Biochemistry and Biophysics Reports 5 (2016) 196–202

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

A soluble form of human nectin-2 impairs exocrine secretion of pancreas and formation of zymogen granules in transgenic mice

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ARTICLE INFO

Article history:
Received 19 August 2015
Received in revised form
7 December 2015
Accepted 8 December 2015
Available online 10 December 2015

Keywords:
Soluble nectin-2
Pancreas
Exocrine abnormality
Endoplasmic reticulum
Transgenic mice

ABSTRACT

Transgenic mouse lines expressing a soluble form of human nectin-2 (hNectin-2Ig Tg) exhibited distinctive elevation of amylase and lipase levels in the sera. In this study, we aimed to clarify the histopathology and to propose the transgenic mouse lines as new animal model for characteristic pancreatic exocrine defects. The significant increase of amylase and lipase levels in sera of the transgenic lines approximately peaked at 8 weeks old and thereafter, plateaued or gradually decreased. The histopathology in transgenic acinar cells was characterized by intracytoplasmic accumulation of abnormal proteins with decrease of normal zymogen granules. The hNectin-2Ig expression was observed in the cytoplasm of pancreatic acinar cells, which was consistent with zymogen granules. However, signals of hNectin-2Ig were very weak in the transgenic acinar cells with the abnormal cytoplasmic accumulaion. The PCNA-positive cells increased in the transgenic pancreas, which suggested the affected acinar cells were regenerated. Acinar cells of hNectin-2Ig Tg had markedly small number of zymogen granules with remarkable dilation of the endoplasmic reticulum (ER) lumen containing abundant abnormal proteins. In conclusion, hNectin-2Ig Tg is proposed as a new animal model for characteristic pancreatic exocrine defects, which are due to the ER stress induced by expression of mutated cell adhesion molecule that is a soluble form of human nectin-2.

1. Introduction

Nectins are Ca^{2+}-independent immunoglobulin (Ig)-like cell–cell adhesion molecules [1]. The nectin family is composed of four members, nectin-1, -2, -3 and -4, all of which, except nectin-4, have two or three splicing variants: nectin-1α, -1β, -1γ, -2α, -2δ, -3α, -3β, and -3γ [2]. Nectin members, except nectin-1γ, consist of an extracellular region with three Ig-like loops, a single transmembrane region, and a cytoplasmic tail. The extracellular regions form homo-cis-dimers and promote homophilic or heterophilic trans-interactions. Extracellular Ig-like domains of each member of the nectin family consisted of a single V-like domain (first Ig-like domain) and two C-like extracellular domains. Nectin-2 forms a variety of cell–cell junctions, including cadherin-based adherens junctions in epithelial cells and fibroblasts in culture, synaptic junctions in neurons [3,4], Sertoli cell-spermatid junctions in the testis [5], and also known to be needed for maintenance of cardiac structure and function [6]. In addition, nectin-2 functions as a receptor for some herpesviruses [7–9].

Recently, we generated transgenic mouse lines expressing a soluble form of human nectin-2 (hNectin-2Ig Tg), to examine the interaction of nectin-2 with herpesviruses. In the process of phenotypic examination of hNectin-2Ig Tg, we incidentally found that the transgenic lines exhibited distinctive elevation of serum amylase and lipase. It has been reported that nectin-2 express in human pancreatic tissue and pancreatic cancer [10,11], though the functions in the pancreas have yet to be determined. Then we did not suppose why the serum pancreatic enzyme levels were

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http://dx.doi.org/10.1016/j.bbrep.2015.12.006

elevated in hNectin-2Ig Tg. In the present study, we analyzed blood biochemical and cytoarchitectonic abnormalities and cellular expression of hNectin-2Ig Tg to understand the phenotype and pathology in the transgenic mouse. We aimed to propose the transgenic mouse lines as new animal model for characteristic pancreatic exocrine defects.

