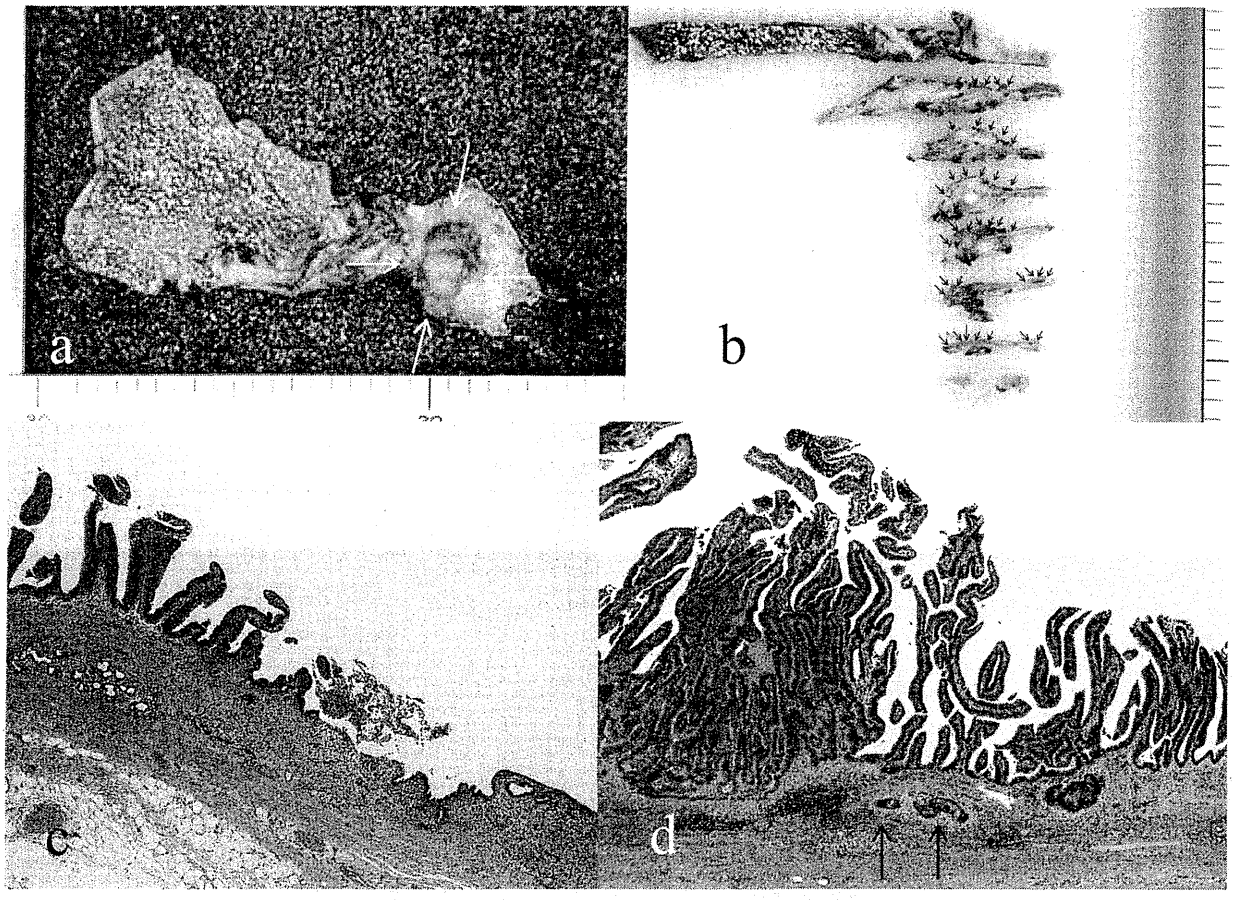

図3　病理所見

a　肉眼所見では上部胆管に乳頭膨張型胆管腫瘍を認めた（矢印）.

b　腫瘍の割面は白色充実性で，境界は比較的明瞭だった（矢印）.

c　HE染色，×40．粘膜平坦部分への癌の進展は認めなかった.

d　HE染色，×100．腫瘍は乳頭状または管状に増殖する乳頭腺癌であった．腫瘍の大部分は粘膜内にとどまるが，一部線維筋層への浸潤を認めた（矢印）.

分・整，体温35.5℃．右上腹部に軽度の自発痛・圧痛・叩打痛を認める．腹部は平坦・軟で筋性防御を認めない．眼瞼結膜に貧血無く，眼球結膜に軽度の黄疸を認める.

第2回入院時検査所見：高度肝機能障害，軽度のCRP上昇ならびに黄疸を認めた（表2）.

腹部CT検査：肝内胆管の軽度拡張を認めた（図2a，矢頭）．胆管左壁がわずかに造影され，その十二指腸側の胆管内には軽度の造影効果を伴う腫瘍が認められた（図2a，b，c）.

ERCP検査：総胆管内に可動性のない陰影欠損を認めた．胆管炎治療のため内視鏡的経鼻胆管ドレナージ（ENBD）を留置した．胆管炎改善後のENBD造影で，上部胆管に乳頭状隆起性病変を認めた．胆嚢管は造影されなかった（図2d）.

管腔内超音波（IDUS）検査：上・中部胆管に乳頭状腫瘍を認め，胆管壁外側高エコー層は保たれていた（図2e）.

第2回入院後経過：腫瘍生検で高分化腺癌の診断が得られた．閉塞性黄疸に対しENBD抜去後7Fr7cm胆管ドレナージチューブステント（オリンパスメディカルシステムズ社，東京）を留置した．IDUSで外側高エコー層が保たれており，IDUSならびに腹部CTで門脈・肝動脈への浸潤を認めず，腫大リンパ節も無いことから術前深達度診断はfmと診断した．ENBD造影で認められた乳頭状腫瘍は圧迫等による変形や可動性を認めず，IDUSで認められた不整エコー領域は腫瘍であると考えられた．IDUSでは乳頭状腫瘍部以外に胆管壁肥厚や不整は認めず，ENBD造影所見でも病変部以外に胆管壁不整あるいは硬化像は認めなかったため術前

表3　総胆管結石に胆管癌を合併した症例の報告（2000～2010年）

報告者	報告年	性別	年齢	胆管癌診断時期	胆石治療～胆管癌診断の期間（月）	胆管癌診断方法	胆管癌存在部位	深達度	治療	病理
山本ら[3]（3例）	2000	NA	NA	胆石と同時	0	細胞診1例，生検2例	肝門部3例	m 3例	手術	腺癌
隈元ら[4]	2003	男	70	胆石と同時	0	生検	下部	m	手術	管状腺癌
春木ら[5]	2004	女	73	胆石後	12	生検	肝門部	NA	放射線治療	癌（生検）
〃	〃	女	83	胆石と同時	0	細胞診	肝門部	NA	経過観察	細胞診のみ
西村ら[6]	2004	男	68	胆石と同時	0	生検	下部	NA	手術	Dysplasia
新倉ら[7]	2004	男	74	胆石後	62	生検	下部	si	手術	中分化腺癌
吉田ら[8]	2005	男	74	胆石と同時	0	生検	上部	T1	手術	管状腺癌
木田ら[9]（4例）	2005	男3例，女1例	58－76歳	胆石と同時	0	細胞診	NA	m 1例，fm 3例	手術	NA
石塚ら[10]	2005	男	94	胆石後	2	生検	中部	m（IDUS）	経過観察	乳頭状腺癌
和唐ら[11]	2006	男	63	胆石と同時	0	細胞診	中部	m	手術	乳頭状腺癌
越知ら[12]	2007	女	70	胆石と同時	0	生検	下部	NA	手術	NA
杉田ら[13]	2008	男	74	胆石後	19	生検	中下部	NA	手術	癌
大西ら[14]	2008	女	72	胆石後	16	生検	下部	膵浸潤	手術	低分化腺癌
比佐ら[15]	2008	女	70歳代	胆石後	14	生検	中下部	膵浸潤	手術	NA
本症例	2010	男	70歳代	胆石後	14	生検	上部	fm	手術	高分化腺癌

