Acyclic retinoid targets platelet-derived growth factor signaling in the prevention of hepatic fibrosis and hepatocellular carcinoma development

Running title: Acyclic retinoid prevents hepatic fibrosis and HCC

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The authors declare that no potential competing interests exist.

Funding:
This work was supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare, and KOWA Co., Ltd., Tokyo, Japan

Abbreviations: ACR, Acyclic Retinoid; CH-C, Chronic Hepatitis C; HCC, Hepatocellular Carcinoma; HCV, Hepatitis C Virus; HSCs, Hepatic Stellate Cells; PDGF, Platelet-Derived Growth Factor; Tg, Transgenic; RTD-PCR, quantitative real-time detection-polymerase chain
reaction; IHC, immuno histochemical; EMT, epithelial-mesenchymal transition.

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Abstract

Hepatocellular carcinoma (HCC) often develops in association with liver cirrhosis, and its high recurrence rate leads to poor patient prognosis. Although recent evidence suggests that peretinoin, a member of the acyclic retinoid family, may be an effective chemo-preventive drug for HCC, published data regarding its effects on hepatic mesenchymal cells, such as stellate cells and endothelial cells, remain limited. Using a mouse model in which PDGF-C is over expressed (Pdgf-c Tg), resulting in hepatic fibrosis, steatosis, and eventually, HCC development, we demonstrate that peretinoin significantly represses the development of hepatic fibrosis and tumors. Peretinoin inhibited the signaling pathways of fibrogenesis, angiogenesis and Wnt/beta-catenin in Pdgf-c transgenic mice. In vitro, peretinoin repressed the expression of PDGF receptors (PDGFR) alpha/beta in primary mouse hepatic stellate cells (HSCs), hepatoma cells, fibroblasts, and endothelial cells. Peretinoin also inhibited PDGF-C-activated transformation of HSCs into myofibroblasts. Together, our findings demonstrate that PDGF signaling is a target of peretinoin in preventing the development of hepatic fibrosis and HCC.
Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with a particularly poor patient outcome (1). It often develops as a result of chronic liver disease associated with hepatitis B (HBV) or hepatitis C virus (HCV) infection or with other etiologies such as long term alcohol abuse, autoimmunity and hemochromatosis (2-5). Despite the recent advances in antiviral therapy for HBV or HCV, these are insufficient to completely prevent the occurrence of HCC. Moreover, the recent increase in non-alcoholic fatty liver disease (NAFLD) associated with metabolic syndrome is a potential high-risk factor for the development of HCC (6).

HCC often develops during the advanced stages of liver fibrosis and is associated with deposits of extracellular matrix synthesized by activated stellate cells. During the course of chronic hepatitis, non-parenchymal cells, including Kupffer, endothelial and activated stellate cells, release a variety of cytokines and growth factors. One of these growth factors is platelet-derived growth factor (PDGF), which is involved in fibrogenesis, angiogenesis and tumorigenesis (7, 8). PDGF expression has been shown to be up-regulated from the early stages of chronic hepatitis, suggesting its association with the development of fibrosis in chronic hepatitis C (CH-C) (9, 10). Over-expression of PDGF-C in mouse liver resulted in the progression of hepatic fibrosis, steatosis and the development of HCC; this mouse model closely resembles the human HCC that is frequently associated with hepatic fibrosis (7).

Peretinoin (generic name; code, NIK-333), developed by the Kowa Company (Tokyo, Japan), is an oral acyclic retinoid (ACR) with a vitamin A-like structure that targets the retinoid nuclear receptor. Oral administration of peretinoin was shown to significantly reduce the incidence of post-therapeutic HCC recurrence and improve the survival rates of patients in a clinical trial (11, 12). A large-scale clinical study including various countries is now planned to confirm its clinical efficacy.

Although peretinoin treatment can suppress HCC-derived cell line growth and inhibit
experimental mouse or rat liver carcinogenesis (13, 14), the detailed mechanism of its effect has not been fully elucidated. Peretinoin has a high binding affinity to cellular retinoic acid-binding protein (CRABP) (15) and may interact with retinoic acid receptor-β (RAR β) and retinoid X receptor-α (RXR α) (16), however the precise molecular targets for preventing HCC recurrence have not yet been elucidated.

In this study, we used PDGF-C transgenic (\textit{Pdgf-c Tg}) mice to show that PDGF-C signaling is a possible target of peretinoin in the prevention of hepatic fibrosis, angiogenesis and the development of HCC.

\textbf{Materials and methods}

\textbf{Chemicals}

The acyclic retinoid peretinoin (generic name; code, NIK-333) [(2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, C20H30O2, molecular weight 302.46 g/mol] was supplied by Kowa Company (Tokyo, Japan).