2. Methods and materials

2.1. Generation of transgenic animals

To construct plasmid expressing a soluble form of human nectin-2 (hNectin-2Ig) that consist of the extracellular domain and the Fc portion of human IgG1, synthesized cDNA of the extracellular domain was amplified by PCR using pENTR221 plasmid containing a complete human nectin-2 gene (pENTR221/nectin-2; GenBank accession number: DQ891829, GeneCopoeia) as a template was cloned into a plasmid carrying IgG1-Fc DNA [12] and then, the chimeric gene fragment encoding each fusion protein was inserted into pCXN2 vector as described previously [13]. The PCR primer set used for the construction of plasmid is as follows: 5′-GGACCCCTCGAGCCACCATGGCCCGGGCCGC-3′ and 5′-CTGAGCGGGATCCTCTCGGACAAAGATGACC-3′. Transgenic mice expressing hNectin-2Ig were generated as described previously [14], by microinjection method of the transgenic fragment containing the CAG promoter (cytomegalovirus IE enhancer and chicken ß-actin promoter), the hNectin-2Ig gene, and the rabbit ß-globin polyA signal. To identify transgenic founders, genomic DNA was prepared by using the FTA elute micro cards (Whatman) and PCR was performed using specific primers (5′-CTCTGCATCTCCAAAGAGGG-3′ and 5′-CTGTGCAGACGAAGGTGGTA-3′ for hNectin-2Ig). All mice were kept with free access to food and water in a standard light/dark cycle and were maintained in the animal facility at our institutes and treated according to Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan. The ethics committees of our institutes approved all experiment protocols.

2.2. Blood biochemical examination

Under deep combination anesthesia (0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, 5.0 mg/kg butorphanol), blood samples (200 μL per sample) were collected from malar venous plexus of the 4-, 6-, 8-, 12-, 16-, and 24-week-old mice; hNectin-2Ig Tg line 2216 male ($n=6$) and female ($n=5$), hNectin-2Ig Tg line 2246 male ($n=6$) and female ($n=6$), non-Tg male ($n=12$) and female ($n=10$). Blood biochemical examination about items including Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), amylase, lipase, total bilirubin, glucose, blood urea nitrogen, total cholesterol, triglyceride, Glutamic Pyruvic Transaminase (GPT), and C-reactive protein (CRP) were performed by using the dry-chemistry system (Fuji DRI-CHEM 7000VZ) and appropriate FUJI DRI-CHEM slides (FUJIFILM).

2.3. Antibodies

For Western blot analysis, we used an anti-human IgG (Fc specific)-alkaline phosphatase antibody (from rabbit, Sigma, 1:3000) for detection of hNectin-2Ig. For immunohistochemistry, we used antibodies against human IgG (from goat, Sigma, 1:2000), nectin-2 (from rabbit, GeneTex, 1:3000, it was not reacted with hNectin-2Ig), proliferating cell nuclear antigen (PCNA) (from mouse, DAKO, 1:1000), carboxypeptidase A (CPA) (from rabbit, Abcam, 1:1000), cleaved caspase-3 (from rabbit, Cell Signaling Technology, 1: 1000), and Grp78 (from rabbit, GeneTex, 1:3000) as primary antibodies.

2.4. Western blot analysis

To confirm the expression of hNectin-2Ig in the transgenic sera, Western blot analysis was performed by the method of Towbin et al. [15]. 10 μL of each serum sample was added protein G-Sepharose 4B (Sigma) to absorb fusion proteins for 3 h at 4 °C. After the beads were washed with phosphate-buffered saline, the bound proteins were fractionated by 10% SDS-polyacrylamide gel. Then the separated proteins were electrophoretically transferred to a Sequi-Blot PVDF membrane (Bio-Rad). Blotted membrane was treated sequentially with blocking solution (5% skim milk in phosphate buffered saline with 0.05% Tween 20), primary antibody, and finally the detection was performed using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega) according to the manufacturer's instructions.

2.5. Histopathological analyses

Under deep combination anesthesia mentioned above, mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB). The pancreases and other organs were excised and further immersed overnight in the same fixative or Bouin's fixative (Wako). The fixed organs were dehydrated and then embedded in paraffin-wax in the usual manner. Paraffin sections (2–4 μm in thick) were cut and stained with haematoxylin and eosin (H. E.). Immunohistochemistry was performed using a Histofine SAB-PO(G) kit for human IgG, Simple Stain MAX-PO(M) kit for PCNA, or Simple Stain MAX-PO(R) kit for other rabbit antibodies, according to the manufacturer's instructions (Nichirei). Briefly, the rehydrated sections were incubated with 0.1% H_2O_2 in methanol for endogenous peroxidase blocking and were subjected to heat-induced antigen retrieval by using ImmunoSaver (Nisshin EM) for PCNA, cleaved caspase-3, CPA, followed by primary antibody incubation overnight 4 °C. Then sections were treated with immunohistochemical detection system mentioned above, followed by a detection with 3,3′-Diaminobenzidine tetrahydrochloride solution (ImmPACT DAB Substrate, Vector Laboratories).