NA：not available

水平診断は腫瘍部に限局していると診断した．以上より術前診断は上部胆管癌，乳頭膨張型，T1，Hinf0，Ginf0，Panc0，N0，Stage Iと診断した．

黄疸・胆管炎改善後，同病変に対し術中に肝側水平断端陰性を確認し肝外胆管切除術，胆管空腸吻合術を施行した．最終診断はBs，乳頭膨張型，ly0 v0 pn0 fm S（－）Ginf0 N0 HM0 DM0 EM0，pT1 N0 M0，fStage IAの高分化腺癌であった（図3）．

考　察

2000年1月から2010年12月の医学中央雑誌で（総胆管結石症/TH or 総胆管結石/AL）or（胆管癌/TH or 胆管癌/AL）を検索（抄録を含む，肝内胆管癌を除く）したところ137件がヒットし，このうち総胆管結石に胆管癌を合併したものは13件19例（男性10例，女性6例，不明3例，平均年齢74.1＋/－8.2歳）であった[3)～15)]（表3）．胆管癌発生部位は肝門部5例，上部胆管1例，中部4例，下部5例であった．また，壁深達度はm 5例（IDUSでの診断1例を含む），fm 4例（T1を含む），ss以深3例であった．治療は手術16例，放射線治療1例，経過観察2例であった．病理組織検査結果の詳細が記載されている7例は腺癌の診断であった．19例中13例で胆管結石と同時に胆管癌と診断され，残りの6例は胆管結石治療後に胆管癌と診断された．この6例の胆管癌診断までの期間は中央値20.8カ月（2-62カ月）であった（表3）．

本症例では上部胆管癌による胆管狭窄部位に総胆管結石を合併した可能性があると考えられたが，第1回入院時には癌の診断は困難であった．EML後に遺残結石や有意な胆管狭窄は認められないと判断したが，EST等の処置により胆管の十分な造影は困難であった（図1d，1e）．

総胆管結石が胆管癌合併のリスク因子か否かは議論があり，現在まで結論は出ていない[1)2)]．三上らは総胆管結石に対するEPBD治療後5年以上経過観察可能であった101例を調査し，胆道癌の合併は1例（肝内胆管癌）のみであったと報告している[1)]．また，Kageokaらは総胆管結石に対してEST治療を行った262例を解析したが，胆道系悪性腫瘍は胆嚢癌1例のみであった[16)]．以上のとおり，総胆管結石に胆管癌が合併する確率は高いとはいえないが，総胆管結石切石術後早期に胆管癌を発見される症例は同時性の胆管癌合併の可能性が高いと推測される．本症例は総胆管結石治療後早期に胆管癌を発見され，かつ，通常と異なる不整形の結石を認めていることから，総胆管結石切石時に同時性の胆管癌が合併していた可能性が高いと考えられた．

本症例は総胆管結石を除去した後の胆管に軽度の硬化が疑われたが，胆管炎による変化と推測されていた．しかし，胆嚢結石を認めないにもかかわらず上・中部胆管に不整形の結石を認めたことから，落下結石や胆管原発の総胆管結石の可能性は低いと考えるべきであった．特異な結石形態から悪性腫瘍の可能性を疑い，初回入院時に遺残結石の確認だけでなくバルーン閉塞下胆管造影，ENBD造影や細胞診，IDUSでの詳細な胆管評価，さらには胆道鏡・細胞診・生検等といった侵襲的検査も考慮する必要があったと考えられた．IDUSによる胆管壁の評価あるいはENBD造影による質の高い胆管像，経口胆道鏡等の精査を行えばより早期に胆管癌を発見できた可能性がある．胆管結石による胆管炎と単純に考えるだけでなく，胆石の発生過程や患者の既往歴，胆石の形態等から，通常の胆石に矛盾する点があれば，早期に精密検査を施行する必要があると考えられた．

まとめ

胆管癌を合併した総胆管結石の1例を経験した．総胆管結石に胆管癌を合併する頻度は少ないが，胆管癌の結石合併は複数の報告がある．不整形総胆管結石切石時や胆石の危険因子が無い患者の総胆管結石の場合は胆管癌の合併に留意する必要があると考えられた．

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CLINICAL STUDIES

Expression of chondroitin-glucuronate C5-epimerase and cellular immune responses in patients with hepatocellular carcinoma

Eishiro Mizukoshi, Kazumi Fushimi, Kuniaki Arai, Tatsuya Yamashita, Masao Honda and Shuichi Kaneko

Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan

Keywords
cancer – CTL – epitope – immunotherapy – peptide vaccine – tumour-associated antigen

Abbreviations
ELISPOT, enzyme-linked immunospot; HCV, hepatitis C virus; HLA, human leucocyte antigen; IFN, interferon; PBMC, peripheral blood mononuclear cells; SART, squamous cell carcinoma antigen recognized by T cells; TIL, tumour infiltrating lymphocytes.

Correspondence
Shuichi Kaneko, MD, Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan
Tel: + 81-76-265-2230
Fax: + 81-76-234-4250
e-mail: skaneko@m-kanazawa.jp

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Abstract

Background & Aims: Chondroitin-glucuronate C5-epimerase is an enzyme that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulphate biosynthesis. It is also identified to be a tumour-associated antigen recognized by cytotoxic T cells (CTLs) and its enhanced expression in many cancers has been reported. In the present study, we investigated the usefulness of this molecule as an immunotherapeutic target in hepatocellular carcinoma (HCC). *Methods:* The expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines and HCC tissues was confirmed by immunofluorescence and immunohistochemical analysis. CTL responses were investigated by several immunological techniques using peripheral blood mononuclear cells (PBMCs) or tumour-infiltrating lymphocytes. To determine the safety of immunotherapy using chondroitin-glucuronate C5-epimerase-derived peptide, 12 patients with HCC were administered s.c. vaccinations of the peptides and analysed. *Results:* Chondroitin-glucuronate C5-epimerase was expressed in HCC cell lines and human tissues including alpha-foetoprotein (AFP)-negative individuals. Chondroitin-glucuronate C5-epimerase-specific CTLs could be generated by stimulating PBMCs of HCC patients with peptides and they showed cytotoxicity against HCC cells expressing the protein. The frequency of CTL precursors investigated by enzyme-linked immunospot (ELISPOT) assay was 0–34 cells/3 $\times$ 10^5 PBMCs and the infiltration of interferon-gamma-producing CTLs into the tumour site was confirmed. In the vaccination study, no severe adverse events were observed and the peptide-specific CTLs were induced in 4 of 12 patients tested. *Conclusions:* Chondroitin-glucuronate C5-epimerase is a potential candidate for tumour antigen with immunogenicity and the peptides derived from this antigen could be useful in HCC immunotherapy.

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and has gained much clinical interest because of its increasing incidence (1). It is treatable by hepatectomy or percutaneous ablation when the lesion is localized to some extent, and radical therapeutic effects can be obtained when resection or cauterization with a safety margin can be performed (2). However, the recurrence rate is very high (3), because active hepatitis and cirrhosis in surrounding non-tumour liver tissues have the potential to generate HCC de novo.

To protect against recurrence, tumour antigen-specific immunotherapy is an attractive option. Many tumour-associated antigens and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades. However, only a few HCC-specific tumour antigens and their antigenic epitopes have been used for human trials (4, 5).

Chondroitin-glucuronate C5-epimerase is an enzyme that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulphate biosynthesis and identical to squamous cell carcinoma antigen recognized by T cells 2 (SART2) (6). It is expressed in many malignant tumour cell lines and various histological types of cancer tissues and function as tumour rejection antigens (7). In addition, peptides containing chondroitin-glucuronate C5-epimerase epitopes are capable of generating CTLs, and therefore, have been used for immunotherapy to treat several kinds of cancers (8, 9). These reports suggest chondroitin-glucuronate C5-epimerase to be useful as a target antigen in HCC immunotherapy. Furthermore, in previous study, we compared T-cell immune responses against the various tumour-associated antigen (TAA)-derived peptides (10). The results of the study showed that CTLs of HCC patients were frequently responsive against a single

chondroitin-glucuronate C5-epimerase-derived peptide. Regarding tumour immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier post-vaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors (11). These results also suggest that chondroitin-glucuronate C5-epimerase is useful as a target for HCC immunotherapy.