\textbf{Animal studies}

The generation and characterization of \textit{Pdgf-c Tg} have been described previously (7). Wild-type and \textit{Pdgf-c Tg} mice on a C57BL/6J background were maintained in a pathogen-free animal facility under a standard 12/12-h light/dark cycle. After weaning at week 4, male mice were randomly divided into the following three groups: 1) \textit{Pdgf-c Tg} or wild-type (WT) mice given a basal diet (CRF-1, Charles River Laboratories Japan, Yokohama, Japan), 2) \textit{Pdgf-c Tg} or WT mice given a 0.03% or 0.06% peretinoin-containing diet, 3) \textit{Pdgf-c Tg} or WT mice given a 0.06% peretinoin-containing diet. Control mice were normal male homozygotes. At week 20, mice were sacrificed to analyze the progression of hepatic fibrosis (n=15 for each of
the three groups). At week 48, mice were sacrificed to analyze the development of hepatic tumors (n=31 for the basal diet group, n=37 for the 0.03% peretinoin group and n=17 for the 0.06% peretinoin group). The incidence of hepatic tumors, maximum tumor size and liver weight were evaluated. None of the treated WT mice given a diet of 0.03% peretinoin died, but death occurred in 5% of WT mice around after 36 weeks of age receiving a 0.06% peretinoin diet, probably because of its toxicity. In Pdgf-c Tg mice, death was observed at similar frequency as WT mice that received 0.06% peretinoin diet.

All animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals at the Takara-Machi Campus of Kanazawa University.

**Cell culture**

Human HCC cell lines Huh-7, HepG2, and HLE, the mouse fibroblast cell line NIH3T3, human umbilical vein endothelial cells (HUVECs) and human stellate cells Lx-2 (kindly provided by Dr. Scott Friedman) were maintained in Dulbecco’s modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco) in a humidified atmosphere of 5% CO₂ at 37 °C. 1–5×10⁴ cells were seeded in each well of a 12-well plate the day before serum starvation in serum-free DMEM for 8 h. The culture medium was then replaced with serum-free medium containing peretinoin. After a further 24 h incubation, cells were harvested for analysis.

**Isolation and culture of mouse HSCs**

Hepatic stellate cells (HSCs) were isolated from C57BL/6J mice and the effect of recombinant human PDGF-C and peretinoin on HSCs was evaluated *in vitro*. Pronase-collagenase liver digestion was used to isolate HSC from wild-type mice. All experiments were replicated at least twice. Freshly isolated HSCs suspended in culture
medium were seeded in uncoated 24-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 72 h. Non-adherent cells were removed with a pipette and the culture medium was replaced with medium containing 80 ng/mL recombinant human PDGF-C (Abnova, Taipei, Taiwan) with or without peretinoin or 9-cis-retinoic acid (9cRA) (5 μM or 10 μM). Cells were harvested for analysis after a further 24-h incubation.

**Isolation of peripheral blood mononuclear cells**

Peripheral blood mononuclear cells were harvested and labeled with FITC-conjugate CD34 (Cell Lab, Fullerton, CA) and R-Phycoerythrin (PE)-conjugated CD31 antibodies (Cell Lab) for 30 min at 4 °C. After washing with 1 ml PBS, CD31 and CD34 surface expression was measured with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). All flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**Gene expression profiling**

Gene expression profiling in mouse liver was evaluated using the GeneChip Mouse Genome 430 2.0 Array (Affymetrix). Liver tissue from WT, Pdgf-c Tg and Pdgf-c Tg with 0.06% peretinoin mice all at week 20 and week 48 was obtained and a total of 34 chip assays were performed as described previously (17). Expression data have been deposited in the Gene Expression Omnibus (GEO; NCBI Accession; GSE31431).

Pathway analysis was performed using MetaCore (GeneGo, St. Joseph, MI). Functional ontology enrichment analysis was performed to compare the Gene Ontology (GO) process distribution of differentially expressed genes (p<0.01) (10, 17). Direct interactions among differentially expressed genes between Pdgf-c Tg mice with or without peretinoin administration were examined as reported previously (10). Each connection represents a direct, experimentally confirmed, physical interaction (MetaCore).
Histopathology and immunohistochemical (IHC) staining

Mouse liver tissues were fixed in 10% formalin and stained with hematoxylin-eosin (H&E). The liver neoplasms (HCC and liver cell adenoma) were diagnosed according to previously described criteria (18, 19). Hepatic fibrosis was evaluated by Azan staining. Percentages of fibrous areas were calculated microscopically using an image analysis system (BIOREVO BZ-9000: KEYENCE Japan, Osaka, Japan). Immunohistochemical (IHC) staining was performed by an immunoperoxidase technique with an Envision kit (DAKO, Carpinteria, CA). Primary antibodies used were: rabbit polyclonal PDGFR-α (1:100 dilution), PDGFR-β (1:100 dilution), VEGFR1 (1:100 dilution), desmin (1:100 dilution), β-catenin (1:200 dilution), mouse monoclonal cyclin D1 (1:400 dilution) (all from Cell Signaling Technology, Beverly, MA), collagen 1 (1:100 dilution), collagen 4 (1:100 dilution), CD31 (1:100 dilution), CD34 (1:100 dilution) (all from Abcam, Cambridge, MA), Tie-2 (1:80 dilution) and Myc (1:100 dilution) (both from Santa Cruz Biotechnology, Santa Cruz, CA).

