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Nephronectin is upregulated in acute and chronic hepatitis and aggravates liver injury by recruiting CD4 positive cells

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ABSTRACT

Nephronectin (Npnt) is an extracellular matrix protein known to play a critical role in kidney development; however, its physiological role in the liver remains elusive. Here we show that Npnt expression is upregulated in mouse models of both acute and chronic hepatitis induced by Concanavalin A (Con A) and 3,5-diethocarbonyl-1,4-dihydrocollidine (DDC), respectively. In both models, Npnt was localized in inflammatory foci and was mainly secreted from mesenchymal cells and in part by cholangiocytes. Interestingly, ectopic expression of Npnt in hepatocytes induced the development of granuloma-like cell clusters mainly composed of $CD4^+$ T cells or NKT cells but did not induce apparent hepatitis. Furthermore, we found that Npnt exacerbated the Con A-induced acute hepatitis. These results indicate that Npnt plays an important role in the initiation of hepatitis by recruiting $CD4^+$ T cells or NKT cells into the foci of inflammation. In addition, we reveal that Npnt expression is also upregulated in human hepatitis. Therefore, Npnt may be a potential therapeutic target for acute and chronic hepatitis.

1. Introduction

Previously we performed a microarray analysis of cholangiocytes derived from a normal liver and 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC)-induced chronic hepatitis liver [1]. We found that the expression of about 50 genes was significantly upregulated in chronic hepatitis liver. Most genes were related to fibrosis, epithelial-mesenchymal transition, hepatic stem/progenitor cell response. Among these genes, *Nephronectin* (*Npnt*) was expressed 5-fold higher in DDC-induced chronic hepatitis liver than in normal liver.

Npnt is an extracellular matirix (ECM) protein, containing an Arg-Gly-Asp (RGD) motif. ECMs are mesh structures composed of proteins and carbohydrates, filling intercellular spaces. The interaction of ECM with cell surface integrins plays crucial roles in development, immunity, inflammation, and homeostasis by regulating cell adhesion, migration, growth, differentiation, and apoptosis [2–4]. The binding of integrin to ECM depends on an RGD motif in ECM proteins [5,6]. The RGD motif is the cell recognition site involved in various ECMs and platelet adhesion proteins, such as fibronectin, vitronectin, osteopontin (OPN), and Npnt [7–10]. Among them, OPN is known to exacerbate hepatitis [11–13].

Although Npnt has been known to play an important role in kidney development [14,15], its functions in the liver remain unknown. Considering that both Npnt and OPN contain an RGD motif and that OPN is a key modulator in hepatitis, it is anticipated that Npnt may also play some roles in liver inflammation. Here we elucidate the roles of Npnt in hepatitis.

2. Materials and methods

See Supplementary materials and methods for more details.

Abbreviations: Npnt, nephronectin; OPN, osteopontin; Con A, concanavalin A; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-collidine; NKT, natural killer T cells; BD, bile duct; PV, portal vein; CV, central vein; ECM, extracellular matrix; HTVi, hydrodynamic tail vein injection; PC, parenchymal cell; CK19, cytokeratin 19; Thy1, thymocyte differentiation antigen 1; EpCAM, epithelial cell adhesion molecule; AST, aspartate amino transferase; ALT, alanine transaminase; RGD, arginine–glycine–aspartate; SCID, severe combined immunodeficiency.

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2.1. Animals

Specific pathogen-free male C57BL/6J mice and FOX CHASE SCID mice were purchased from Nihon CLEA (Tokyo, Japan). All experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the University of Tokyo (approval number is 24003).

2.2. Con A-induced acute hepatitis and DDC-induced chronic hepatitis

Concanavalin A (Con A) was purchased from Sigma–Aldrich (St. Louis, MO) and was injected intravenously into mice through the tail vein. The dose of Con A was 20 mg/kg or 15 mg/kg. A diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) was purchased from Nihon CLEA (Tokyo, Japan).

2.3. Overexpression by hydrodynamic tail vein injection (HTVi) method

Mouse Npnt cDNA was amplified by PCR (primers (5′ – 3′): GCTAGCGCAGCTGGCTTTCTTCGAGGC and CTCGAGGCTCAAGCCAGAGCCAATGGC) and was inserted into the NheI and XhoI sites of a pLIVE vector (Mirus, Madison, WI). pLIVE-Npnt (50 μg) was diluted with TransIT-EE Hydrodynamic Delivery Solution (Mirus, Madison, WI) and injected into the tail vein of 7-week-old male C57BL/6 mice. pLIVE-SEAP (secreted alkaline phosphatase) (50 μg) was used as a control. One week after injection, mice were sacrificed for histological analysis.