2.6. Transmission electron microscope

Ultrastructural analyses of pancreatic tissue from 28-week-old hNectin-2Ig Tg and non-Tg were performed using transmission electron microscopy (TEM). For TEM, sections were fixed with 2% PFA-2.5% glutaraldehyde (GA) in 0.1 M PB and ultrathin sections were prepared and stained with 2% uranyl acetate according to standard procedures. The ultrathin sections on the grids were observed by a TEM (JEM-1200EX; JEOL) at an acceleration voltage of 80 kV.

2.7. Statistics

The values were expressed as the averages of at least three times ± standard deviations. Statistical analysis was performed by Student's *t*-test.

3. Results

3.1. Significant increase in serum amylase and lipase in hNectin-2Ig Tg

Two transgenic mouse lines (2216 and 2246) expressing hNectin-2Ig were generated. Western blot analysis of their sera

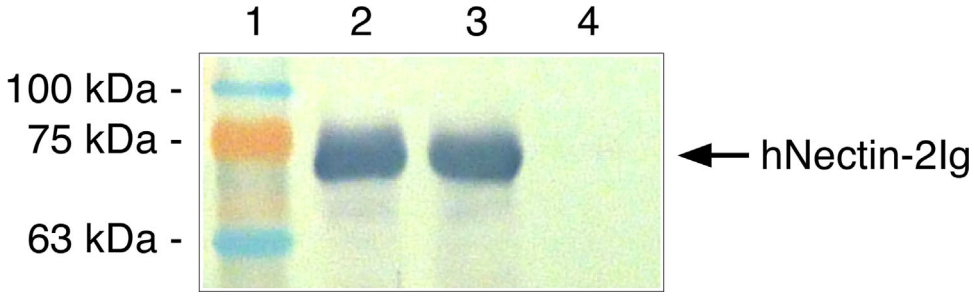

Fig. 1. Detection of hNectin-2Ig secreted in the sera of transgenic mouse lines by Western blot analysis. Lane 1: molecular weight marker, lane 2: hNectin-2Ig Tg line 2216, lane 3: hNectin-2Ig Tg line 2246, lane 4: non-Tg.

confirmed expression of hNectin-2Ig (Fig. 1). The transgene was under the control of CAG promoter, which allows an expression in all cell types. The expression was observed all of tissues tested by immunohistochemical staining (data not shown). There were no significant differences between hNectin-2Ig Tg and non-Tg in appearance and body weight until at least six months after birth (Fig. 2A). Concentrations of amylase in sera were significantly increased in hNectin-2Ig Tg lines, from 6- to 16- week-old male and female animals (Fig. 2B). Significant increases of lipase in sera were also observed in 6- to 24- week-old hNectin-2Ig Tg mice (Fig. 2C). The maximum averages of the amylase and lipase concentrations in hNectin-2Ig Tg were approximately 2-fold (line 2216, 8-week-old, female) and 5-fold (line 2216, 8-week-old, female), respectively, higher than those in non-Tg. The concentrations of amylase and lipase in sera of hNectin-2Ig Tg approximately peaked at 8 weeks old and thereafter, plateaued or gradually decreased (Fig. 2B and C). Whereas, there was no significant difference in concentrations of glucose between sera of hNectin-2Ig Tg and non-Tg (Fig. 2D). In other items of blood biochemical examination, significant differences were not observed between hNectin-2Ig Tg and non-Tg (data not shown). These results suggest that hNectin-2Ig Tg were characterized by pancreatic exocrine dysfunction, although the apparent clinical signs were not observed.