Quantitative real-time PCR (RTD-PCR)

Total RNA was isolated from frozen liver tissue samples using a GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO), according to the manufacturer’s protocol. cDNA was synthesized from 100 ng total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) then mixed with the TaqMan Universal Master Mix (Applied Biosystems) and each TaqMan probe. TaqMan probes used were PDGFR-α/β, VEGFR1/2, α-SMA, collagen 1/4, β-catenin, CyclinD1, and Myc (Applied Biosystems). Relative expression levels were calculated after normalization to GAPDH.

Western blotting

Western blotting was performed as described previously (20). Whole-cell lysates
from mouse liver were prepared and lysed by CellLyticTM MT cell lysis reagent (Sigma-Aldrich) containing Complete Mini EDTA-free Protease Inhibitor cocktail tablets (Roche, Mannheim, Germany). Cytoplasmic and nuclear protein extracts were prepared using the NE-PER nuclear extraction reagent kit (Pierce Biotechnology, Rockford, IL). Primary antibodies used were PDGFR-α (1:1000 dilution), PDGFR-β (1:1000 dilution), VEGFR2 (1:1000 dilution), p44/42 MAPK (1:1000 dilution), total AKT(1:1000 dilution), p-p44/42 MAPK (1:1000 dilution), p-AKT (Ser473) (1:1000 dilution), p-AKT (Thr308) (1:1000 dilution), β-catenin (1:2000 dilution), cyclin D1 (1:400 dilution), lamin A/C (1:1000 dilution) (all Cell Signaling Technology), α-SMA (1:200 dilution; DAKO Japan), 4-HNE (1:200 dilution; NOF, Tokyo, Japan), GAPDH (1:1000 dilution) and Myc (1:1000 dilution) (both Santa Cruz).

Statistical analysis
Results are expressed as means ± S.D. Significance was tested by one-way analysis of variance with Bonferroni’s method, and differences were considered statistically significant at \( P<0.05 \).

Results

Peretinoin prevented the development of hepatic fibrosis in \( \text{Pdgf-c Tg} \)
To evaluate the HCC chemo-preventive effects of peretinoin, we utilized a mouse model of \( \text{Pdgf-c Tg} \) in which PDGF-C is expressed under the control of the albumin promoter (7). Experimental mice were male mice expressing the PDGF-C transgene (\( \text{Pdgf-c Tg} \)) while male mice not expressing the transgene were considered wild-type (WT). After weaning at week 4, \( \text{Pdgf-c Tg} \) or non-transgenic WT mice were fed a basal diet or a diet containing 0.03 or 0.06% peretinoin. At week 20, mice were sacrificed to analyze the progression of hepatic
fibrosis. At week 48, mice were sacrificed to analyze the development of hepatic tumors (Fig. 1A). At week 20, azan staining showed that predominant pericellular fibrosis had developed in Pdgf-c Tg mice (Fig. 1B). Densitometric analysis showed a significant dose-dependent reduction in the size of the fibrotic area in mice that received a diet containing peretinoin at both week 20 and week 48 (Fig. 1C). Peretinoin therefore efficiently repressed the development of hepatic fibrosis in Pdgf-c Tg mice.

The expression of fibrosis-related genes in Pdgf-c Tg mice was evaluated by IHC staining, RTD-PCR and western blotting. The expression of PDGFR-α and PDGFR-β, essential receptors for intra-cellular PDGF-C signaling, was up-regulated mainly in the intracellular or portal area in Pdgf-c Tg mice livers (Fig. 2), but was significantly repressed by peretinoin after weaning at week 4. Similarly, the expression of collagen 1, collagen 4 and desmin was significantly up-regulated in Pdgf-c Tg mice, but repressed by peretinoin (Fig. 2 and Supplemental Fig 1A).

RTD-PCR results confirmed that these genes were substantially up-regulated in Pdgf-c Tg mice and significantly repressed by both 0.03% and 0.06% peretinoin (Fig. 3A). Western blotting analysis showed that the expression of phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2 and cyclin D1, representative markers of the cell proliferation signaling pathway, was up-regulated in Pdgf-c Tg mice, and repressed by peretinoin (Fig. 3B). Thus, peretinoin could partially but significantly prevent the development of hepatic fibrosis in Pdgf-c Tg mice during the study observation period of 48 weeks.

**Peretinoin prevented the development of hepatocellular carcinoma in Pdgf-c Tg mice**

At week 48, Pdgf-c Tg mice developed hepatic tumors with an incidence of 90% (Fig. 4A). Histological assessment of these tumors verified that 54% (15/28) were adenomas and 46% (13/28) were HCC (Fig. 4A, C, and Supplemental Fig. 2) (21). Peretinoin (0.03%) dose-dependently repressed the incidence of hepatic tumors to 53% (19/36) and to 29%
Correlating with tumor incidence, maximum tumor size and liver weight were also significantly repressed by peretinoin (Fig. 4B). Thus, peretinoin repressed the development of hepatic tumors in Pdgf-c Tg mice.