2.4. Statistical analysis

Student's *t*-tests with equal variance and two-tailed distribution were used to determine the significance of differences between two groups (Excel statistical analysis software, Microsoft Japan, Tokyo, Japan). A *P*-value of 0.05 or lower was considered significant. Results where indicated are expressed as mean ± standard error.

3. Results

3.1. Expression of Npnt in mice hepatitis models

To investigate the correlation of Npnt expression with hepatitis, we examined Npnt mRNA levels in both the acute hepatitis model induced by Con A administration and the chronic hepatitis model induced by DDC diet (Fig. 1A and B) [16,17]. Npnt expression was rapidly induced at 12 h and decreased gradually thereafter in Con A-induced acute hepatitis. While, in chronic hepatitis, Npnt expression levels increased 2 weeks after feeding of DDC diet. These results suggested that Npnt expression correlated with the severity of hepatitis. Immunohistochemistry (IHC) also revealed that Npnt was strongly expressed in areas around portal veins, bile ducts, and central veins in Con A-induced acute hepatitis livers, while it was weakly expressed in areas around portal veins and central veins in normal livers (Fig. 1C–F). Strong expression of Npnt was also observed in the livers of DDC-induced chronic hepatitis in

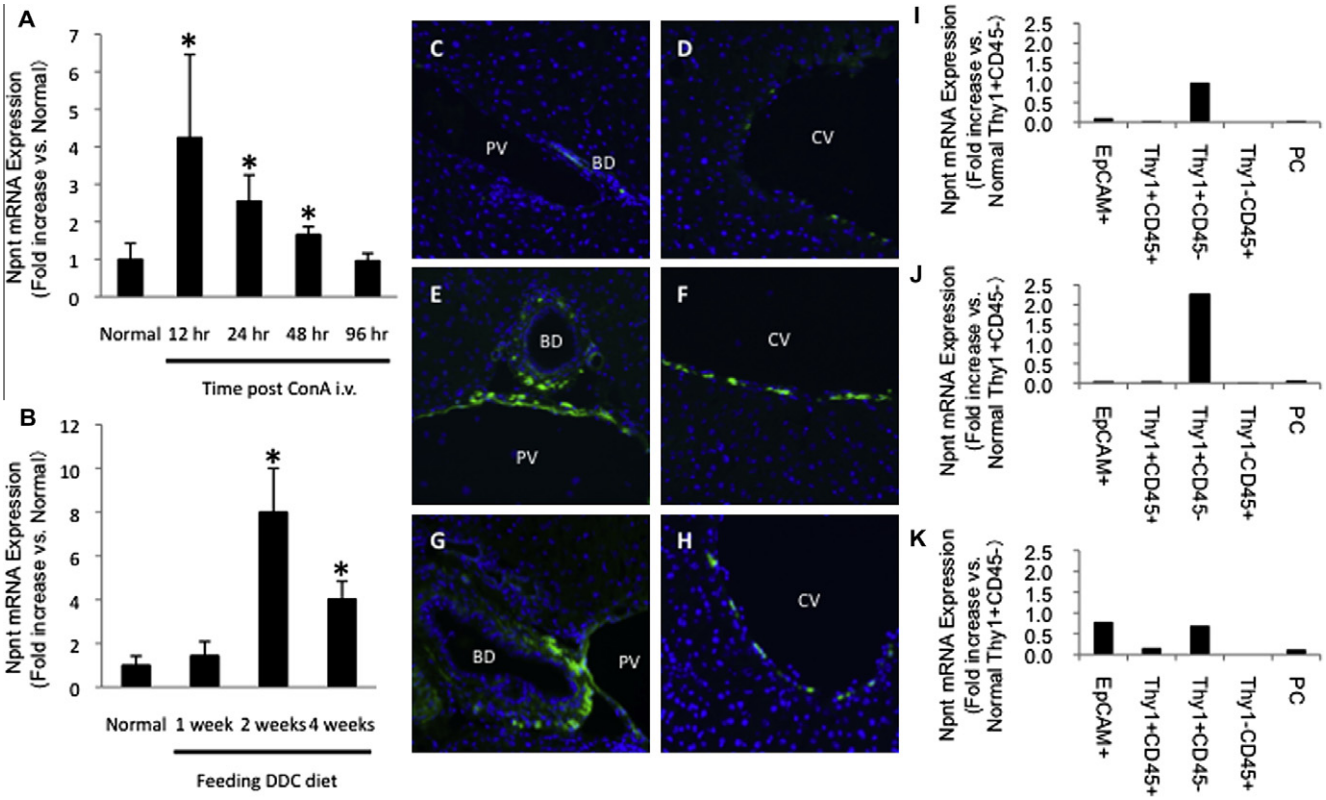

Fig. 1. Npnt expression in normal, acute and chronic hepatitis livers and identification of liver cells expressing Npnt. (A, B) Expression of mouse liver Npnt mRNA was analyzed in Con A-induced acute liver injury (A) and DDC-induced chronic liver injury (B). Con A (20 mg/kg) was injected into mice intravenously. The fold increase of Npnt mRNA in injured livers relative to normal livers is shown. Beta-actin mRNA was used as an internal control. $n = 5$ per group. $^{*}p < 0.05$. (C–H) IHC of Npnt in a normal liver (C, D), acute hepatitis liver 12 h after intravenous injection of Con A (20 mg/kg) (E, F), and chronic hepatitis liver 2 weeks after feeding of the DDC diet (G, H). Liver sections were immunostained with anti-Npnt antibody (green). Nuclei were counterstained with Hoechst (blue). Original magnification, 200×. PV: portal vein, BD: bile duct, CV: central vein (I–K) Analyses of Npnt mRNA expression in various types of cells from a normal liver (I), acute hepatitis liver 12 h after Con A (20 mg/kg) injection (J), and chronic hepatitis liver 2 weeks after feeding of the DDC diet (K). Each type of cell was isolated from livers based on the expression of EpCAM, Thy1, and CD45 by a cell sorter.

areas similar to acute hepatitis, especially around portal veins and bile ducts (Fig. 1G and H).

