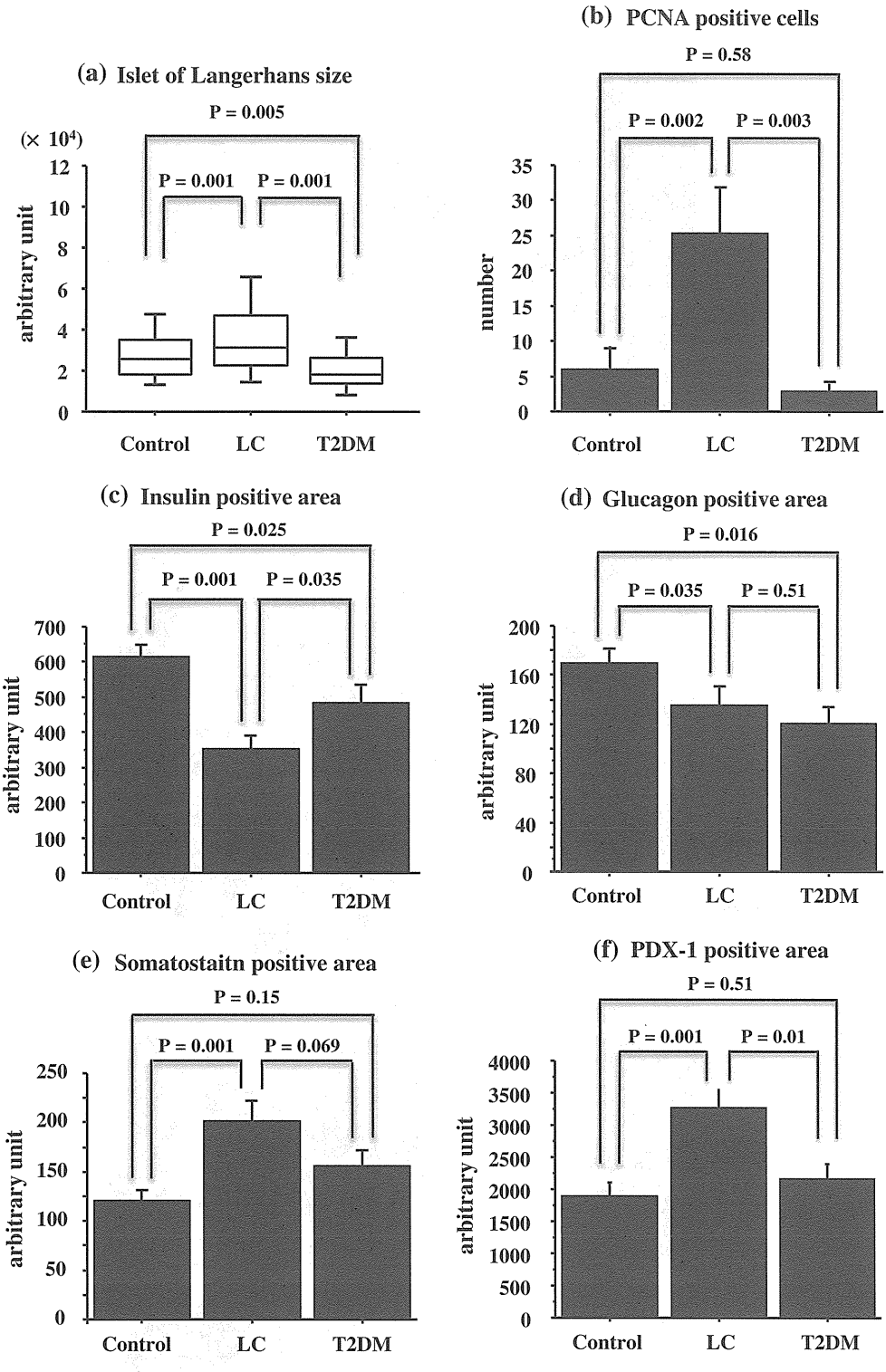

Fig. 1 Comparison of islet size and staining area. **a** Islet of Langerhans size, **b** number of PCNA-positive cells, **c** insulin-positive area, **d** glucagon-positive area, **e** somatostatin-positive area, **f** PDX-1-positive area

insulin-positive cells were located mainly in central regions of islets while glucagon-positive cells were located mainly at the periphery in control and DM groups; a disorganized distribution of these cells was apparent in the LC group (Fig. 2).