**Serial gene expression profiling in the liver of Pdgf-c Tg mice that developed hepatic fibrosis and tumors**

To examine which signaling pathways were altered during the progression of hepatic fibrosis and tumor development, we analyzed gene expression profiling in the liver of Pdgf-c Tg mice using Affymetrix gene chips. By filtering criteria for $p < 0.001$ and more than 2-fold differences, 538 genes were selected as differentially expressed. One-way hierarchical clustering analysis of differentially expressed genes is shown in Supplemental Fig. 3.

Of the three main clusters, two were up-regulated (cluster A and cluster B) and one was down-regulated (cluster C). Cluster A consisted of immune-related (chemokine (C-C motif) receptor (CCR)4, CCR2, toll-like receptor (TLR)3 and TLR4), apoptosis-related (caspase (CASP)1 and CASP9), angiogenesis and/or growth factor-related (PDGF-C, VEGF-C, osteoponitin, HGF), oncogene-related (v-ets erythroblastosis virus E26 oncogene homolog (Ets)1, Ets2, CD44, N-myc downstream regulated (NDRG)1) and fibrosis-related (tubulin) genes. The expression of cluster A genes was further up-regulated in tumors at week 48. Cluster B consisted mainly of connective tissue- and/or fibrosis-related (vascular cell adhesion molecule (VCAM)1, collagen I, III, IV, V, VI, integrin, decorin, TGF-βRII, PDGFR-α and PDGFR-β) genes, the expression of which declined slightly at week 48. In contrast, Cluster C, containing differentiation- and liver function-related genes (cytochrome P450, family 2, subfamily c (CYP2C)), were down-regulated during the course of hepatic fibrosis and tumor development (Supplemental Fig. 4). Cluster C included xenobiotic and metabolic process-related genes, which are potential targets of peretinoin. Peretinoin treatment prevented hepatic fibrosis and it preserved liver function. In addition, peretinoin might induce
its target genes. Thus, peretinoin reduced the expression of up-regulated genes (cluster A and cluster B) and restored the expression of down-regulated genes (cluster C) at both week 20 and week 48 (Supplemental Fig. 3 and Supplemental Fig. 4).

To examine the molecular network consisting of differentially expressed genes in Pdgf-c Tg mice with or without peretinoin administration, the direct interactions of 513 genes were analyzed by MetaCore (i.e. 413 genes were down-regulated and 100 genes were up-regulated in Pdgf-c Tg mice treated with peretinoin compared with untreated mice; \( p<0.002 \)). A core gene network consisting of 41 genes was obtained (Supplemental Fig. 5) including interactions between representative growth factors, receptors (PDGFR and TGFβR) and transcriptional factors. Of these genes, the transcriptional factors Sp1 and Ap1 appear to be key regulators in the network (Supplemental Fig. 5).

**Peretinoin inhibits PDGFR in vitro**

Gene expression profiling landscaped the dynamic changes of signaling pathways in Pdgf-c Tg mice. To determine the effects of peretinoin in vitro, primary hepatic stellate cells (HSCs) from normal C57BL/6J mice were stimulated by PDGF-C (Fig. 5) to induce the expression of PDGFR-α, PDGFR-β, alpha smooth muscle actin (α-SMA) and collagen 1a2; activated HSCs thus transformed into myofibroblasts (Fig. 5A, B). Peretinoin significantly reduced the expression of these genes and inhibited HSC activation.

We next evaluated the effects of peretinoin on human hepatoma cell lines (Huh-7, HepG2 and HLE), mouse embryonic fibroblast cells (NIH3T3), HUVECs and Lx-2 (22) (Supplemental Fig. 6A). Experimental conditions were optimized so that more than 90% of cells were variable at 20 μM peretinoin, as determined by an MTS cell proliferation assay (data not shown). Peretinoin dose-dependently inhibited the expression of PDGFR-α and PDGFR-β in Huh-7, HepG2, HLE, NIH3T3, HUVEC and Lx-2 cells, while no obvious expression of PDGFR-α was observed in HepG2 cells and HUVECs (Supplemental Fig. 6A).
Peretinoin also inhibited VEGFR2 expression in HUVEC. These results were confirmed by RTD-PCR (data not shown). Correlating with these results, the expression of phosphorylated serine/threonine kinase AKT (p-AKT) and p-ERK1/2, downstream signaling molecules of PDGFR-α, PDGFR-β and VEGFR2, was also dose-dependently repressed. The expression of collagen 1a2 was significantly repressed by peretinoin in Lx-2, HLE and Huh-7 cells (Supplemental Fig. 6B). These results suggest that peretinoin may inhibit hepatic fibrosis, angiogenesis and tumor growth through reduction of the PDGF and VEGF signaling pathway.