Inflammatory liver diseases are known to be accompanied by an increase in the number of neutrophils, NKT cells, and T cells [18,19]. Histological examinations consistently showed the infiltration of blood cells into periportal and pericentral areas in Con A-induced acute hepatitis livers, whereas a few blood cells were detected in normal livers (Supplementary Fig. 1A and B). Livers in DDC-induced chronic hepatitis exhibited a marked infiltration of blood cells into the periportal area (Supplementary Fig. 1C). These results raise the possibility of a close relationship between the infiltration of blood cells and expression of Npnt in both acute and chronic hepatitis.

3.2. Identification of Npnt-producing cells

In order to identify Npnt-producing cells, we isolated intrahepatic cell components from normal, acute and chronic hepatitis liver, respectively. We sorted 5 cell populations; EpCAM$^+$, EpCAM$^-$ Thy1$^+$ CD45$^+$, EpCAM$^-$ Thy1$^+$ CD45$^-$, EpCAM$^-$ Thy1$^-$ CD45$^+$, and parenchymal cells (PC), which correspond to cholangiocytes, T lymphocytes, mesenchymal cells (fibroblast and/or myofibroblast), hematopoietic cells except for T lymphocytes, and hepatocytes, respectively. Npnt was expressed only in mesenchymal cells in normal liver (Fig. 1I) and increased in Con A-induced hepatitis (Fig. 1J). Immunostaining revealed that Thy1$^+$ mesenchymal cells around portal and central veins were more abundant in Con A-induced hepatitis liver compared to normal liver (Supplementary Fig. 2A and B). In the DDC-fed liver, Npnt was mainly expressed in mesenchymal cells and cholangiocytes (Fig. 1K) and the number of cholangiocytes and mesenchymal cells increased markedly compared to that in normal liver (Supplementary Fig. 2A and C).

3.3. Overexpression of Npnt in liver induces granuloma-like cell clusters

To reveal the physiological role of Npnt in the liver, we ectopically expressed Npnt in livers using the hydrodynamic tail vein injection (HTVi) method. This is a simple method to achieve hepatocyte-specific gene expression by injecting expression vectors with a large amount of buffer, into the mice tail vein and is suitable for assessment of *in vivo* function of secreted proteins [20,21]. Histological examination showed that granuloma-like cell clusters developed in livers expressing Npnt (Fig. 2A and B), whereas such clusters were not found in livers that received the control plasmid vector (Fig. 2C). Granuloma-like cell clusters (White arrow) are formed around Npnt positive area (Fig. 2E and F), suggesting that Npnt plays an important role in the recruitment of immune cells.