In the present study, we examined chondroitin-glucuronate C5-epimerase expression in various hepatoma cell lines and HCC tissues of patients, and analysed immune responses to the antigen using peripheral blood mononuclear cells (PBMCs) and tumour-infiltrating lymphocytes (TILs). Furthermore, to investigate the usefulness of HCC immunotherapy targeting chondroitin-glucuronate C5-epimerase, we analysed the safety and cellular immune responses in the patients vaccinated with chondroitin-glucuronate C5-epimerase-derived peptide.

Materials and methods

Patients

Forty-four HLA-A24-positive HCC patients were examined for the expression of chondroitin-glucuronate C5-epimerase and cellular immune responses. Twelve HCC patients were enrolled in vaccination study. Informed consent was obtained from each patient included in the present study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the regional ethics committee.

The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens in 17 cases, surgical resection in nine cases, and autopsy in five cases. For the remaining 13 patients, the diagnosis was based on typical hypervascular tumour staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT (12). The pathological grading of tumour-cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer (13). The severity of liver disease was evaluated according to the criteria of Desmet *et al.* using biopsy specimens of liver tissue (14). Eleven normal blood donors and 23 chronic hepatitis C patients (11 cirrhosis) with HLA-A24, who were diagnosed by liver biopsy, served as controls.

Cell lines

Four human hepatoma cell lines (HLF, Hep3B, HLE and Huh7) and Paca-2, which is a pancreatic cancer cell line, were cultured in DMEM (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FCS) (Gibco). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/ml of hygromycin B (Sigma, St Louis, MO, USA), and K562 was cultured in RPMI 1640 medium containing 10% FCS (15).

Immunofluorescence and immunohistochemical analysis

The expression of chondroitin-glucuronate C5-epimerase was examined in four different Hepatoma cell lines. A pancreatic cancer cell line (Paca 2) was used as a positive control. They were fixed in acetone with methanol for 5 min and incubated with rabbit anti-human chondroitin-glucuronate C5-epimerase (ProteinTech Group, Inc., Chicago, IL, USA; diluted 1:50) or mouse anti-human AFP (Nichirei Bioscience, Tokyo, Japan) antibody overnight at 4°C. For immunofluorescence analyses, Alexa Fluor 488-conjugated anti-rabbit and anti-mouse IgG (Invitrogen, Tokyo, Japan) were used for chondroitin-glucuronate C5-epimerase and AFP detection respectively.

The expression in HCC tissue was examined in 26 patients. Non-cancerous tissues were also obtained by a paired liver biopsy or surgical resection from the non-neoplastic liver tissue. The tissues were fixed in buffered zinc formalin (Anatech Ltd, Battle Creek, MI, USA), embedded in paraffin, sectioned (at 3 μm), and stained with haematoxylin and eosin. The sections were deparaffinized, treated in a pressure cooker for 1–4 min, and incubated with rabbit anti-human chondroitin-glucuronate C5-epimerase or AFP (DakoCytomation, Inc, Carpinteria, CA, USA) antibody overnight at 4°C. The tissue sections were visualized using the DAKO EnVision^{TM}+ System (DakoCytomation, Inc.). The expression levels were semi-quantitatively classified into four categories (negative to low, moderate and high; negative: no staining, low: <20% of the area stained, moderate: 20%-80% of the area stained, high: >80% of the area stained).

ELISPOT assay

The PBMCs and TILs were isolated as described previously (16). ELISPOT assays were performed as reported previously with the following modifications (16). Three different peptides (Peptide 1; DYSARWNEI, Peptide 2; AYDFLYNYL, Peptide 3; SYTRLFLIL) derived from chondroitin-glucuronate C5-epimerase were used for the detection of CTLs. Negative controls consisted of a HIV envelope-derived peptide ($HIVenv_{584}$) (17). Positive controls consisted of 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$) (18). The peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). The coloured spots were counted with a KS ELISPOT Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of an antigen from the number in its presence. Responses to peptides derived form chondroitin-glucuronate C5-epimerase in HCC

patients were considered positive if the number of specific spots was more than the mean + 3SD of that in normal donors and if the number of spots in the presence of an antigen was at least two-fold greater than the number in its absence. Responses to peptides HIVenv$_{584}$ and CMVpp65$_{328}$ were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least two-fold that in its absence. ELISPOT assays were also performed in 12 patients whose PBMCs were available for analysis at 2–4 weeks after RFA.

CTL induction and Cytotoxicity assay

Peptide 3 (SYTRLFLIL), which corresponds to HLA-A24-restricted CTL epitope (7, 19), was used to produce chondroitin-glucuronate C5-epimerase-specific T cells. CTLs were expanded from PBMCs as detailed previously (16). C1R-A24 cells and human hepatoma cell lines were used as targets. Cytotoxicity assay was performed by chromium-release assay. Percent cytotoxicity was calculated as previously described (16). For the assay using hepatoma cell lines, cytotoxicity was considered positive when it was higher than that of CTLs against K562 which shows non-specific lysis.

Vaccination study

Twelve HLA-A24-positive HCC patients who were treated by radiofrequency ablation (RFA) and obtained complete necrosis of tumour with safety margin were enrolled in this vaccine study (Trial registration: UMIN000004540). They were vaccinated with peptide 3 (SYTRLFLIL) into the subcutaneous tissue of the armpit 4 weeks after RFA. The peptide utilized in the present study was prepared under conditions of Good Manufacturing Practice (NeoMPS, San Diego, CA, USA). One millilitre of the peptide, which was supplied in vials containing 0.04–4 mg/ml sterile solution, was mixed with an equal volume of incomplete Freund's adjuvant (Montanide ISA-51; Seppic, Paris, France) and emulsified in 5-ml syringes. 1.5 ml of the preparing peptide was injected and the patients received three biweekly vaccinations. Toxicity was assessed every 2 weeks using the National Cancer Institute's Common Toxicity Criteria. To evaluate the immunological effect, ELISPOT assay was performed before and 4 weeks after the final vaccination. Responses to vaccination were considered positive if more than 10 specific spots were detected and if the number of spots after vaccination was at least two-fold than before vaccination. After final vaccination, HCC recurrence was evaluated by dynamic CT or MRI every 3 months.

Statistical analysis

Data are expressed as the mean ± SD. The Mann–Whitney's *U*-test was used for statistical analyses of chondroitin-glucuronate C5-epimerase expression in HCC and non-cancerous liver tissues. The χ^2 test with Yates' correction and the unpaired *t*-test were used for univariate analysis of the effect of variables on the T-cell response against chondroitin-glucuronate C5-epimerase. A level of $P < 0.05$ was considered significant.

Results

Expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines and HCC tissues

Chondroitin-glucuronate C5-epimerase was expressed in all hepatoma cells (Fig. 1a) and its cellular distribution was cytoplasmic, similar to that in Paca-2, a pancreatic cancer cell line reported to express the protein (7). The expression was observed even in the hepatoma cell lines not expressing AFP, namely HLF and HLE.

The expression of chondroitin-glucuronate C5-epimerase in HCC tissues was examined in 26 HCC patients. A representative result for one HCC patient is shown in Fig. 1b. In this case, the expression of chondroitin-glucuronate C5-epimerase was observed in HCC tissue but not in non-cancerous areas. In addition, AFP was not detected in HCC tissue. To compare the expression levels of this protein between cancerous and non-cancerous tissues, the expression was semi-quantitatively classified into four categories as described in materials and methods, and analysed. The expression levels were higher in HCC tissue than in the non-cancerous tissue ($P < 0.0001$) (Fig. 1c). The expression in liver tissue was also observed in the patients with chronic hepatitis and cirrhosis (Control), however, the expression levels were lower than those in HCC tissue ($P = 0.0137$). The expression of chondroitin-glucuronate C5-epimerase and AFP in HCC tissue was observed in 26 (100%) and 12 (46%) of 26 patients respectively (Fig. 1d). The expression of chondroitin-glucuronate C5-epimerase was observed even in the HCC tissues without AFP expression.