We examined the expression of two key regulators in peretinoin signaling, Sp1 and Ap1, in Huh-7 cells. Interestingly, the expression of Sp1 was decreased, which correlates with that of PDGFR-α, while expression of phosphorylated c-Jun (p-c-Jun) was increased in Huh-7 cells (Supplemental Fig. 6C). Therefore, peretinoin appears to repress the expression of PDGFR, partially through the inhibition of Sp1.

**Peretinoin inhibits hepatic angiogenesis in Pdgf-c Tg mice**

The effect of peretinoin on liver angiogenesis in Pdgf-c Tg mice was further analyzed. IHC staining of Pdgf-c Tg mouse livers at week 20 and week 48 revealed over-expression of the endothelial markers CD31 and CD34 and the endothelial growth factors VEGFR1 and endothelium-specific receptor tyrosine kinase 2 (Tie2) in the mesenchymal region (Fig. 6 and Supplemental Fig. 1A). This expression was significantly repressed by peretinoin as determined by the densitometric area (Supplemental Fig. 1B). RTD-PCR results revealed significant up-regulation of VEGFR1 (Flt-1) in Pdgf-c Tg mice compared with WT mice at both week 20 and week 48, while the expression of VEGFR2 (Flk-1) and Tie2 was only up-regulated at week 48. The expression of these genes was significantly repressed by peretinoin (Fig. 6A). Western blotting analysis confirmed the up-regulation of CD31 and VEGFR1 (Flk-1) at week 48 (Fig. 6B). In addition, p-AKT (Thr 308 and Ser 473) and 4-hydroxy-2-nonenal (4-HNE), an oxidative stress marker, were up-regulated in Pdgf-c Tg
mice and repressed by peretinoin (Fig. 6B).

We also assessed circulating endothelial cells (CEC), a useful biomarker for angiogenesis in the blood, and found that the CD31⁺/CD34⁺ CEC population was significantly up-regulated in Pdgf-c Tg mice at week 48 but significantly repressed by peretinoin (Fig. 6C, D). Thus, peretinoin appears to inhibit angiogenesis in the liver of Pdgf-c Tg mice, which might prevent the development of hepatic tumors.

**Peretinoin inhibits canonical Wnt/β-catenin signaling in Pdgf-c Tg mice**

The activation of the Wnt/β-catenin signaling pathway is seen in 17–40% of patients with primary HCC (23, 24). Moreover, recent reports suggested an interaction between PDGF signaling and Wnt/β-catenin signaling (25-27). We evaluated Wnt/β-catenin signaling in Pdgf-c Tg mice and showed by IHC staining that β-catenin was overexpressed in the submembrane at week 48 (Fig. 7A). Peretinoin significantly reduced this expression (Fig. 7A, B), and western blotting revealed that accumulation of β-catenin in the nuclear fraction of liver tumor tissues was more preferentially repressed by peretinoin than in the cytoplasmic fraction, although expression was repressed in both fractions (Fig. 7C). Wnt ligand (Wnt5a) and frizzled receptor (Fzd1) expression was significantly up-regulated in hepatic tumors compared with normal liver (Fig. 7D). These results together suggest that canonical Wnt/β-catenin signaling is activated in hepatic tumors and repressed by peretinoin.

Growth factors such as PDGF or HGF potentially activate Wnt/β-catenin signaling (26, 28), which promotes cancer progression and metastasis. We evaluated whether such growth factor signaling could be repressed by peretinoin in hepatic tumors. The expression of c-myc, β-catenin, Tie2, Fit-1 and Flk-1 were significantly up-regulated from 1.5- to 4-fold in hepatic tumors compared with normal liver, and this expression was significantly repressed by peretinoin. Similarly, the expression of PDGFR-α, PDGFR-β, collagen 1a2, collagen 4a2, tissue inhibitor of metalloproteinase 2 (TIMP2) and cyclin D1 was substantially up-regulated.
from 5- to 15-fold in hepatic tumors, and significantly repressed by peretinoin (Fig. 7D). Thus, growth factor signaling as well as canonical Wnt/β-catenin signaling in hepatic tumors appears to be repressed by peretinoin. These results explain the inhibitory effect of peretinoin in the development of HCC in \textit{Pdgf-c Tg} mice.

**Discussion**

HCC often develops in association with liver cirrhosis and its high recurrence rate leads to poor patient prognosis. Indeed, the 10-year recurrence-free survival rate after liver resection for HCC with curative intent was shown to be only 20\% (29). Therefore, there is a pressing need to develop effective preventive therapy for HCC recurrence in order to improve its prognosis.

Peretinoin, a member of the acyclic retinoid family, is expected to be an effective chemo preventive drug for HCC (11, 12, 30) as shown by a previous phase II/III trial in which 600 mg peretinoin per day in the Child-Pugh A subgroup reduced the risk of HCC recurrence or death by 40\% \text{[HR=0.60 (95\% CI: 0.40-0.89)]} (31). However, further clinical studies are needed to confirm the clinical efficacy of peretinoin, and a large scale study involving several countries is currently being planned.