It should be noted that upregulation of Npnt expression and infiltration of blood cells were observed in the two hepatitis models (Supplementary Fig. 1). In general, granulomas are mainly comprised of macrophages, neutrophils, and lymphocytes. Interestingly, Npnt overexpression by HTVi in SCID mice did not induce the formation of granuloma-like cell clusters (Fig. 2D). Because SCID mice lack T cells, B cells, and NKT cells, the clusters of cells recruited by Npnt overexpression in C57BL/6 mice are likely

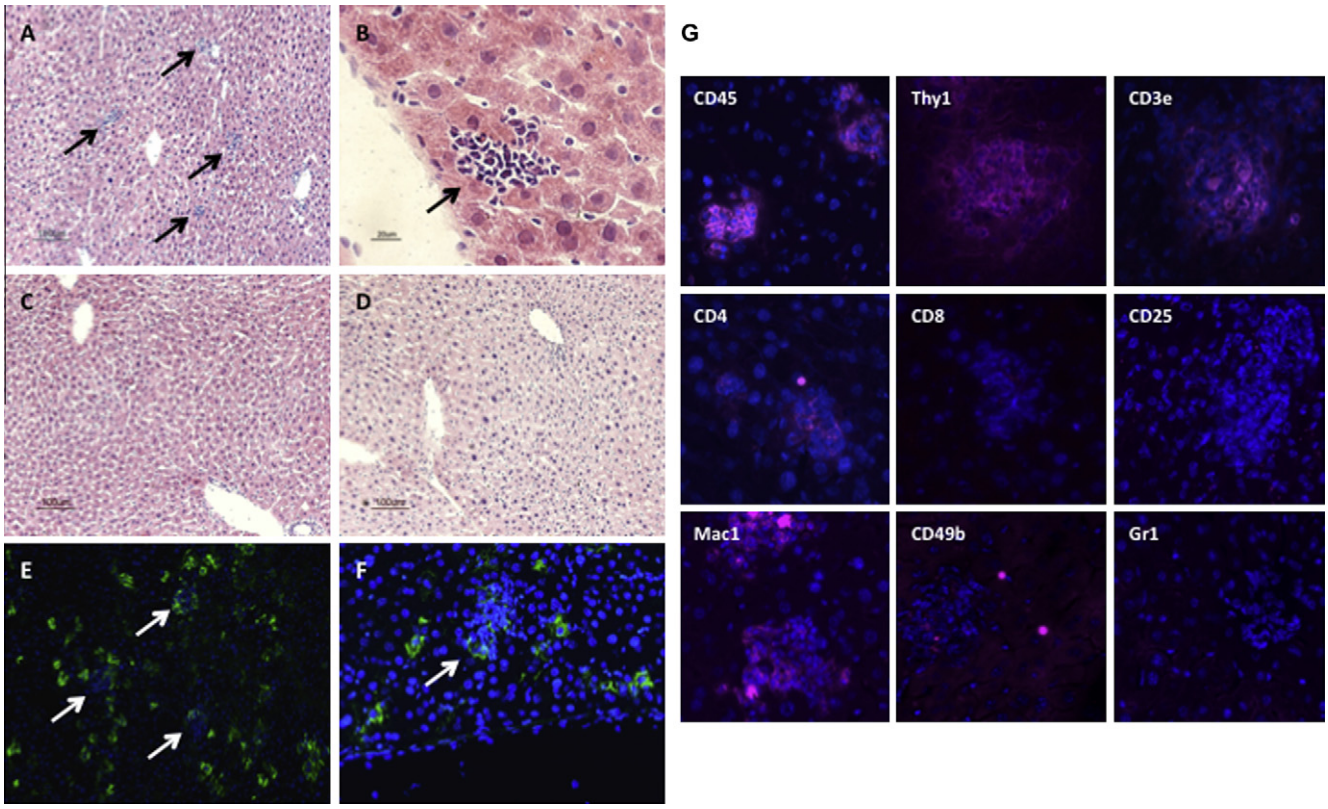

Fig. 2. *In vivo* overexpression of Npnt induces granuloma-like cell cluster formation. (A–D) Histological analysis of mouse livers overexpressing Npnt or SEAP (used as control). (A, B) C57BL/6J mice were analyzed 1 week after HTVi of pLIVE-Npnt. (C) C57BL/6J mice were analyzed 1 week after HTVi of pLIVE-SEAP. (D) SCID mice were analyzed 1 week after HTVi of pLIVE-Npnt. Granuloma-like cell clusters (black arrow) are observed only in Npnt overexpressing C57BL/6J mice. Sections were stained with hematoxylin and eosin. Original magnification, 100× (A, C and D) and 400× (B). (E, F) IHC of Npnt in Npnt overexpressing liver. Npnt producing hepatocytes and secreted Npnt are stained green. Granuloma-like cell clusters (white arrow) are formed around highly-concentrated area of secreted Npnt. Original magnification, 100× (E) and 400× (F). (G) IHC of granuloma-like cell clusters by various cell surface markers in C57BL/6J mice after HTVi of pLIVE-Npnt plasmid. Liver sections were stained with anti-CD45, Thy1, CD3e, CD4, CD8, CD25, Mac1, CD49b, Gr1 antibody, respectively (magenta), and nuclei were counterstained with Hoechst (blue). Original magnification, 400×.

to be composed of these immune cells, consistent with recent reports that the numbers of NKT cells and T cells were increased in inflammatory liver disease such as Con A-induced hepatitis [18,19].