3.2. Degeneration with accumulation of abnormal proteins in acinar cells of hNectin-2Ig Tg

Since abnormal concentrations of amylase and lipase in sera of hNectin-2Ig Tg were observed, their pancreases were pathologically analyzed. Histologically, degenerated pancreatic acinar cells with increased eosinophilic cytoplasm were diffusely scattered in the transgenic pancreas at 4 weeks old (Fig. 3B). The degenerations of pancreatic acinar cells were progressed, and most of them had intracytoplasmic accumulation of weakly eosinophilic materials with decrease of normal zymogen granules in hNectin-2Ig Tg at 8 weeks old (Fig. 3C). These histological changes were furthermore progressive and the more than half of acinar cells had nuclei pushed away by accumulation of eosinophilic materials increased in the transgenic pancreas at 16 weeks old (Fig. 3D). Pyknotic and fragmented nuclei were rarely observed in the degenerated acinar cells. Immunohistochemistry with an anti-human IgG goat antibody revealed that hNectin-2Ig was mainly expressed in the cytoplasm of pancreatic acinar cells, which was consistent with zymogen granules (Fig. 3F–H). The materials with weakly eosinophilic in H. E. accumulated in cytoplasm of the acinar cells were stained very weak with an anti-human IgG antibody to detect hNectin-2Ig (Fig. 3G and H).

The expression of exocrine enzyme CPA in transgenic pancreas was sparse relative to that of the non-Tg control (Fig. 3I and L, 16-week-old). The CPA positive signals were hardly detected in the intracytoplasmic accumulated material of the transgenic acinar cells (Fig. 3L). A very few number of cleaved caspase-3-positive cells, with the classical signs of apoptotic death, were observed in the pancreatic acinar cells of hNectin-2Ig Tg (Fig. 3M, 16-week-old). Cleaved caspase-3-positive cells were not observed in non-Tg acinar cells. The PCNA-positive cells increased in the transgenic pancreas at 6 to 24 week old, comparison with non-Tg, which suggested the affected acinar cells were regenerated (Fig. 3N).

3.3. Expression of ER stress marker and ultrastructural anomaly of ER in hNectin-2Ig Tg pancreas

To investigate ER anomaly of transgenic pancreas, immunohitochemistry for Grp78, ER stress marker, and ultrastructural analyses were performed. A few acinar cells positive for Grp78 were scattered in the both of non-Tg and Tg pancreas at 4 weeks old (Fig. 4A, D). In the transgenic pancreas at 8 weeks old, the intracytoplasmic accumulation was frequently positive for Grp78 (Fig. 4E). Such Grp78-positive acinar cells were sequentially increased in transgenic pancreas (Fig. 4F, 28-week-old). The ultrastructure of pancreas at 28 weeks old, when highly expressed ER stress marker, was examined by TEM. The acinar cells from non-Tg mice contained abundant zymogen granules (Fig. 4G and H), whereas acinar cells of hNectin-2Ig Tg have markedly small number of zymogen granules with remarkable dilation of the ER lumen (Fig. 4K and L). The dilated ER in hNectin-2 Ig Tg contained less electron-dense materials compared with that of normal zymogen granules in the non-Tg (Fig. 4I and M). In the transgenic acini which zymogen granules were relatively left, the granules were apically located around the lumen in pancreatic acini of hNectin-2Ig Tg as well as Non-Tg (Fig. 4J and N). However, the zymogen granules of hNectin-2Ig Tg showed lower electron density than that of non-Tg, and the acinar lumens tended to become narrower by exclusion due to dilation of the ER.

4. Discussion

In the present study, we generated transgenic mouse lines expressing a soluble form of human nectin-2 (hNectin-2Ig), the fusion protein consisting of the extracellular domain of human nectin-2 and Fc portion of human IgG1, under the control of CAG promoter (hNectin-2Ig Tg). Histopathologically, it was suggested that hNectin-2Ig was continuously expressed in the cytoplasm including the zymogen granules, resulting in impairment of zymogen granule formation. The most likely intracytoplasmic accumulation of abnormal proteins disrupts cytoarchitectures and functions of the acinar cells, following the elevating of amylase and lipase levels in the sera.