Detection of chondroitin-glucuronate C5-epimerase-specific T cells by IFN-γ ELISPOT analysis

The clinical profiles of the 11 healthy normal donors, 12 patients with chronic hepatitis C, 11 patients with cirrhosis and 44 patients with HCC analysed in the present study are shown in Table 1.

To determine whether a significant number of T cells specifically reacted with the chondroitin-glucuronate C5-epimerase-derived peptides (peptide 1, 2 and 3) in HCC patients, ELISPOT assays were performed using PBMCs from 11 healthy donors (Fig. 2a). The number of specific spots was 1.0 ± 1.3, 1.5 ± 1.3 and 1.0 ± 1.4/3×10^5 PBMCs respectively. Similarly, cells that specifically reacted with the peptides were counted among chronic hepatitis C and cirrhosis patient-derived PBMCs. Regarding a value larger than the mean + 3SD

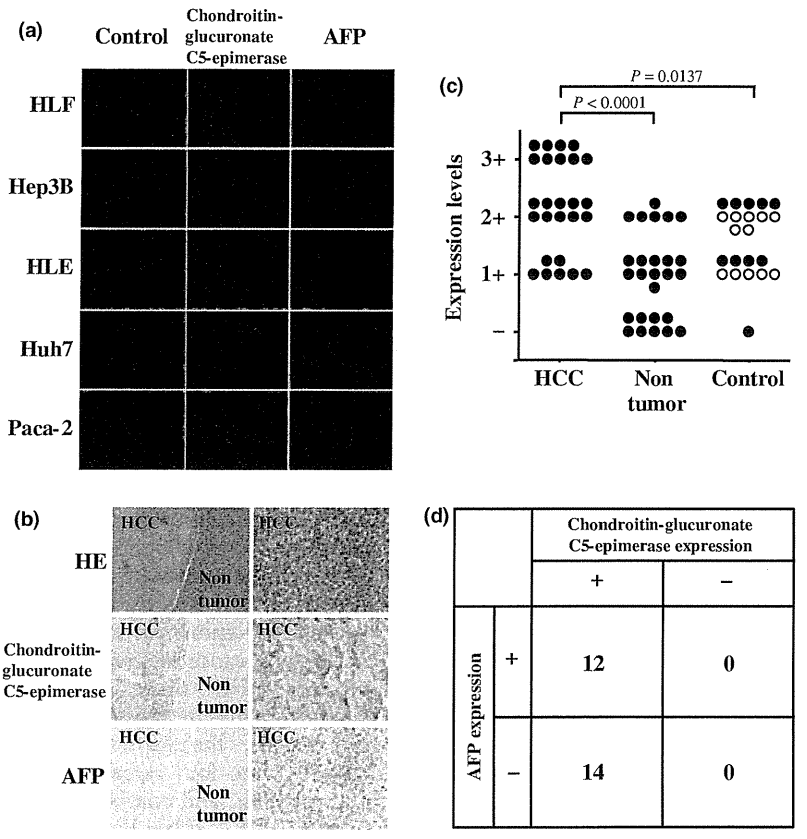

Fig. 1. Expression of chondroitin-glucuronate C5-epimerase. (a) immunofluorescence analysis for the expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines. Original magnification, ×400. (b) Immunohistochemical analysis for the expression of chondroitin-glucuronate C5-epimerase and AFP in sequential non-cancerous and HCC tissue sections. Original magnification, ×200 (left) and ×400 (right). (c) Analysis of chondroitin-glucuronate C5-epimerase expression levels among the three groups (HCC; tumour tissue in HCC patients, Non-tumour; non-tumour tissue in HCC patients, Control; liver tissue in disease control groups). Closed and open circles show the level of chondroitin-glucuronate C5-epimerase expression in the patients with cirrhosis and chronic hepatitis respectively. (d) The expression of chondroitin-glucuronate C5-epimerase was also compared with AFP expression in HCC tissues.

of the number of T cells that specifically reacted with the peptide in healthy donor-derived PBMCs as a significant response, 1 of 23 (4.3%) patients showed a significant response to each of the chondroitin-glucuronate C5-epimerase-derived peptides (Fig. 2a).

In the same analysis of HCC patients, 10.8, 16.2 and 27.0% of the patients showed significant responses to peptide 1, 2 and 3 respectively (Fig. 2b). A significant response specific to $CMVpp65_{328}$ was detected in 36.4%, 34.8% and 45.9% of healthy donors, disease control groups and HCC patients, respectively, with no significant difference among the three groups. On the other hand, no significant response to $HIVenv_{584}$ was observed in all groups.

To clarify the clinical characteristics of chondroitin-glucuronate C5-epimerase-specific T-cell responses in HCC patients, the clinical background was compared between patients who showed positive responses to chondroitin-glucuronate C5-epimerase-derived peptides and those who did not. The clinical features of both groups were not statistically different in terms of age, gender, serum AFP levels, differentiation of HCC, tumour multiplicity, vascular invasion, TNM factors and stages, histology of the non-tumour liver, liver function and the type of viral infection (Table 2). Chondroitin-glucuronate C5-epimerase-specific T cells had been generated even in the early stages of HCC.

Next, to examine the existence of chondroitin-glucuronate C5-epimerase-specific T cells among TILs, we performed a similar analysis in another seven patients from whom samples of both PBMCs and TILs could be obtained. In the assay using PBMCs and TILs, four of seven (57.1%) and five of seven (71.4%) patients, respectively, showed significant responses to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) (Fig. 3a). A positive T-cell response in TILs was observed even in one patient without a positive T-cell response in PBMCs (patient 39).

Table 1. Characteristics of the patients studied

Clinical diagnosis	No. of patients	gender M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Aetiology (B/C/Others)	Child-Pugh (A/B/C)	Diff. degree[a] (wel/mod /por/ND)	Tumour size[b] (large/small)	Tumour multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM stage (I/II/IIIA/IIIB/ IIIC/IV)
Normal donors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chronic hepatitis	12	7/5	54 ± 11	104 ± 119	12 ± 4	0/12/0	12/0/0	ND	ND	ND	ND	ND
Liver cirrhosis	11	5/6	60 ± 11	83 ± 73	79 ± 140	1/7/3	6/5/0	ND	ND	ND	ND	ND
HCC	44	35/9	66 ± 8	67 ± 32	1629 ± 7874	8/34/2	28/14/2	11/17/3/13	29/15	25/19	12/32	13/17/5/ 1/2/6

[a]Histological degree of HCC; wel: well-differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
[b]Tumour size was divided into either 'small' (≤2 cm) or 'large' (>2 cm).

Cytotoxic activity of chondroitin-glucuronate C5-epimerase-specific CTLs against hepatoma cell lines

Whether the chondroitin-glucuronate C5-epimerase-derived peptides used were capable of generating peptide-specific CTLs from PBMCs was investigated in 18 HCC patients. The CTLs specific to chondroitin-glucuronate C5-epimerase could be induced in 8 of 18 (44.4%) patients (Fig. 3b and c). They exhibited cytotoxicity against hepatoma cell lines with the HLA-A24 molecule and expression of chondroitin-glucuronate C5-epimerase, that correspond to HLF and HLE, but not against Hep3B and Huh7 cells without HLA-A24 (Fig. 3d).

Clinical safety of chondroitin-glucuronate C5-epimerase-derived peptide and its immunological effects

The clinical profiles of the 12 HCC patients with vaccination are shown in Table 3. The treatment was well-tolerated and there were no treatment-related serious adverse events. The most common adverse event was grade 1 injection-site reaction manifesting as pain, pruritus, skin induration and rubor. The worsening of hepatitis or liver function was not observed in any of the vaccinated patients.