3.4. Characterization of blood cells recruited by Npnt

We next characterized the immune cells forming granuloma-like cell clusters in livers expressing Npnt. Immunostaining of liver sections with several cell surface markers revealed that the cells forming granuloma-like clusters were almost all positive for CD45, Thy1, and CD3e. $CD4^+$, $Mac1^+$ and $CD49b^+$ cells were included in some cell clusters, whereas none of the clusters examined contained $CD8^+$, $CD25^+$ and $Gr1^+$ cells (Fig. 2G). These results suggested that Npnt recruited either $CD4^+$ T cells or NKT cells.

3.5. Overexpression of Npnt exacerbates Con A-induced acute hepatitis

It is known that NKT cells and T cells play a critical role in hepatitis including the Con A-induced acute hepatitis model, while the depletion of liver NK cells fails to inhibit Con A-induced hepatic injury [22]. It should be noted that overexpression of Npnt in livers induced the formation of granuloma-like cell clusters consisting of $CD4^+$ T cells or NKT cells, but did not augment liver injury as measured by serum markers such as AST and ALT (data not shown). These results suggest that Npnt plays a crucial role in the recruitment of immune cells to inflammatory foci, but does not activate the recruited cells, raising the possibility that Npnt may be mainly involved in the initial step of the pathogenesis of hepatitis and that $CD4^+$ T cells or NKT cells recruited to inflammatory foci are activated by other stimuli such as inflammatory cytokines.

In order to investigate the effect of Npnt in hepatitis, we injected Con A (15 mg/kg) intravenously into mice seven days after HTVi of Npnt expression or control vector. In control mice, the severity of liver damage, as reflected by serum AST and ALT levels, peaked at 12 h and then declined 72 h after Con A injection. In contrast, mice injected with the Npnt expression vector exhibited more severe liver injury with a time course similar to that in control mice (Fig. 3A and B). These data suggested that $CD4^+$ T cells or NKT cells recruited by Npnt are involved in liver injury by the activation of their cytotoxicity.

3.6. The RGD motif is responsible for the formation of granuloma-like cell clusters

The RGD motif has been known to be a prerequisite for the integrin binding activity of Npnt [9,10]. In order to reveal whether the interaction between integrin and Npnt is responsible for the formation of granuloma-like cell clusters or not, we constructed an Npnt mutant lacking the RGD motif (Fig. 3C middle) and expressed