Immunohistochemistry for Grp78 suggested that sequential changes in enzyme levels and histology of hNectin-2Ig Tg were correlated to the ER stress in acinar cells. The ultrastructural findings observed in the transgenic acinar cells, which is

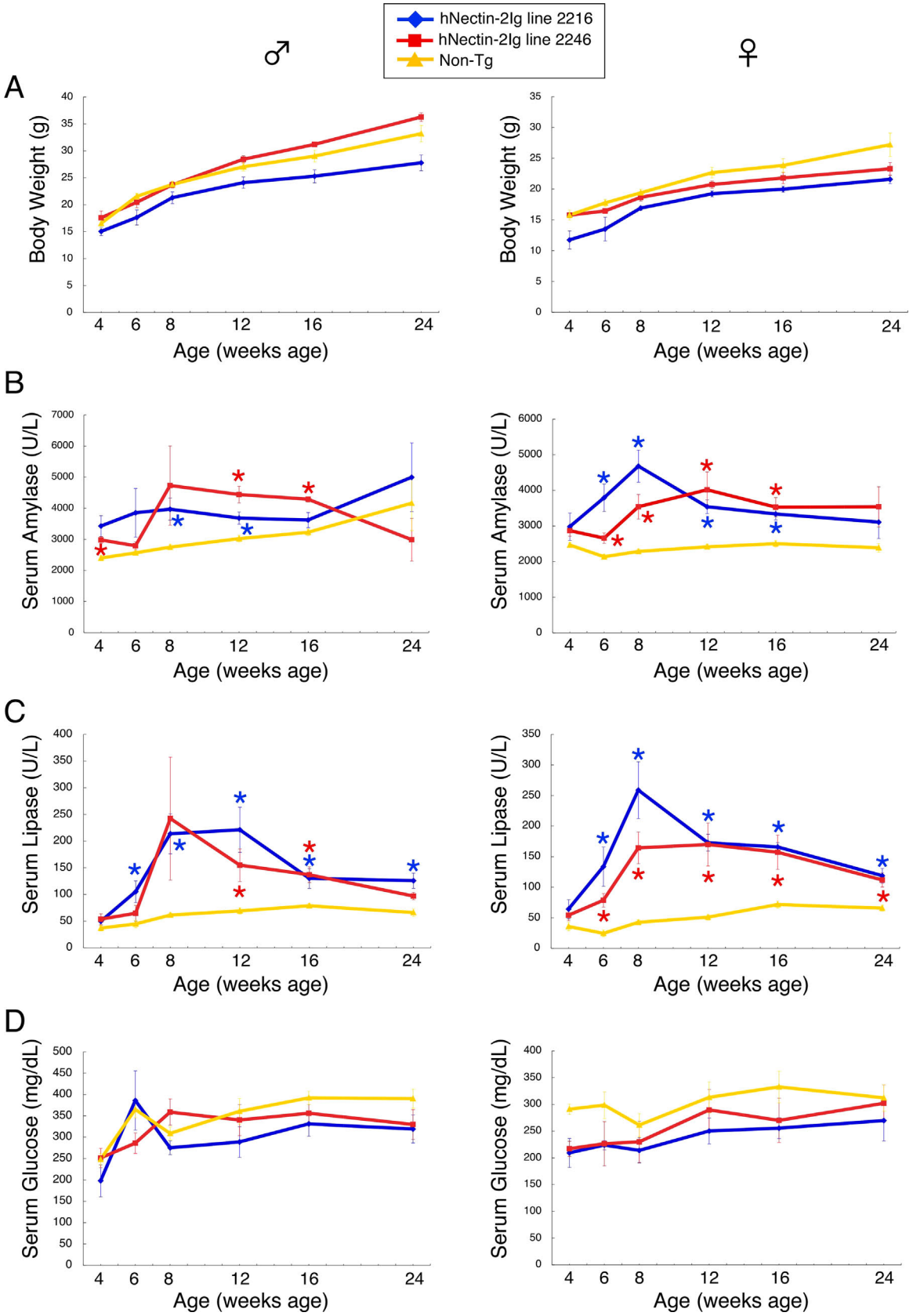

Fig. 2. Significant increase of amylase and lipase levels in sera of hNectin-2Ig Tg. (A–D) Left, male; right, female; blue, hNectin-2Ig Tg line 2216; red, hNectin-2Ig Tg line 2246; yellow, non-Tg litter mates. (A) Body weight of hNectin-2Ig Tg and non-Tg mice. No significant difference was observed at all timepoints ($P > 0.01$). (B) Concentrations of serum amylase in hNectin-2Ig Tg and non-Tg mice (*$P < 0.01$). (C) Concentrations of serum lipase in hNectin-2Ig Tg and non-Tg mice (*$P < 0.01$). (D) Concentrations of serum glucose in hNectin-2Ig Tg and non-Tg mice. No significant difference was observed at all timepoints ($P > 0.01$).