During the course of chronic hepatitis, non-parenchymal cells including Kupffer, endothelial and activated stellate cells release a variety of cytokines and growth factors that might accelerate hepatocarcinogenesis. Although peretinoin has been shown to suppress the growth of HCC-derived cells by inducing apoptosis and differentiation (32-35), increasing p21 and reducing cyclin D1 (13), limited data have been published about its effects on hepatic mesenchymal cells such as stellate cells and endothelial cells (14).

In parallel with a phase II/III trial, we conducted a pharmacokinetics study of peretinoin focusing on 12 patients with CH-C and HCC to monitor the biological behavior of
peretinoin in the liver. Gene expression profiling during peretinoin administration revealed that HCC recurrence within 2 years could be predicted and that PDGF-C expression was one of the strongest predictors. In addition, other genes related to angiogenesis, cancer stem cell and tumor progression were down-regulated, while expression of genes related to hepatocyte differentiation and tumor suppression was up-regulated by peretinoin (data not shown). Moreover, a recent report revealed the emerging significance of PDGF-C-mediated angiogenic and tumorigenic properties (7, 8, 36). In this study, we therefore utilized the mouse model of *Pdgf-c Tg* which displays the phenotypes of hepatic fibrosis, steatosis and HCC development that resemble human HCC arising from chronic hepatitis usually associated with advanced hepatic fibrosis.

We demonstrated that peretinoin effectively inhibits the progression of hepatic fibrosis and tumors in *Pdgf-c Tg* mice (Figs. 1 and 4). Affymetrix gene chips analysis revealed dynamic changes in hepatic gene expression (Supplemental Fig. 3), which were confirmed by IHC staining, RTD-PCR and western blotting. Pathway analysis of differentially expressed genes suggested that the transcriptional regulators Sp1 and Ap1 are key regulators in the peretinoin inhibition of hepatic fibrosis and tumor development in *Pdgf-c Tg* mice (Supplemental Fig. 5).

We clearly showed that peretinoin inhibited PDGF signaling through the inhibition of PDGFRs (Fig. 2 and Fig. 3). In addition, we showed that PDGFR repression by peretinoin inhibited primary stellate cell activation (Fig. 5). Interestingly, this inhibitory effect was more pronounced than the effects of 9cRA (Fig. 5B). Normal mouse and human hepatocytes neither express PDGF receptors (JS Campbell and N. Fausto, unpublished data), nor proliferate in response to treatment with PDGF ligands (7). However, peretinoin inhibited the expression of PDGFRs, collagens and their downstream signaling molecules in cell lines of hepatoma (Huh-7, HepG2 and HLE), fibroblast (NIH3T3), endothelial cells (HUVEC) and stellate cells (Lx-2) (Supplemental Fig. 6). Furthermore, Sp1 but not Ap1, might be involved in
the repression of PDGFR-α in Huh-7 cells (Supplemental Fig. 6C). The over-expression of Sp1 activated PDGFR-α promoter activity, while siRNA knockdown of Sp1 repressed PDGFR-α promoter activity in Huh-7 cells (data not shown). Therefore, this appears to confirm that Sp1 is involved in the regulation of PDGFR, as reported previously (37, 38), although these findings should be further investigated in different cell lines. A recent report showed the involvement of transglutaminase 2, caspase3 and Sp1 in peretinoin signaling (35).

Peretinoin was shown to inhibit angiogenesis in the liver of Pdgf-c Tg mice in the present study, as shown by the decreased expression of VEGFR1/2 and Tie 2 (Figs. 2, 6 and Supplemental Fig. 1). Moreover, peretinoin inhibited the number of CD31* and CD34* endothelial cells (CEC) in the blood and liver (Fig. 6C, D), while also inhibiting the expression of EGFR, c-kit, PDGFRs and VEGFR1/2 in Pdgf-c Tg mice (data not shown). We also showed that peretinoin inhibited the expression of multiple growth factors such as HGF, IGF, VEGF, PDGF and HDGF, which were up-regulated from 3- to 10-fold in Pdgf-c Tg mice (Supplemental Fig. 3). These activities collectively might contribute to the anti-tumor effect of peretinoin in Pdgf-c Tg mice. The inhibition of both PDGFRs and VEGFR signaling by peretinoin was previously shown to have a significant effect on tumor growth (36), and we confirmed herein that peretinoin inhibited the expression of VEGFR2 in HUVECs (Supplemental Fig. 6) (39). Finally, we demonstrated that peretinoin inhibited canonical Wnt/β-catenin signaling by showing the decreased nuclear accumulation of β-catenin (Fig. 7). These data confirm the previous hypothesis of trans-repression of the β-catenin promoter by 9cRA in vitro (40).

Although we demonstrated that the PDGF signaling pathway is a target of peretinoin for preventing the development of hepatic fibrosis and tumors in mice, retinoid-inducing genes such as G0S2 (41), TGM2 (35), CEBPA (42), ATF, TP53BP, metallothionein 1H (MT1H), MT2A and hemopexin (HPX) were up-regulated in peretinoin-treated mice (data not shown).
These canonical retinoid pathways are likely to participate in preventing disease progression in conjunction with anti-PDGF effects.