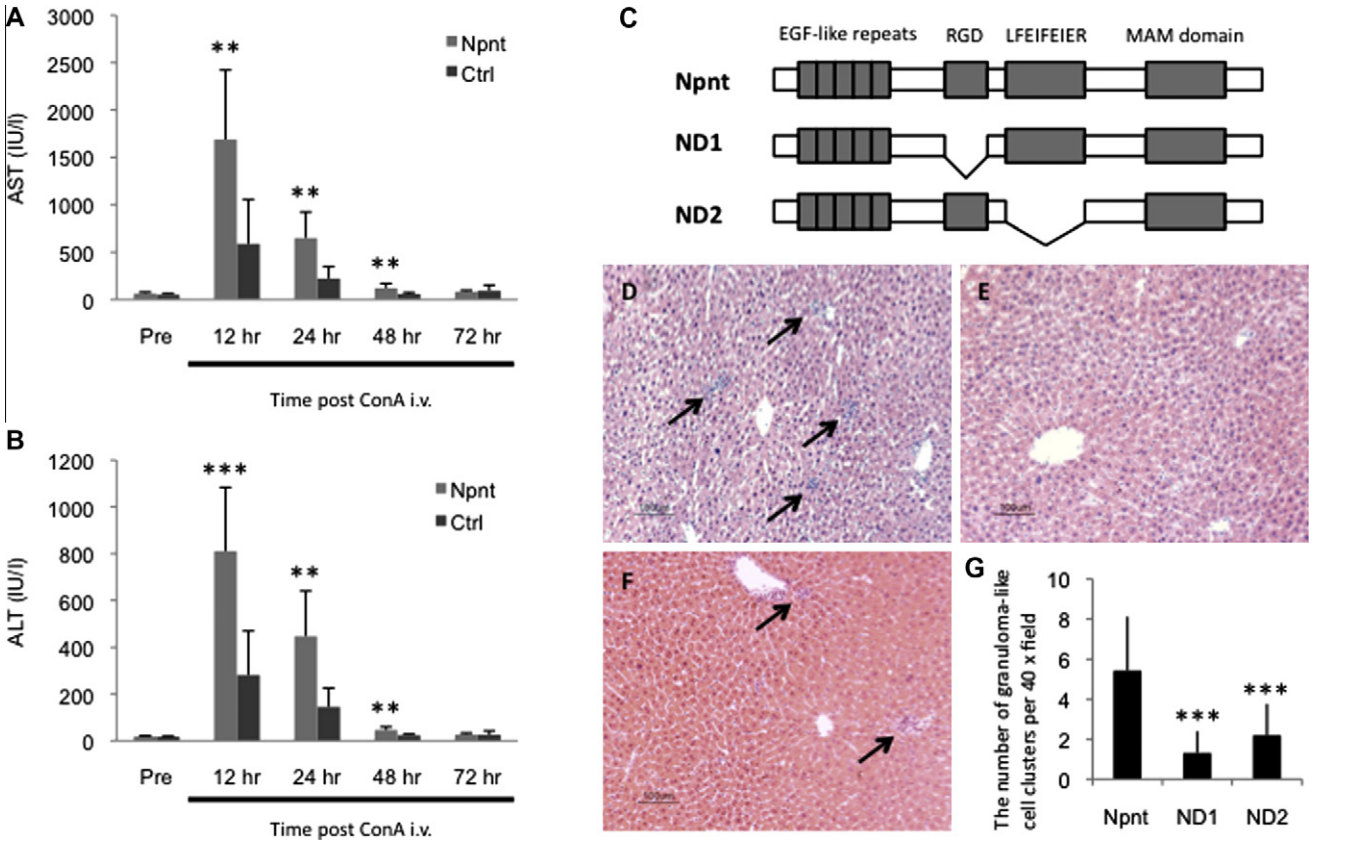

Fig. 3. *In vivo* overexpression of Npnt exacerbates Con A-induced acute hepatitis and RGD motif is necessary for the formation of granuloma-like cell clusters. Plasma aspartate aminotransferase (AST) levels (U/L) (A) and plasma alanine aminotransferase (ALT) levels (U/L) (B) were analyzed in pLIVE-SEAP (Ctrl vector) HTVi or pLIVE-Npnt HTVi mice at 0 (Pre), 12, 24, 48 and 72 h after intravenous injection of Con A (15 mg/kg). $n = 6$ (Ctrl), 5 (Npnt HTVi) per time point. $^{**}p \leqq 0.01$, $^{***}p \leqq 0.001$ vs. Ctrl vector HTVi group. (C) Schematic of wild type Npnt (top: Npnt), RGD deletion mutant (middle: ND1), and LFEIFEIER deletion mutant (lower: ND2). (D–F) Representative histology of livers. Livers of C57BL/6J mice were analyzed 1 week after injection of pLIVE-Npnt (wild type) (D), pLIVE-Npnt RGD deletion mutant (E), or pLIVE-Npnt LFEIFEIER deletion mutant (F) plasmids by HTVi. Sections were stained with hematoxylin and eosin. Granuloma-like cell clusters are observed (black arrow). Original magnification, 100×. (G) Quantification of granuloma-like clusters. The average number of granuloma-like clusters per 40× field of view is shown. Twenty-five random fields from 5 mice per group were used for analysis. $^{***}p \leqq 0.001$ vs. native form of Npnt.

it in the liver by HTVi method. The number of granuloma-like cell clusters for the deletion mutant in livers was significantly lower than that for the native form of Npnt (Fig. 3D, E and G). Sato et al. reported that the LFEIFEIER sequence at the C-terminal side of the RGD motif plays a supportive role in high-affinity binding to α8β1 integrin [23]. We therefore constructed a deletion mutant lacking the LFEIFEIER sequence (Fig. 3C lower) and expressed it in the liver by HTVi method. The deletion mutant induced the formation of granuloma-like cell clusters at an intermediate level between the native form of Npnt and the RGD deletion mutant (Fig. 3D, F and G). These results strongly suggested that the RGD motif mediated the recruitment of $CD4^+$ T cells or NKT cells and that the LFEIFEIER sequence partly contributed to the recruitment.