Number of PCNA-positive and Ki67-positive cells in islets

The number of PCNA-positive cells in islets was significantly increased in the LC group compared to the control

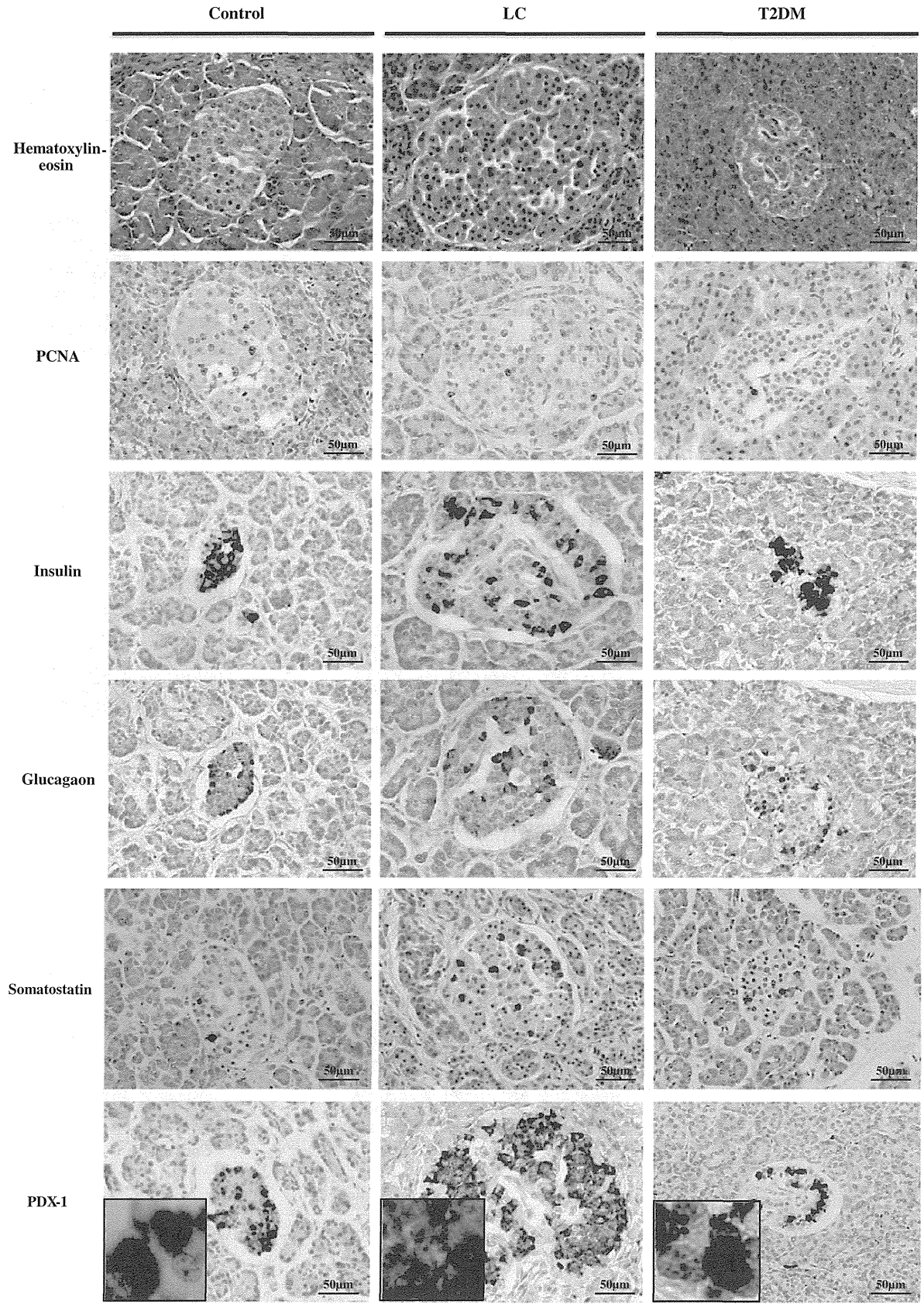

Fig. 2 Representative images of islets; magnification, ×200

group ($P = 0.002$, Fig. 1b). There was no significant difference in the number of PCNA-positive cells between the T2DM and control groups. Ki67-positive cells were detected in just one tissue sample of the LC group and were not observed in the other groups.