In the analysis of ELISPOT assay using PBMCs of patients with vaccination, 4 patients demonstrated an immune response (Fig. 4a and Table 3). All of the patients that responded were immunized with 3.0 mg of peptide. None of the patients immunized with 0.03 or 0.3 mg of peptide showed an enhancement of peptide-specific immune response. The enhancement of immunological response to $HIVenv_{584}$ and $CMVpp65_{328}$ was not observed in any patients except patient A2.

To examine whether similar occurs for the immune response in HCC patients with only RFA, we analysed chondroitin-glucuronate C5-epimerase-derived peptide-specific T-cell responses in 12 HCC patients without vaccination, whose PBMCs were available for analysis at 2–4 weeks after RFA. In this analysis, we observed an increase of the frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific T cells in 2 of 12 patients (Fig. 4b). The frequency of the patients who showed an increase in the number of chondroitin-glucuronate C5-epimerase-derived peptide-specific T cells was higher in the patients with vaccination of 3 mg of peptide (66.7%) than in those without vaccination (16.7%).

Finally, we examined the HCC recurrence rate after RFA between the patients with and without the peptide-specific CTL response to examine the clinical effect of an increase of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs after vaccination. In the analysis, the recurrence rate in the patients with an increase in the peptide-specific CTLs after vaccination (two of four patients, 50%) was lower than that in the patients without immune response (six of eight patients, 75%) at 300 days after RFA, although there was no sta-

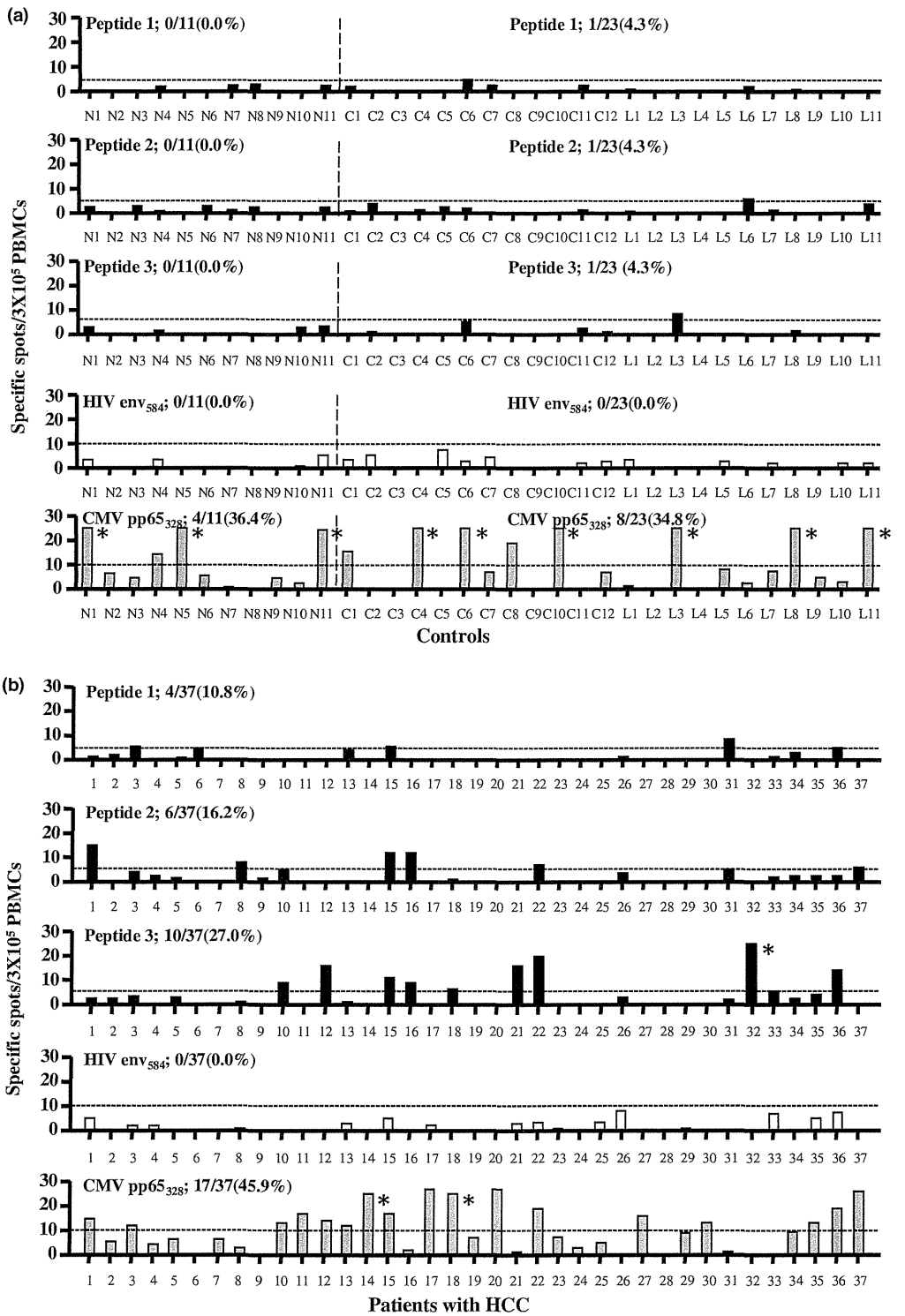

Fig. 2. Immune responses of chondroitin-glucuronate C5-epimerase-specific T cells. (a) IFN-γ ELISPOT assay of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptides (peptides 1, 2 and 3: solid bars) or control peptides (peptides HIVenv$_{584}$ and CMVpp65$_{328}$: open and grey bars respectively) in normal donors and disease control groups. "N" denotes normal donors. "C" denotes the patients with chronic hepatitis. "L" denotes the patients with cirrhosis. % shows the ratio of the patients who showed positive responses. *denotes more than 30 specific spots. (b) IFN-γ ELISPOT assay in HCC patients. *denotes more than 30 specific spots.

Table 2. Univariate analysis of the effect of variables on the T-cell response against chondroitin-glucuronate C5-epimerase

	Patients with positive T-cell response	Patients without positive T-cell response	*P*-value[a]
No. of patients	15	22	
Age (years)[b]	64.6 ± 9.8	68.7 ± 5.9	NS
gender(M/F)	14/1	15/7	NS
AFP (ng/ml)	3569.7 ± 13070.0	580.7 ± 2394.2	NS
Diff. degree of HCC (well/moderate or poor/ND)[c]	3/7/5	8/6/8	NS
Tumour multiplicity (multiple/solitary)	10/5	13/9	NS
Vascular invasion (+/-)	5/10	6/16	NS
TNM factor			
(T1/T2–4)	4/11	8/14	NS
(N0/N1)	14/1	22/0	NS
(M0/M1)	13/2	20/2	NS
TNM stage (I/II–IV)	4/11	8/14	NS
Histology of non-tumour liver (LC/Chronic hepatitis)	12/3	20/2	NS
Liver function (Child A/B/C)	11/4/0	13/7/2	NS
Aetiology (HCV/HBV/Others)	11/3/1	20/1/1	NS
T-cell response against to CMV $pp65_{328}$ (+/-)	9/6	9/13	NS

[a]NS: not significant.
[b]Data are expressed as the mean ± SD.
[c]ND: not determined.

tistical significance owing to the small number of patients.

Discussion

Many tumour-associated antigens and their epitopes capable of inducing HLA-class I-restricted CTLs have been identified in various cancers. Some of the epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials (11, 20–22).

With regard to immunotherapy for HCC, AFP is considered a useful tumour-associated antigen and AFP-derived peptides have actually been used in clinical trials (5, 23–25). However, in general, the production of AFP depends on the size of the tumour, with AFP expressed in only 0–40% of HCCs less than 30 mm in size (26). Therefore, for immunotherapy for HCC in cases where AFP is not expressed in tumour tissue, it is necessary to identify other tumour-associated antigens.