The precise mechanism of peretinoin toxicity, in which 5% of mice treated with 0.06% peretinoin died after 24 weeks of treatment, is currently under investigation. These mice showed severe osteopenia and we speculate that the toxicity might be caused by retinoid-induced osteopenia, as observed in a hypervitaminosis A rat model (43). However, the toxicity of prolonged treatment with oral retinoids in humans remains controversial (44) and severe osteopenia has so far only been seen in a rodent model.

In summary, we show that peretinoin effectively inhibits hepatic fibrosis and HCC development in \( \text{Pdgf-c Tg} \) mice. Further studies are needed to elucidate the detailed molecular mechanisms of peretinoin action and the effect of peretinoin on PDGF-C in human HCC. The recently developed multikinase inhibitor Sorafenib (BAY 43-9006, Nexavar) was shown to improve the prognosis of patients with advanced HCC (45). Promisingly, a phase II/III trial of peretinoin showed it to be safe and well tolerated (46). Therefore, combinatorial therapy that incorporates the use of small molecule inhibitors with peretinoin may be beneficial to some patients. The application of peretinoin during pre- or early-fibrosis stage could be beneficial in preventing the progression of fibrosis and subsequent development of HCC in patients with chronic liver disease.

**Acknowledgements**

The authors are grateful to Dr. Scott Friedman, Mount Sinai School of Medicine, for providing Lx-2 cell lines. The authors thank Mina Nishiyama and Masayo Baba for their excellent technical assistance. This work was funded by NIH grants CA-23226, CA-174131 and CA-127228 (to N. Fausto and J.S. Campbell). This work was also supported in part by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (to Honda et al).
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Figure Legends

Figure 1
A: Feeding schedule of Pdgf-c Tg and WT mice. After weaning, male mice were randomly divided into three groups: (1) Pdgf-c Tg or WT mice receiving basal diet, (2) Pdgf-c Tg or WT mice receiving 0.03% peretinoin-containing diet, and (3) Pdgf-c Tg or WT mice receiving 0.06% peretinoin-containing diet.
B: Azan staining of WT or PDGF-C Tg mouse livers fed with different diets at 20 weeks (20w) and 48 weeks (48w).
C: Densitometric analysis of Pdgf-c Tg mouse liver fibrotic areas at 20 weeks (20w) (n=15) and 48 weeks (48w) (n=15).

Figure 2
Immunohistochemical staining of PDGFR-α, PDGFR-β, collagen 1, CD34 and VEGFR1 expression in Pdgf-c Tg or WT mouse livers fed a basal diet or 0.06% peretinoin.

Figure 3
A: RTD-PCR analysis of PDGFR-α, PDGFR-β, collagen 1a2, collagen 4 a2 and TIMP2 expression in Pdgf-c Tg (n=5) or WT mouse livers (n=15).
B: Western blotting analysis of PDGFR-α, PDGFR-β, α-SMA, p-ERK, ERK, cyclin D1 and GAPDH expression in PDGF-C Tg or WT mouse livers fed a basal diet or 0.06% peretinoin at 20 (20w) or 48 weeks (48w) (n=3).

Figure 4
A: Incidence of hepatic tumors (adenoma or HCC) in \textit{Pdgf-c Tg} mouse livers fed with different diets.

B: Tumor sizes and liver weights of \textit{Pdgf-c Tg} and WT mice fed with basal diet (n=31 for \textit{Pdgf-c Tg}, n=15 for WT mice) or 0.03% (n=36 for \textit{Pdgf-c Tg}, n=15 for WT mice) and 0.06% (n=17 for \textit{Pdgf-c Tg}, n=15 for WT mice) peretinoin at 48 weeks.

C: Macroscopic findings of \textit{Pdgf-c Tg} or WT mouse livers.

No obvious change was observed in the liver of WT mice fed with 0.06% peretinoin for 48 weeks (upper left panel). Fibrosis and steatosis was observed in the liver of \textit{Pdgf-c Tg} mice fed a basal diet for 20 weeks (upper right panel). Multiple tumors developed (white arrows) in the liver of \textit{Pdgf-c Tg} mice fed a basal diet for 48 weeks (lower left panel). Suppression of tumor development in the liver of \textit{Pdgf-c Tg} mice fed a 0.06% peretinoin diet for 48 weeks (lower right panel).

**Figure 5**

A: Microscopic view of freshly isolated primary mouse hepatic stellate cells (HSCs), after (left) PDGF-C transformation into myofibroblasts. Peretinoin inhibited the transformation of HSCs by PDGF-C.

B: RTD-PCR analysis of PDGFR-\(\alpha\), PDGFR-\(\beta\), \(\alpha\)-SMA and collagen 1a2 expression in HSCs treated with or without PDGF-C, peretinoin and 9cRA (n=4). PDGF-C(+), 80 ng/mL; peretinoin (+), 5 \(\mu\)M; (++), 10 \(\mu\)M; 9cRA (+), 5 \(\mu\)M; (++), 10 \(\mu\)M.