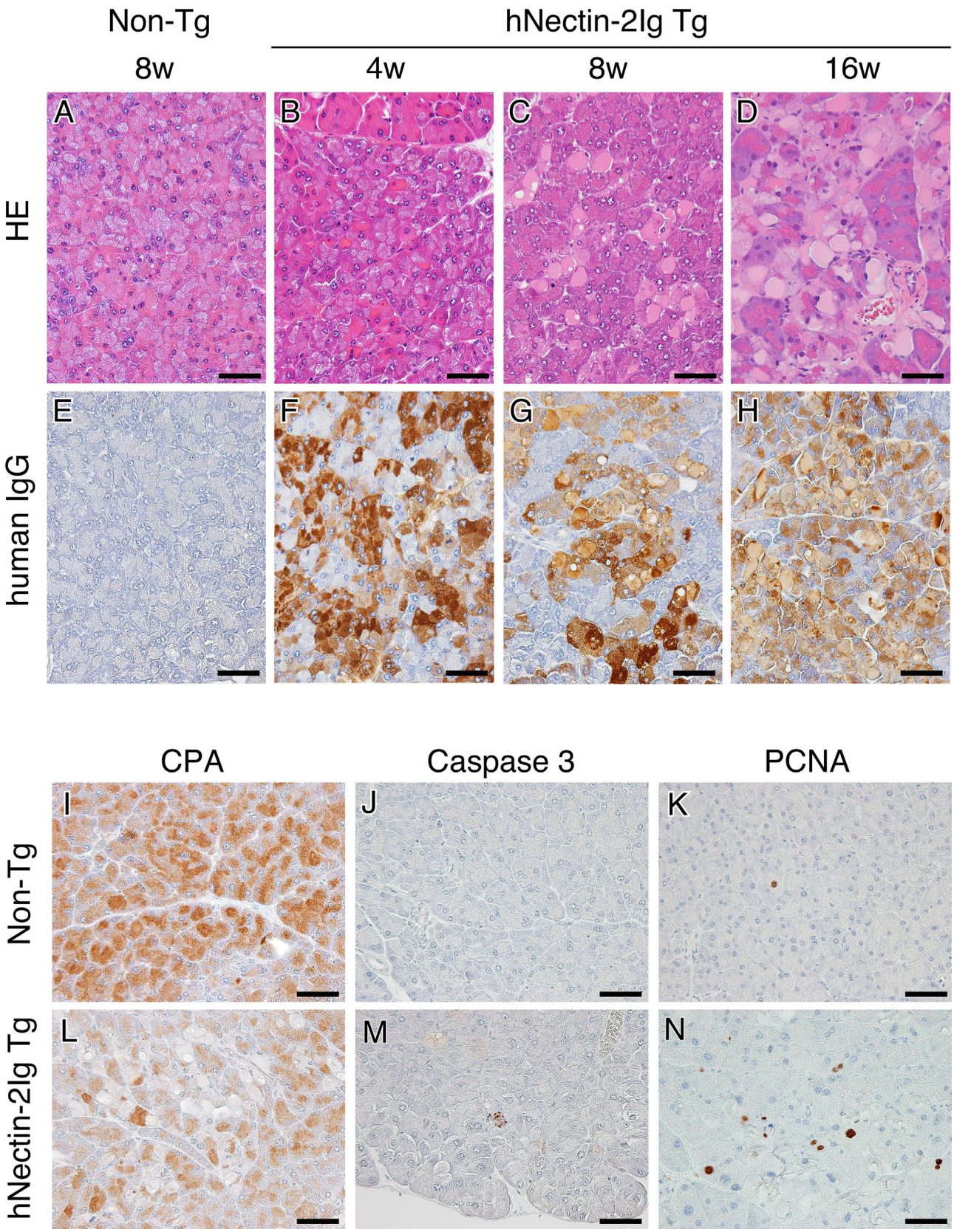

Fig. 3. Degeneration with accumulation of abnormal proteins in pancreatic acinar cells from hNectin-2Ig Tg, with dicrease of normal zymogen granules and acinar regeneration. (A–D) Histopathology of pancreatic acini in non-Tg (A, 8-week-old) and hNectin-2Ig Tg (B, 4-week-old; C, 8-week-old; D, 16-week-old). (E-H) Immunohistochemistry for the hNectin-2Ig of pancreatic acini in non-Tg (E, 8-week-old) and hNectin-2Ig Tg (F, 4-week-old; G, 8-week-old; H, 16-week-old). (I, L) Immunohistochemistry for CPA of pancreatic acini in non-Tg (I) and hNectin-2Ig Tg (L). (J, M) Immunohistochemistry for cleaved caspase-3 of pancreatic acini in non-Tg (J) and hNectin-2Ig Tg (M). (K, N) Immunohistochemistry for PCNA of pancreatic acini in non-Tg (K) and hNectin-2Ig Tg (N). (I–N) 16-week-old. (A–N) Bars, 50 μm.