In the present study, the expression of chondroitin-glucuronate C5-epimerase was observed in all of the HCC tissues examined and independent of differential degree, size, TNM stage and the expression of AFP in the tumour. These results suggest the advantage of this antigen as a target for immunotherapy of HCC.

On the other hand, the expression of this protein was also observed in non-cancerous tissue of HCC patients, although less frequently and at lower levels than in HCC tissue. Our results are consistent with the recent finding that chondroitin-glucuronate C5-epimerase is expressed in some normal tissues including liver tissue (6). Such results imply that immunotherapy targeting chondroitin-glucuronate C5-epimerase may have adverse effects on liver tissue expressing the protein. Therefore, we next examined the existence and specificity of chondroitin-glucuronate C5-epimerase-specific CTLs in HCC patients.

The presence of chondroitin-glucuronate C5-epimerase-recognizing CTLs has been reported as SART2-specific CTLs in lung, gastric and pancreatic cancer patients (7, 27, 28). However, to our knowledge, there has been no report of the presence of chondroitin-glucuronate C5-epimerase-specific CTLs in HCC patients except our recent study using only one SART2-derived peptide (10). In this study, we used three different HLA-A24 restricted peptides which were previously identified and derived from naturally processed squamous cell carcinoma antigen. The HLA-A24 allele is found in 60% of Japanese (29), and therefore, to use HLA-A24-restricted peptides has the advantage of analysing CTL responses to tumour-associated antigens in Japanese patients.

We showed that chondroitin-glucuronate C5-epimerase-specific CTLs could be generated by stimulating PBMCs with peptides, and the CTLs were cytotoxic to hepatoma cell lines. Chondroitin-glucuronate C5-epimerase-specific immune responses were observed frequently only in HCC patients and the frequency of CTLs was higher in HCC patients than control groups, indicating that the immune responses are specific to HCC. Furthermore, the CTLs were also detected among TILs, suggesting that they infiltrate the tumour. Based on these findings, we confirmed that chondroitin-glucuronate C5-epimerase-specific CTL precursors exist in HCC patients and the immune responses are specific for HCC.

In previous study, we reported that the frequency of TAA-derived peptide-specific CTLs in HCC patients was 0–92 cells/3 × 10^5 PBMCs and the frequency of the

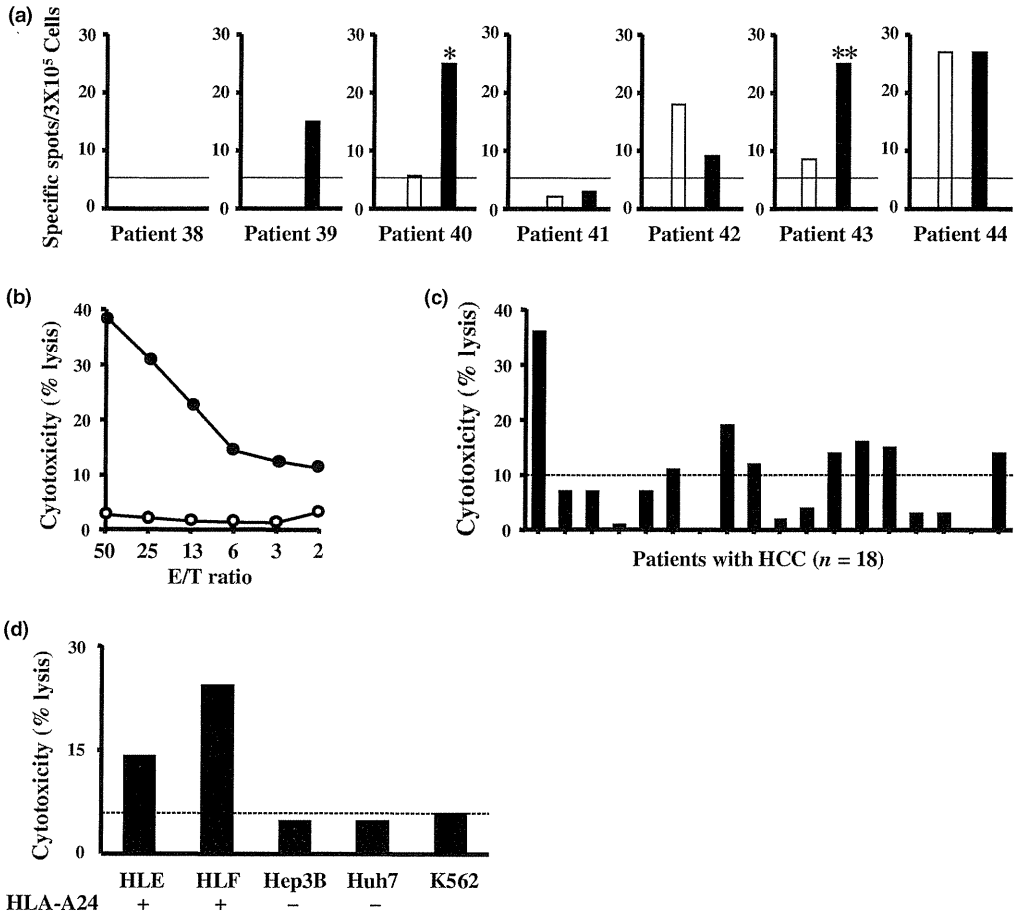

Fig. 3. Characteristics of chondroitin-glucuronate C5-epimerase-specific CTLs. (a) IFN-γ ELISPOT assay of PBMCs and TILs to one of the chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) in seven HCC patients. Open and solid bars show the frequency of chondroitin-glucuronate C5-epimerase-specific T cells in PBMCs and TILs respectively. *denotes 114 specific spots. **denotes 42 specific spots. (b) Representative results of the CTL assay. The closed and open circles show the cytotoxicity against C1R-A*2402 cells pulsed with and without a peptide respectively. (c) CTL assays (E/T ratio of 50:1) were performed in 18 HCC patients. Solid bars show the result for one patient. The results are shown as specific cytotoxic activity, which was calculated as follows: (cytotoxic activity in the presence of peptide) - (cytotoxic activity in the absence of peptide) and considered positive when higher than 10%. (d) Cytotoxicity of chondroitin-glucuronate C5-epimerase-specific T-cell lines derived with peptides was also measured against hepatoma cell lines. The cytotoxicity was considered positive when it was higher than that against K562 which shows non-specific lysis (E/T ratio of 50:1).

patients who showed immune responses to each peptide was 0–19% (10). In the present study, the frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs in HCC patients was 0–30 cells/ 3×10^5 PBMCs and the frequency of the patients who showed immune responses to the peptides was 11–27%. These results show that the frequencies of chondroitin-glucuronate C5-epimerase-specific CTLs in PBMCs and the patients with CTLs responsive to the TAA are very similar to those of previously identified immunogenic TAA-derived epitopes and suggest that the antigen and its CTL epitope are immunogenic. In addition, the CTLs were generated even in the early stages of HCC. These results suggest the advantages of using chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for immunotherapy of HCC.