Expression of PDX-1

The PDX-1-positive area in islets was significantly increased in LC groups compared with the control (Fig. 1f). There was no significant difference in PDX-1 expression between the T2DM and control groups (Fig. 1f). In addition, nucleocytoplasmic translocation of PDX-1 was observed in the LC group, whereas PDX-1 was mainly expressed in the nuclei in the T2DM and control groups (Fig. 2, inset).

Discussion

Our findings demonstrate hypertrophy of islets in LC. However, insulin-positive area in islets was significantly decreased in the LC group compared with the control and T2DM groups. In contrast, the PDX-1-positive area was significantly increased in LC, and nucleocytoplasmic translocation of PDX-1 was seen in LC.

The area of islets was reduced in the T2DM group. Half of our cases had a history of diabetic therapy such as sulfonylurea. While compensatory islet hypertrophy is seen in early stages of diabetes, which results in maintenance of normal plasma glucose levels, collapse of the compensatory system and elevated β cell apoptosis results in reduced islet hypertrophy [37, 38]. Thus, the β cells might have been exhausted in the subjects we studied, which resulted in their need for antidiabetic therapy including sulfonylurea.

In contrast, we observed islet hypertrophy and an increase in PCNA-positive cells of islets in the LC group. These findings have a good correlation with previous reports [16, 31–33]. Compensatory insulin hypersecretion and expansion of β cell mass are caused by insulin resistance, obesity, and pregnancy [37, 39–42]. Notably, an interaction between the liver and pancreas in the regulation of glucose metabolism has been recently proposed. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and islet hypertrophy [43–45]. In addition, pancreatic beta cell mass is regulated by neuronal signals from the liver [46]. Although we could not demonstrate insulin resistance in the LC group due to limited laboratory data, we excluded patients with obesity. The liver failure could also affect islet hypertrophy. Since we have no autopsy specimens in patients with HCV-related cirrhosis who died of non-hepatic diseases, there is a possibility that the liver failure rather than LC induced islet hypertrophy. However, Saitoh et al. [31] reported the islet hypertrophy in patients with LC and increases PCNA-positive cell counts in islet. Therefore, it seems that the LC rather than liver failure affected the changes in islets.

The discrepancy between PCNA and Ki-67 expressions in islets were seen in this study. Although Ki-67 is known to be a useful marker in cell proliferation with rapid turnover cells such as cancer, limited information is available for the usefulness in cells except cancer [47]. On the other hand, PCNA expressions have been previously used for evaluation of the islet cell cycle [31, 48]. Taken together, the discrepancy might be caused by the difference of characteristics between rapid and slowly growing cells.

Having examined the pathological changes in pancreatic endocrine cells, we now discuss several related issues.

Liver cirrhosis

Insulin and PDX-1

In this study, we observed islet hypertrophy and an increase in PCNA-positive cells of islets in the LC. Since islet size is generally defined by β cell mass [38, 49] and plasma insulin levels and C-peptide levels were maintained in our subjects, we expected insulin staining to delineate islets in LC. However, insulin-positive area in the islets of LC patients was decreased despite our observations of islet hypertrophy and disorganization of β cells. To characterize the islet cells which were negative for insulin, we focused on the PDX-1 which associated with compensatory β cell expansion and insulin secretory function. The result was that the PDX-1-positive area in islets of LC was increased. Therefore, these cells may have the characteristics of β cells. Since PDX-1 occurs in differentiated endocrine cells and is upregulated specifically in β cells, resulting in insulin secretary function [29, 50], increased PDX-1 expression may be an adaptive response to glucose intolerance in the LC. We also found that nucleocytoplasmic translocation of PDX-1 occurred in the LC groups. Kawamori et al. [51] demonstrated that nucleocytoplasmic translocation of PDX-1 indicates increased oxidative stress in islets. Therefore, it is possible that functional impairments, including defects in insulin synthesis, occur in LC despite compensatory hypertrophy of islets. We propose that there is unknown cross-talk between the liver and pancreas in LC.