3.7. Expression of Npnt in human livers

To examine the possibility that Npnt is involved in human hepatitis, we investigated Npnt expression in human liver specimens. In normal livers, Npnt was weakly expressed in periportal areas (Fig. 4A and B). In contrast, Npnt was strongly and extensively expressed in periportal fibrotic areas of chronic hepatitis livers where the infiltration of numerous immune cells was observed (Fig. 4C and D). These data indicate that Npnt is upregulated and involved in the pathogenesis of hepatitis in humans as well as mice.

4. Discussion

In this study, we have investigated the pathogenic role of Npnt in liver injury. The expression of Npnt mRNA was transiently upregulated in our mouse models of acute and chronic hepatitis. In Con A-induced acute hepatitis, inflammatory foci were found in the portal area and around the central vein [24]. In this model, Npnt-expressing cells were $Thy1^+CD45^-$ cells, possibly mesenchymal cells, in periportal and pericentral areas. On the other hand, in DDC-induced chronic hepatitis, also known as cholangitis, inflammatory foci were observed within Glisson's capsules, especially around bile ducts [17]. In this model, Npnt expressing cells were $Thy1^+CD45^-$ cells and $EpCAM^+$ cholangiocytes. In both injury models, Npnt expressing cells were clearly increased and localized in inflammatory foci. Overexpression of Npnt by the HTVi method induced the development of granuloma-like cell clusters in the parenchymal area of the liver. Since plasmid DNA is mainly introduced into hepatocytes by HTVi, it is suggested that the ectopic expression of Npnt in hepatocytes contributes to the recruitment of immune cells and formation of cell clusters in the parenchymal area. IHC revealed that granuloma-like cell clusters consist of cells of the T cell lineage ($Thy1^+$ $CD3e^+$ $CD4^{+/-}$ $CD8^-$ $CD25^-$ $Mac1^{+/-}$ $CD49b^{+/-}$), mainly $CD4^+$ T cells and NKT cells, as the latters are $CD49b^+$ and partially $CD4^+$ and $Mac1^+$ [25,26]. A previous study using T cell-deficient mice and T cell depletion with specific antibodies revealed that Con A-induced liver injury was mediated by $CD4^+$ T cells [27,28]. In addition, Takeda et al. [29] reported that CD1d knockout mice lacking NKT cells were resistant to Con A, indicating a role for NKT cells in acute hepatitis. Consistently, overexpression of Npnt by HTVi in SCID mice, which lack T cells, B cells, and NKT cells, failed to induce granuloma-like cell clusters, indicating that these cells are involved in the development of granuloma-like cell clusters. Furthermore we showed that $CD4^+$ T cells and NKT cells were recruited by the interaction of cell surface integrin and the RGD motif.