For the next step to investigate the usefulness of chondroitin-glucuronate C5-epimerase as an immunotherapeutic target in HCC, we examined the safety and efficacy of chondroitin-glucuronate C5-epimerase-derived peptide as a cancer vaccine. In previous studies using chondroitin-glucuronate C5-epimerase-derived peptides for several cancers, they were reported to be safe. However, most patients with HCC have chronic liver disease. Therefore, safety of the peptide vaccine should be confirmed in the patients with chronic hepatitis or cirrhosis. The present vaccination study included nine patients with chronic liver diseases (four chronic hepatitis and five cirrhotic patients) confirmed by histological examination and there was no severe adverse event in all patients vaccinated. The induction of chondroitin-glucuronate C5-epimerase-specific CTLs

Table 3. Patient characteristics

Patient	Peptide Dose (mg)	Age	gender	Aetiology	Stage of HCC	ALT (IU/L)	AFP (ng/ml)	Child-Pugh (A/B/C)	Histology of liver	Treatment	Immune response	Toxicity (grade)
A1	0.03	73	F	HCV	I	26	12	A	F4A2	RFA	–	Pa(1)
A2	0.03	78	F	HCV	I	45	10	B	F4A2	RFA	–	P(1)
A3	0.03	59	M	NBNC	II	30	10	A	ND	RFA	–	None
B1	0.3	79	M	HCV	I	40	61	A	F3A1	RFA	–	R(1), S(1)
B2	0.3	72	M	NBNC	II	24	66	A	ND	RFA	–	R(1), S(1), P(1), H(1)
B3	0.3	78	M	HCV	II	45	10	A	F3A2	RFA	–	P(1)
C1	3.0	67	M	HCV	I	111	49	A	F3A1	RFA	+	P(1), S(1)
C2	3.0	73	M	NBNC	I	30	5	A	ND	RFA	–	None
C3	3.0	78	F	HCV	I	23	24	A	F4A2	RFA	+	P(1)
C4	3.0	75	M	HBV	I	21	15	A	F3A1	RFA	+	R(1), P(1)
C5	3.0	49	M	HBV	I	18	14	A	F4A1	RFA	+	None
C6	3.0	69	F	HBV	II	42	84	A	F4A2	RFA	–	Pa(1)

H, headache; Pa, pain; P, pruritus; R, rubor; S, skin induration.

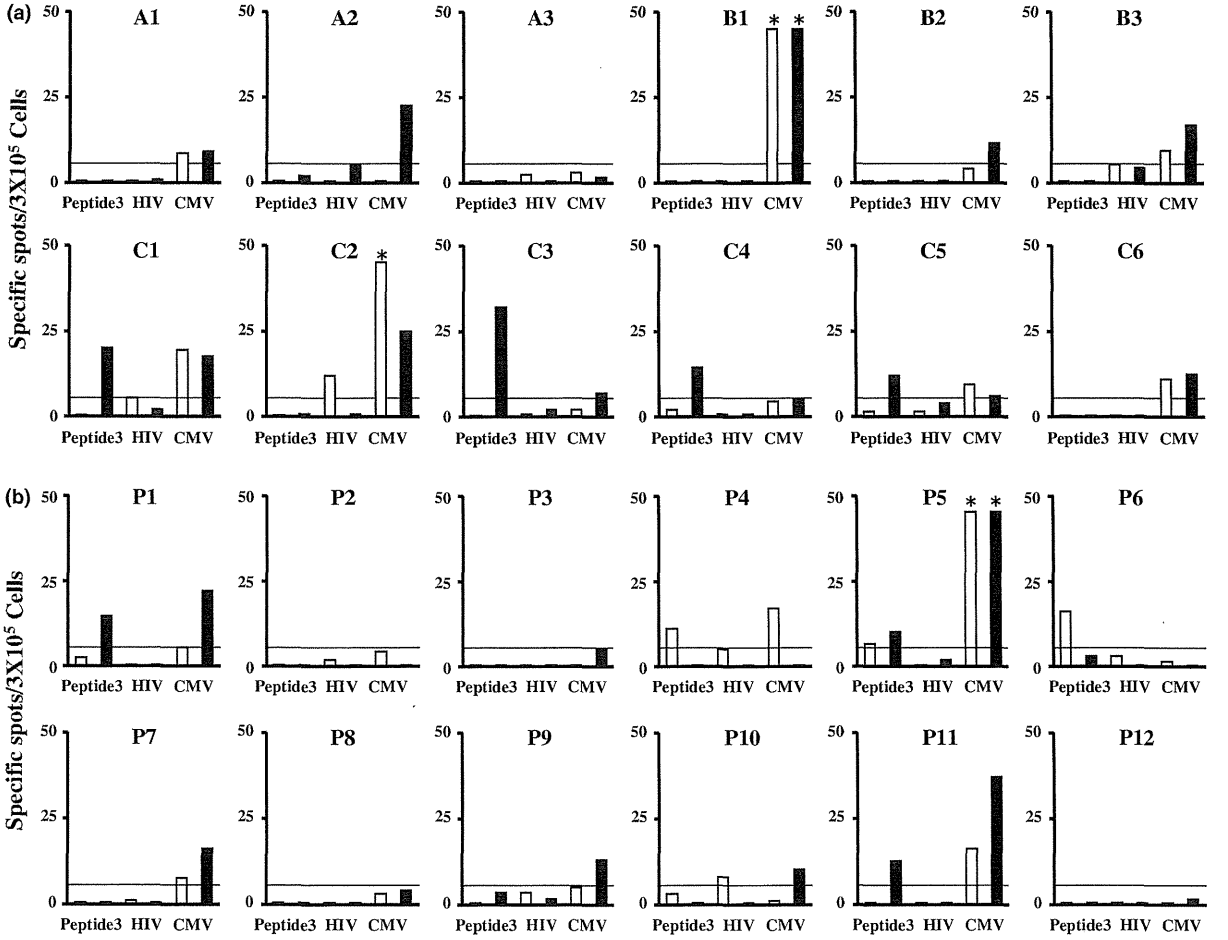

Fig. 4. IFN-γ ELISPOT assays of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) or control peptides (peptides $HIVenv_{584}$ and $CMVpp65_{328}$) in HCC patients with RFA. (a) The assays were performed in the patients with peptide 3 vaccination. White and black bars show the T-cell responses before and after vaccination respectively. (b) The assays were also performed in the patients without vaccination. White and black bars show the T-cell responses before and after RFA respectively. *denotes more than 50 specific spots.

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was observed in four of six (66.7%) patients vaccinated with 3 mg of peptide, which is similar to the frequency of responded patients reported in other peptide vaccination studies (11, 20).

Apart from induction of CTLs, the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for advanced HCC is still unclear. In previous vaccine studies for advanced HCC, AFP, hTERT and glypican-3 have been targeted as tumour-associated antigens for the treatment (25, 30–32). In these studies, peptide-specific CTLs were reported to be induced in 10–80% of vaccinated patients. However, in spite of the induction of peptide-specific CTLs, it has been reported that the anti-tumour effect was very limited. Recent studies have shown that the frequency of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) is increased in HCC patients and the cells inhibit the function of T cells (33, 34). Therefore, controlling their function might be important to develop more effective vaccination for advanced HCC.

In contrast, other recent studies using chondroitin-glucuronate C5-epimerase-derived peptides for other advanced cancers have shown the induction of cellular immune responses and clinical responses for certain patients (9, 11). In the analysis of the prognosis of patients with RFA and chondroitin-glucuronate C5-epimerase-derived peptide vaccination in the present study, the recurrence rate in the patients with an increase in the peptide-specific CTLs after vaccination was lower than that in the patients without immune response. Although further studies are necessary to evaluate the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides for HCC, the results of our study suggest that chondroitin-glucuronate C5-epimerase is a potential candidate for a target of HCC immunotherapy.

In conclusion, chondroitin-glucuronate C5-epimerase is a potential candidate for a tumour antigen with immunogenicity, and peptides derived from the protein would be useful for immunotherapy in cases of HCC.