Glucagon

In our study, glucagon-positive area in islets of LC patients was also reduced, accompanied by abnormal distribution. Although we could not measure the plasma glucagon levels

of our subjects, elevated plasma glucagon levels in LC have been reported [52, 53]. The reason for this discrepancy remains unclear. However, Antoniello et al. [54] reported no difference in glucagon degradation activity between healthy and cirrhotic subjects, using biopsy specimens. In addition, there are no reports of changes in α cells in LC. Further investigation should be focused on pathophysiology of hyperglucagonemia, in LC with and without a portosystemic shunt.

Diabetes

Decreased β cell mass and elevated plasma glucagon levels have been reported in advanced T2DM [37, 55]. In the T2DM group, we found that the area of islets was reduced and the insulin-positive area in islets were significantly decreased compared to the control group. Although we could not investigate serum glucagon levels, we also found that the glucagon-positive area in islets was decreased in T2DM. In addition, we examined the expression of PDX-1, an important transcription factor regulating islet cell function. Although overexpression of PDX-1 is reported in hyperinsulinemic obese mice, limited information is available regarding PDX-1 expression in β cells of diabetic patients [56]. We first demonstrated that PDX-1-positive area was maintained in T2DM patients including those who received antidiabetic medications. These findings indicate that PDX-1-positive cells, which have characteristics of β cells, are maintained even in medicated diabetic patients. Since α cells in islets are known to play an important role in insulin secretion [57], the insulin secretion capacity of β cells (which is one of their functions) might have been impaired in this study.

Somatostatin

Somatostatin, secreted by islet δ cells and the hypothalamus, is unique in its ability to suppress glucagon and insulin release from pancreatic endocrine cells [54, 55]. Exogenous somatostatin inhibits insulin and glucagon secretion, and somatostatin secretion from δ cells is stimulated by increased glucose [58]. However, the impact of δ cells on hepatogenous diabetes as well as T2DM is poorly understood. Here, we showed that the somatostatin-positive area in islets was significantly increased in LC groups. The increased somatostatin content of islets in diabetic rats has been reported [59, 60], and our data showed similar changes in islets of diabetic patients.

Conclusion

Here, we showed that in LC, the islets of Langerhans were enlarged, and insulin-positive area and glucagon-positive area in islets were decreased accompanied with abnormal distributions. The PDX-1-positive area in islets of cirrhotic patients was increased, accompanied by nucleocytoplasmic translocation of PDX-1. Thus, we have demonstrated impaired β cell function in LC. However, it remains unclear how long insulin secretory malfunction had been going on during the progress of liver diseases in the subjects of our study. Further studies focusing on the histopathological changes of pancreatic endocrine cells, associated with disease progression and age, might point to better diabetic treatments in liver diseases.

Acknowledgments This study was supported in part by a Grant-in-Aid for Young Scientists (B) (No. 19790643 to T.K.) and a Grant-in-Aid for Scientific Research (C) (No. 21590865 to M.S.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by Health and Labour Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan, and by a Grant for Cancer Research from Fukuoka Cancer Society.

Conflict of interest The authors declare that they have no conflict of interest.

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Japanese Encephalitis Virus Core Protein Inhibits Stress Granule Formation through an Interaction with Caprin-1 and Facilitates Viral Propagation

Hiroshi Katoh,[a] Toru Okamoto,[a] Takasuke Fukuhara,[a] Hiroto Kambara,[a] Eiji Morita,[b] Yoshio Mori,[d] Wataru Kamitani,[c] Yoshiharu Matsuura[a]

Department of Molecular Virology,[a] International Research Center for Infectious Diseases,[b] and Global COE Program,[c] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; Department of Virology III, National Institute of Infectious Diseases, Tokyo, Japan[d]

Stress granules (SGs) are cytoplasmic foci composed of stalled translation preinitiation complexes induced by environmental stress stimuli, including viral infection. Since viral propagation completely depends on the host translational machinery, many viruses have evolved to circumvent the induction of SGs or co-opt SG components. In this study, we found that expression of Japanese encephalitis virus (JEV) core protein inhibits SG formation. Caprin-1 was identified as a binding partner of the core protein by an affinity capture mass spectrometry analysis. Alanine scanning mutagenesis revealed that Lys^{97} and Arg^{98} in the α-helix of the JEV core protein play a crucial role in the interaction with Caprin-1. In cells infected with a mutant JEV in which Lys^{97} and Arg^{98} were replaced with alanines in the core protein, the inhibition of SG formation was abrogated, and viral propagation was impaired. Furthermore, the mutant JEV exhibited attenuated virulence in mice. These results suggest that the JEV core protein circumvents translational shutoff by inhibiting SG formation through an interaction with Caprin-1 and facilitates viral propagation *in vitro* and *in vivo*.