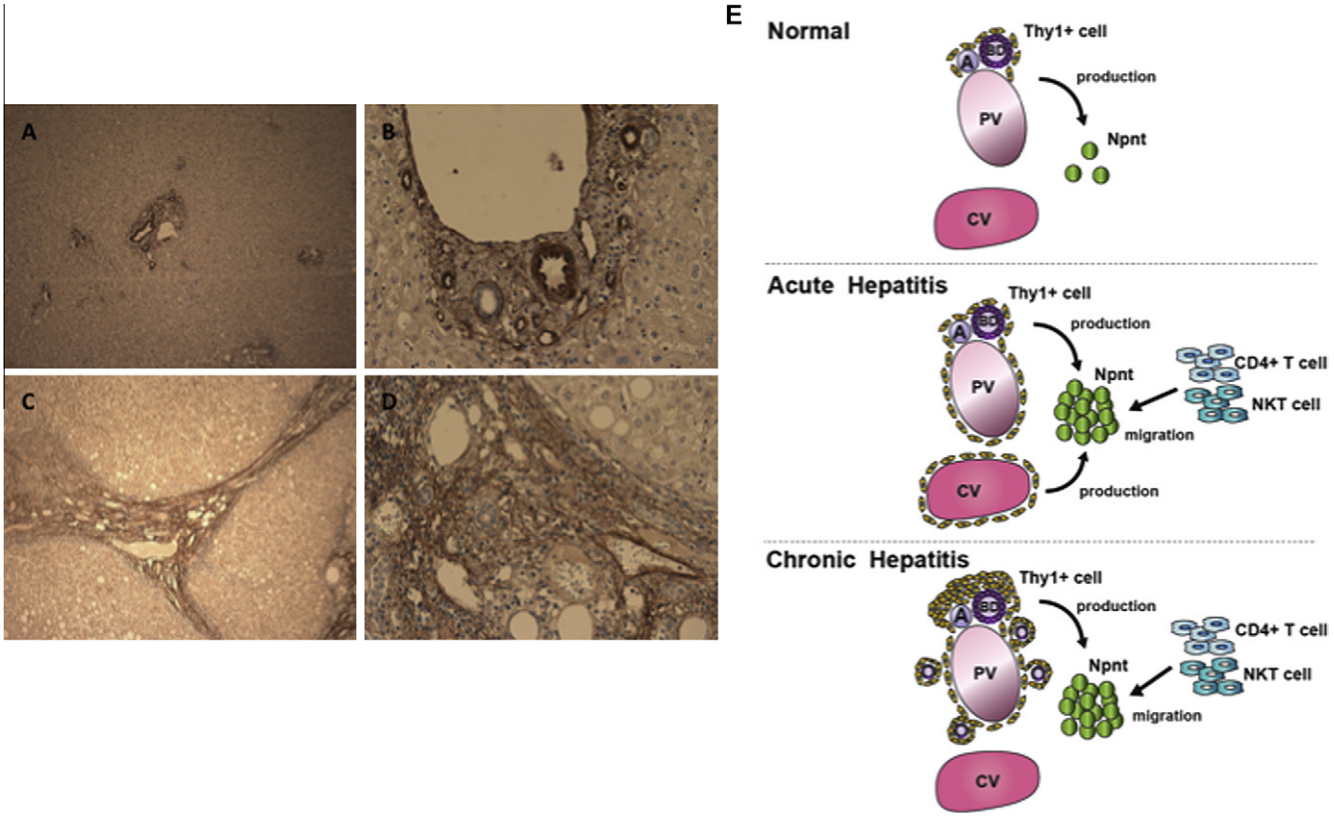

Fig. 4. Npnt expression in human liver sections and a model of the Npnt role in hepatitis. (A–D) Npnt immunostaining of normal (A, B) and cirrhotic (C, D) human liver tissue. Color was developed using metal enhanced diaminobenzidine as a substrate. Counterstaining was performed with hematoxylin. Original magnifications, 50× (A, C); 200× (B, D). (E) In a normal liver, $Thy1^+$ cells produce a small amount of Npnt. In Con A-induced acute hepatitis, $Thy1^+$ cells proliferate in both periportal and pericentral areas and produce a large amount of Npnt. In DDC-induced chronic hepatitis, the outgrowth of bile ducts and surrounding stromal tissue is induced in the portal area. In addition to $Thy1^+$ cells, cholangiocytes produce Npnt. In both cases, secreted Npnt promotes the migration of NKT cells and $CD4^+$ T cells and these cells finally contribute to the exacerbation of hepatitis. PV: portal vein, BD: bile duct, A: hepatic artery, CV: central vein.

Con A-activated NKT cells are known to secrete osteopontin (OPN), another matrix protein containing the RGD motif [11]. However, the mechanism of recruiting NKT cells into the inflammatory foci in injured liver has remained unknown. Our study revealed that Npnt played a crucial role for recruiting $CD4^+$ T cells or NKT cells into the liver and tethering them in inflammatory foci. Furthermore, Npnt expression by HTVi exacerbated Con A-induced acute hepatitis. These results suggest the synergistic action of Npnt, OPN, and Con A for the recuitment and activation of NKT cells, which also explains why the administration of non-peptide mimetics of the RGD motif ameliorates Con A-induced liver injury [30].

In summary, our study revealed the pathogenic role of Npnt in hepatitis as shown in Fig. 4E. In normal livers, mesenchymal cells produce a small amount of Npnt to maintain the immune system around portal veins in livers. In Con A-induced acute hepatitis, mesenchymal cells proliferate in periportal and pericentral areas and produce a large amount of Npnt. In DDC-induced chronic hepatitis, bile ducts together with the surrounding stromal tissue proliferate and cholangiocytes, in addition to mesenchymal cells, produce Npnt, leading to cholangitis. In both cases, Npnt promotes the infiltration of $CD4^+$ T cells or NKT cells via an interaction between the RGD motif and integrin. This is the first report showing the role of Npnt in hepatitis via the recruitment of $CD4^+$ T cells or NKT cells. NKT cells also secrete OPN, contributing to the progression of hepatitis as described previously [11]. Since Npnt knockout mice display renal agenesis or kidney hypoplasia at birth and are lethal, further investigation using conditional knockout mice would help us better understand the roles of Npnt in liver diseases. Because mouse and human Npnt share 88% sequence homology [31], functions of Npnt may be conserved in human and we, in fact, showed that Npnt was expressed more extensively in human chronic hepatitis livers than in normal livers. It would be interesting to study whether the expression level of Npnt is correlated with the severity of hepatitis or the prognosis of patients. While further clinical studies to confirm our present findings are necessary, Npnt may become a potential therapeutic target for acute and chronic hepatitis.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.076.

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