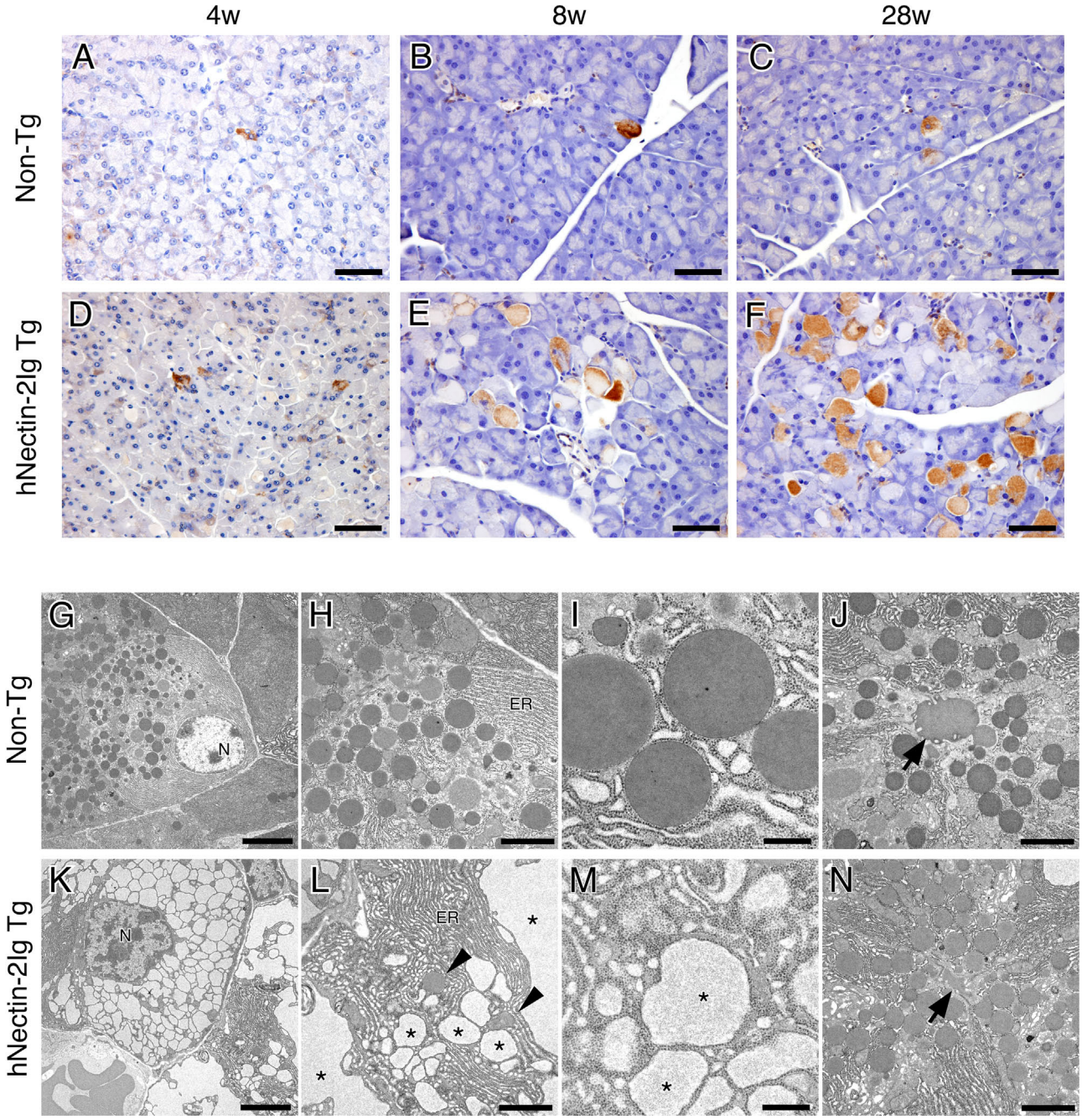

Fig. 4. High expression of ER stress marker, Grp78, and ultrastructural anomaly of ER in hNectin-2Ig Tg pancreas. Grp78-positive acinar cells were sequentially increased in hNectin-2Ig Tg (A–C, non-Tg; D–F, Tg. A, D, 4-week-old; B, E, 8-week-old; C, F, 28-week-old). Severe dilation of the ER lumen with accumulation of abundant, less electron-dense materials as compared with that of normal zymogen granules, in pancreatic acinar cells of hNectin-2Ig Tg. Non-Tg (G–J) and hNectin-2Ig Tg (K–N), 28-week-old. Remarkable dilation of the ER lumen containing less electron-dense material (asterisks) and severe decrease of zymogen granules (arrowheads) in acinar cells from hNectin-2Ig Tg (K–M), whereas non-Tg acinar cells have abundant zymogen granules (G–I). N, nuclei. Bars: 5 μm (G, K), 2 μm (H, L), 500 nm (I, M). Zymogen granules were apically located around the lumen (arrows) in pancreatic acini of hNectin-2Ig Tg (N) as well as Non-Tg (J). Bar, 2 μm.

characterized by the ER dilation with accumulation of abnormal proteins, were exactly in agreement with the ER stress. Pancreatic acinar cells are especially susceptible to ER stress and require the unfolded protein response (UPR) for recovery from the ER stress, because they need to produce and process the abundant and various proteins [16–18]. In fact, the ER stress in pancreatic acinar cells, promoted by disruption of Xbp1 that is an important transcription factor for UPR, induces extensive acinar apoptosis followed by regenerative response [19]. In hNectin-2Ig Tg acinar cells, more slight but continuous ER stress, which can hardly induce apoptosis, may cause mild tissue injury and regenerative changes, as evidenced by expression of PCNA herein.