In eukaryotic cells, environmental stresses such as heat shock, oxidative stress, UV irradiation, and viral infection trigger a sudden translational arrest, leading to stress granule (SG) formation (1). SGs are cytoplasmic foci composed of stalled translation preinitiation complexes and are postulated to play a critical role in regulating mRNA metabolism during stress via so-called "mRNA triage" (2). The initiation of SG formation results from phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) at Ser^{51} by various kinases, including protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control nonrepressed 2 (GCN2), and heme-regulated translation inhibitor (HRI), which are commonly activated by double-stranded RNA (dsRNA), endoplasmic reticulum (ER) stress, nutrient starvation, and oxidative stress, respectively. Phosphorylation of eIF2α reduces the amount of eIF2-GTP-tRNA complex and inhibits translation initiation, leading to runoff of elongating ribosomes from mRNA transcripts and the accumulation of stalled translation preinitiation complexes. Thus, SGs are defined by the presence of components of translation initiation machinery, including 40S ribosome subunits, poly(A)-binding protein (PABP), eIF2, eIF3, eIF4A, eIF4E, eIF4G, and eIF5. Then, primary aggregation occurs through several RNA-binding proteins (RBPs), including T-cell intracellular antigen-1 (TIA-1), TIA-1-related protein 1 (TIAR), and Ras-Gap-SH3 domain-binding protein (G3BP). These RBPs are independently self-oligomerized with the stalled initiation factors and with other RBPs, such as USP10, hnRNP Q, cytoplasmic activation/proliferation-associated protein-1 (Caprin-1), and Staufen and with nucleated mRNA-protein complex (mRNP) aggregations (3, 4). SG assembly begins with the simultaneous formation of numerous small mRNP granules which then progressively fuse into larger and fewer structures, a process known as secondary aggregation (5). The aggregation of TIA-1 or TIAR is regulated by molecular chaperones, such as heat shock protein 70 (Hsp70) (3), whereas that of G3BP is controlled by its phosphorylation at Ser^{149} (4). SG formation and disassembly in response to cellular stresses are strictly regulated by multiple factors.

Viral infection can certainly be viewed as a stressor for cells, and SGs have been reported in some virus-infected cells. Since the propagation of viruses is completely reliant on the host translational machinery, stress-induced translational arrest plays an important role in host antiviral defense. To antagonize this host defense, most viruses have evolved to circumvent SG formation during infection. For example, poliovirus (PV) proteinase 3C cleaves G3BP, leading to effective SG dispersion and virus propagation (6). Influenza A virus nonstructural protein 1 (NS1) has been shown to inactivate PKR and prevent SG formation (7). In the case of human immunodeficiency virus 1 (HIV-1) infection, Staufen1 is recruited in ribonucleoproteins for encapsidation through interaction with the Gag protein to prevent SG formation (8). In contrast, some viruses employ alternative mechanisms of translation initiation and promote SG formation to limit cap-dependent translation of host mRNA (9, 10). In addition, vaccinia virus induces cytoplasmic "factories" in which viral translation, replication, and assembly take place. These factories include G3BP and Caprin-1 to promote transcription of viral mRNA (11).

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*, which includes other mosquito-borne human pathogens, such as dengue virus (DENV), West Nile virus (WNV), and yellow fever virus, that frequently cause significant morbidity and mortality in mammals and birds (12). JEV has

Received 15 August 2012 Accepted 15 October 2012
Published ahead of print 24 October 2012
Address correspondence to Yoshiharu Matsuura, matsuura@biken.osaka-u.ac.jp.