**Figure 6**

A: RTD-PCR analysis of Tie2, Flk-1 and Flt-1 expression in the liver of \textit{Pdgf-c Tg} and WT mice fed with different diets (n=15).

B: Western blotting analysis of Flk-1, CD31, p-AKT (Thr 308, Ser473), AKT, 4-HNE and GAPDH expression in the liver of \textit{Pdgf-c Tg} or WT mice fed a basal diet or 0.06% peretinoin at
48 weeks (n=3).

**C**: FACS analysis of CD31- and CD34-positive CEC in blood of *Pdgf-c Tg* or WT mice fed a basal diet or 0.06% peretinoin at 48 weeks.

**D**: Frequency of CD31- and CD34-positive CEC in blood of *Pdgf-c Tg* or WT mice fed a basal diet or 0.06% peretinoin at 48 weeks (n=10).

**Figure 7**

**A**: Immunohistochemical staining of β-catenin expression in HCC tissues of *Pdgf-c Tg* mice fed a basal diet or 0.06% peretinoin at 48 weeks.

**B**: Densitometric analysis of β-catenin expression in the liver of *Pdgf-c Tg* mice fed with different diets (n=15 for basal diet, n=15 for 0.03% peretinoin, n=5 for 0.06% peretinoin).

**C**: Western blotting analysis of β-catenin expression in cytoplasmic and nuclear fractions of *Pdgf-c Tg* mouse livers fed with different diets. GAPDH was used to standardize cytoplasmic protein and lamin A/C to standardize nuclear protein (n=3).

**D**: RTD-PCR analysis of myc, β-catenin, Tie2, Flt-1, Flk-1, Wnt5a, Fzd1, PDGFR-α, PDGFR-β, collagen 1a2, collagen 4a2, TIMP2 and cyclin D1 expression in HCC tissues of *Pdgf-c Tg* mice fed with different diets (n=15 for basal diet, n=15 for 0.03% peretinoin, n=5 for 0.06% peretinoin). Relative fold expressions compared to WT mice are shown.
**Fig. 3**

**A**

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT 20w</th>
<th>WT 48w</th>
<th>Pdgf-c Tg 20w</th>
<th>Pdgf-c Tg 48w</th>
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<tr>
<td><strong>PDGFRα</strong></td>
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<td><strong>TIMP2</strong></td>
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*significance levels: *P < 0.05 and **P < 0.01 and ***P < 0.001.

**B**

<table>
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<th>Condition</th>
<th>20w</th>
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<td>Pdgf-c Tg + basal diet</td>
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<tr>
<td>Pdgf-c Tg + 0.06% peretinoin</td>
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- **PDGFR-α**
- **PDGFR-β**
- **α-SMA**
- **p-ERK1/2**
- **ERK1/2**
- **Cyclin D1**
- **GAPDH**
**Fig. 4**

**A**

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Tumors</th>
<th>Total Incidence (%)</th>
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<tr>
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<tr>
<td>Basal diet</td>
<td>31</td>
<td>15 Adenoma, 13 HCC</td>
<td>28/31 (90%)</td>
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<tr>
<td>Peretinoin 0.03%</td>
<td>36</td>
<td>13 Adenoma, 6 HCC</td>
<td>19/36 (53%)***</td>
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<tr>
<td>Peretinoin 0.06%</td>
<td>17</td>
<td>4 Adenoma, 1 HCC</td>
<td>5/17 (29%)***</td>
</tr>
</tbody>
</table>

**B**

- Tumor size (mm)
- Liver weight (mg)

- **P**dgc-Tg 48w
- **WT 48w**
- **P**dgc-Tg 48w

**C**

- WT 48w + 0.06% peretinoin
- Pdgc-Tg 20w
- Pdgc-Tg 48w + 0.06% peretinoin

*P < 0.05 and **P < 0.01 and ***P < 0.001.
Fig. 5

A

No treatment | PDGF-C | PDGF-C+Peretinoin

day3 | day5 | day5

B

PDGFRα

PDGFRβ

α-SMA

Collagen 1α2

#: P < 0.05 and ##: P < 0.01 versus NC,
*: P < 0.05 and **: P < 0.01 and ***: P < 0.001 versus PDGF-C.
Fig. 6

A

Tie2

Flk-1

Flt-1

WT 20w, 48w

Pdgf-c Tg 20w, 48w

basal diet, peretinoin 0.03%, peretinoin 0.06%

*P < 0.05, **P < 0.01 and ***P < 0.001

B

VEGFR2 (Flk-1)

CD31

p-AKT (Thr308)

p-AKT (Ser473)

AKT

4-HNE

GAPDH

C

WT 48w, Pdgf-c Tg 48w, Pdgf-c Tg 48w + basal diet, Pdgf-c Tg 48w + 0.06% NIK-333

D

CD31+CD34+ cells (48w)

WT  Pdgf-c Tg

* * *  **