Although we had generated transgenic mouse lines expressing a series of soluble form of transmembrane proteins such as nectin-1 and the deletion mutants, herpesvirus entry mediator (HVEM), and sialic acid-binding Ig-like lectins (siglecs), which were fused to the Fc portion of IgG [14,20–23], the pancreatic histopathology and/or findings suggesting the ER stress in hNectin-2Ig Tg have not been observed. On the other hand, it has been reported that mutated cell adhesion molecule-1 (CADM1), which has analogy and interactivity to nectins, induced ER stress which may be involved in autism spectrum disorder [24]. In addition, Struyf et al. reported

that mutant nectin-1 deleted for the second immunoglobulin-like domain accumulated in the ER and reduced ability to mediate HSV entry [25]. Perhaps there may be such other biological phenomena and disease pathogenesis, induced by the relationship between cell adhesion molecules and ER stress.

In the human pancreas, it has been reported that nectin-2 weakly express in the ductal epithelium and islet cells [11]. In our immunohistochemistry with antibodies against endogenous nectin-2 that did not react with transgenic hNectin-2Ig, the ductal epithelium and islet cells were positive for nectin-2, whereas there was no endogenous nectin-2 staining in acinar cells of non-Tg as well as hNectin-2Ig Tg (data not shown). Thereby it is unlikely that hNectin-2Ig interferes with the expression and function of endogenous nectin-2 to cause the pancreatic pathogenesis.

In conclusion, we propose hNectin-2Ig Tg as a new animal model for characteristic pancreatic exocrine defects, which are due to the ER stress induced by accumulation of mutated cell adhesion molecules.

Acknowledgments

We thank Dr. J. Miyazaki for providing pCXN2 vector. This study was supported partly by the Grant for Joint Research Program of the Institute for Genetic Medicine, Hokkaido University.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.12.006.

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