doi:10.1128/JVI.02186-12

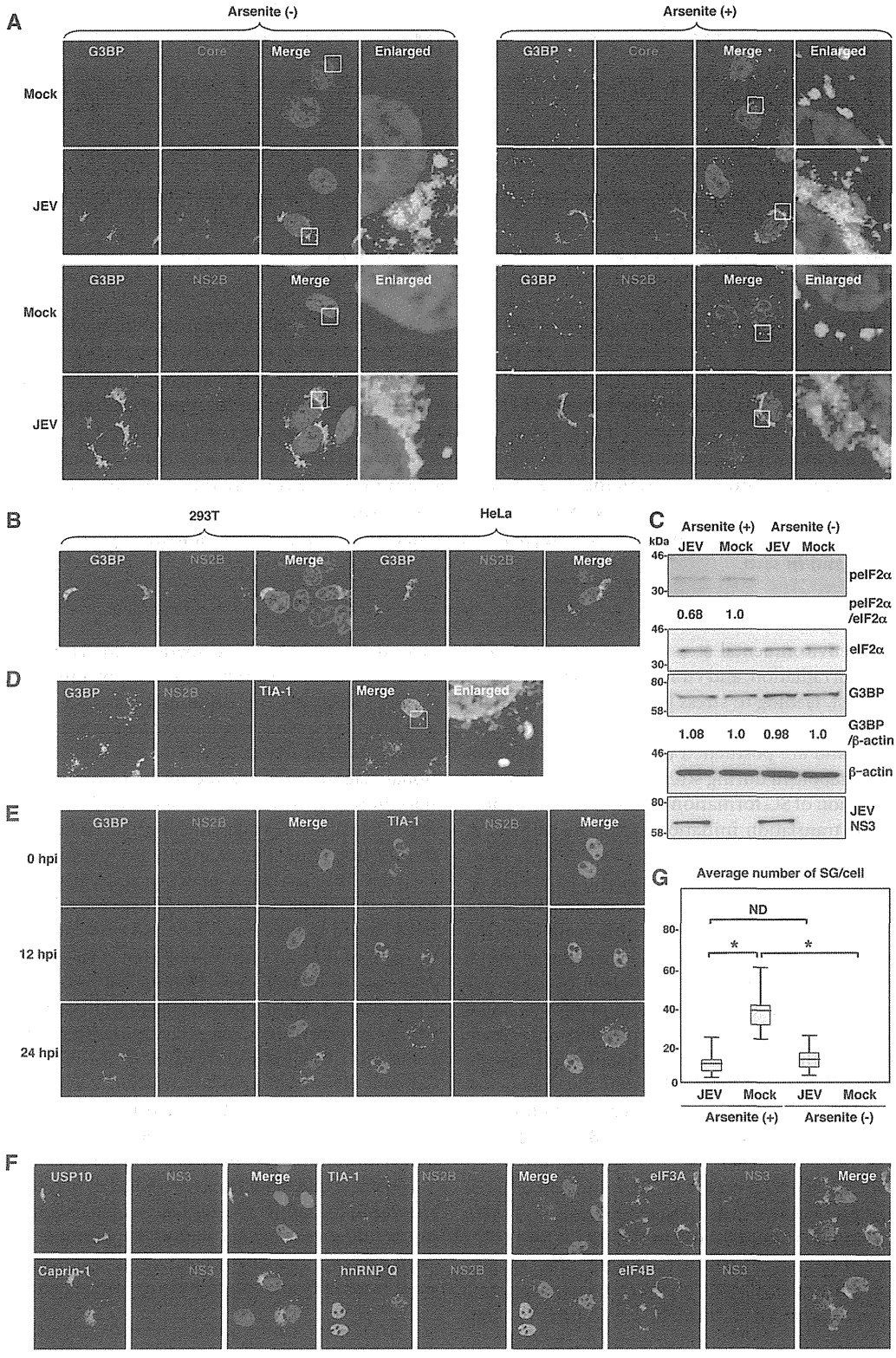

FIG 1 Dynamics of SG-associated factors during JEV infection. (A) Huh7 cells infected with JEV at an MOI of 0.5 were treated with or without 1.0 mM sodium arsenite for 30 min at 37°C, and the levels of expression of G3BP and JEV core protein/NS2B were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-core protein or anti-NS2B PAb, followed by AF488-conjugated anti-mouse IgG (Invitrogen) and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (B) Cellular localizations of G3BP and JEV NS2B in 293T and HeLa cells infected with JEV were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-NS2B PAb, followed by AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (C) Phosphorylation of eIF2α in cells prepared as described in panel A was determined by immunoblotting using the indicated antibodies. The band intensities were quantified by ImageJ