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Therapeutics, Targets, and Chemical Biology

Cancer Research

Acyclic Retinoid Targets Platelet-Derived Growth Factor Signaling in the Prevention of Hepatic Fibrosis and Hepatocellular Carcinoma Development

Hikari Okada[1], Masao Honda[1,2], Jean S. Campbell[4], Yoshio Sakai[1], Taro Yamashita[1], Yuuki Takebuchi[1], Kazuhiro Hada[1], Takayoshi Shirasaki[1], Riuta Takabatake[1], Mikiko Nakamura[1], Hajime Sunagozaka[1], Takuji Tanaka[3], Nelson Fausto[4], and Shuichi Kaneko[1]

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 chemopreventive drug for HCC, published data about its effects on hepatic mesenchymal cells, such as stellate cells and endothelial cells, remain limited. Using a mouse model in which platelet-derived growth factor (PDGF)-C is overexpressed (*Pdgf-c Tg*), resulting in hepatic fibrosis, steatosis, and eventually, HCC development, we show that peretinoin significantly represses the development of hepatic fibrosis and tumors. Peretinoin inhibited the signaling pathways of fibrogenesis, angiogenesis, and Wnt/β-catenin in *Pdgf-c* transgenic mice. *In vitro*, peretinoin repressed the expression of PDGF receptors α/β in primary mouse hepatic stellate cells (HSC), hepatoma cells, fibroblasts, and endothelial cells. Peretinoin also inhibited PDGF-C–activated transformation of HSCs into myofibroblasts. Together, our findings show that PDGF signaling is a target of peretinoin in preventing the development of hepatic fibrosis and HCC. *Cancer Res; 72(17); 4459–71. ©2012 AACR.*

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 or hepatitis C virus infection or with other etiologies such as long-term alcohol abuse, autoimmunity, and hemochromatosis (2–5). Despite the recent advances in antiviral therapy for hepatitis B or hepatitis C virus, these are insufficient to completely prevent the occurrence of HCC. Moreover, the recent increase in nonalcoholic 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, nonparenchymal 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 upregulated from the early stages of chronic hepatitis, suggesting its association with the development of fibrosis in chronic hepatitis C (CH-C; refs. 9 and 10). Overexpression 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, which is frequently associated with hepatic fibrosis (7).

Peretinoin (generic name; code, NIK-333), developed by the Kowa Company, is an oral acyclic retinoid with a vitamin A–like structure, which targets the retinoid nuclear receptor. Oral administration of peretinoin was shown to significantly reduce the incidence of posttherapeutic 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 (15) and may interact with retinoic acid receptor-β and retinoid X receptor-α (16); however, the precise molecular targets for preventing HCC recurrence have not yet been elucidated.

Authors' Affiliations: [1]Department of Gastroenterology, Kanazawa University Graduate School of Medicine; [2]Department of Advanced Medical Technology, Kanazawa University Graduate School of Health Medicine; [3]Department of Oncologic Pathology, Kanazawa Medical University, Kanazawa, Japan; and [4]Department of Pathology, University of Washington School of Medicine, Seattle, Washington

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Corresponding Author: Masao Honda, Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa 920-8641, Japan. Phone: 81-76-265-2235; Fax: 81-76-234-4250; E-mail: mhonda@m-kanazawa.jp

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In this study, we used PDGF-C transgenic (*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.

Materials and Methods

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.

Animal studies

The generation and characterization of *Pdgf-c Tg* have been described previously (7). Wild-type and *Pdgf-c Tg* mice on a C57BL/6J background were maintained in a pathogen-free animal facility under a standard 12-hour/12-hour light/dark cycle. After weaning at week 4, male mice were randomly divided into the following 3 groups: (1) *Pdgf-c Tg* or wild-type (WT) mice given a basal diet (CRF-1, Charles River Laboratories Japan), (2) *Pdgf-c Tg* or WT mice given a 0.03% peretinoin-containing diet, (3) *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 3 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, Japan.

Cell culture

Human HCC cell lines Huh-7, HepG2, and HLE, the mouse fibroblast cell line NIH3T3, human umbilical vein endothelial cells (HUVEC), and human stellate cells Lx-2 (kindly provided by Dr. Scott Friedman, Mount Sinai School of Medicine, New York, NY) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS (Gibco), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. 1 to 5×10^4 cells were seeded in each well of a 12-well plate the day before serum starvation in serum-free DMEM for 8 hours. The culture medium was then replaced with serum-free medium containing peretinoin. After 24-hour incubation, cells were harvested for analysis.

Isolation and culture of mouse hepatic stellate cells

Hepatic stellate cells (HSC) 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% CO_2 for 72 hours. Nonadherent cells were removed with a pipette and the culture medium was replaced with medium containing 80 ng/mL recombinant human PDGF-C (Abnova) with or without peretinoin or 9-*cis*-retinoic acid (9cRA; 5 or 10 μmol/L). Cells were harvested for analysis after 24-hour incubation.

Isolation of peripheral blood mononuclear cells

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

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 weeks 20 and 48 was obtained and a total of 34 chip assays were conducted as described previously (17). Expression data have been deposited in the Gene Expression Omnibus (GEO; NCBI Accession; GSE31431).

Pathway analysis was conducted using MetaCore (GeneGo). Functional ontology enrichment analysis was conducted to compare the Gene Ontology (GO) process distribution of differentially expressed genes ($P < 0.01$; refs. 10 and 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 staining

Mouse liver tissues were fixed in 10% formalin and stained with hematoxylin and eosin. 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). Immunohistochemical (IHC) staining was conducted by an immunoperoxidase technique with an Envision kit (DAKO). 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), and mouse monoclonal cyclin D1 (1:400 dilution; all from Cell Signaling Technology); collagen 1 (1:100 dilution), collagen 4 (1:100 dilution), CD31 (1:100 dilution), and CD34 (1:100 dilution; all from Abcam, Cambridge, MA); and Tie-2 (1:80 dilution) and Myc (1:100 dilution; both from Santa Cruz Biotechnology).

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Quantitative real-time detection PCR

Total RNA was isolated from frozen liver tissue samples using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's protocol. cDNA was synthesized from 100 ng total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting

Western blotting was conducted as described previously (20). Whole-cell lysates from mouse liver were prepared and lysed by CelLytic MT cell lysis reagent (Sigma-Aldrich) containing Complete Mini EDTA-free Protease Inhibitor cocktail tablets (Roche). Cytoplasmic and nuclear protein extracts were prepared using the NE-PER nuclear extraction reagent kit (Pierce Biotechnology). Primary antibodies used were PDGFR-α (1:1,000 dilution), PDGFR-β (1:1,000 dilution), VEGFR2 (1:1,000 dilution), p44/42 MAPK (1:1,000 dilution), total AKT (1:1,000 dilution), p-p44/42 MAPK (1:1,000 dilution), p-AKT (Ser473; 1:1,000 dilution), p-AKT (Thr308; 1:1,000 dilution), β-catenin (1:2,000 dilution), cyclin D1 (1:400 dilution), and lamin A/C (1:1,000 dilution; all Cell Signaling Technology); α-SMA (1:200 dilution; DAKO); 4-HNE (1:200 dilution; NOF); and GAPDH (1:1,000 dilution) and Myc (1:1,000 dilution; both Santa Cruz).

Statistical analysis

Results are expressed as mean ± SD. Significance was tested by 1-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 *Pdgf-c Tg*

To evaluate the HCC chemopreventive effects of peretinoin, we used a mouse model of *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 (*Pdgf-c Tg*); whereas male mice not expressing the transgene were considered WT. After weaning at week 4, *Pdgf-c Tg* or nontransgenic 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 weeks 20 and 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, quantitative real-time detection PCR (RTD-PCR), and Western blotting. The expression of PDGFR-α and PDGFR-β, essential receptors for intracellular PDGF-C signaling, was upregulated 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 upregulated in *Pdgf-c Tg* mice, but repressed by peretinoin (Fig. 2 and Supplementary Fig. S1A).

RTD-PCR results confirmed that these genes were substantially upregulated in *Pdgf-c Tg* mice and significantly repressed by both 0.03% and 0.06% peretinoin (Fig. 3A). Western blotting 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 upregulated 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 HCC in *Pdgf-c Tg* mice

At week 48, *Pdgf-c Tg* mice developed hepatic tumors with an incidence of 90% (Fig. 4A). Histologic assessment of these tumors verified that 54% (15/28) were adenomas and 46% (13/28) were HCC (Fig. 4A and C and Supplementary Fig. S2; ref. 21). Peretinoin (0.03%) dose-dependently repressed the incidence of hepatic tumors to 53% (19/36) and to 29% (5/17) at 0.06%. 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 Supplementary Fig. S3.

Of the 3 main clusters, 2 were upregulated (clusters A and B) and 1 was downregulated (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, osteopontin, HGF), oncogene-related [v-ets erythroblastosis virus E26 oncogene homologue (Ets)1, Ets2, CD44, N-myc downstream-regulated (NDRG)1], and fibrosis-related (tubulin) genes. The expression of cluster A genes was further upregulated in tumors at week

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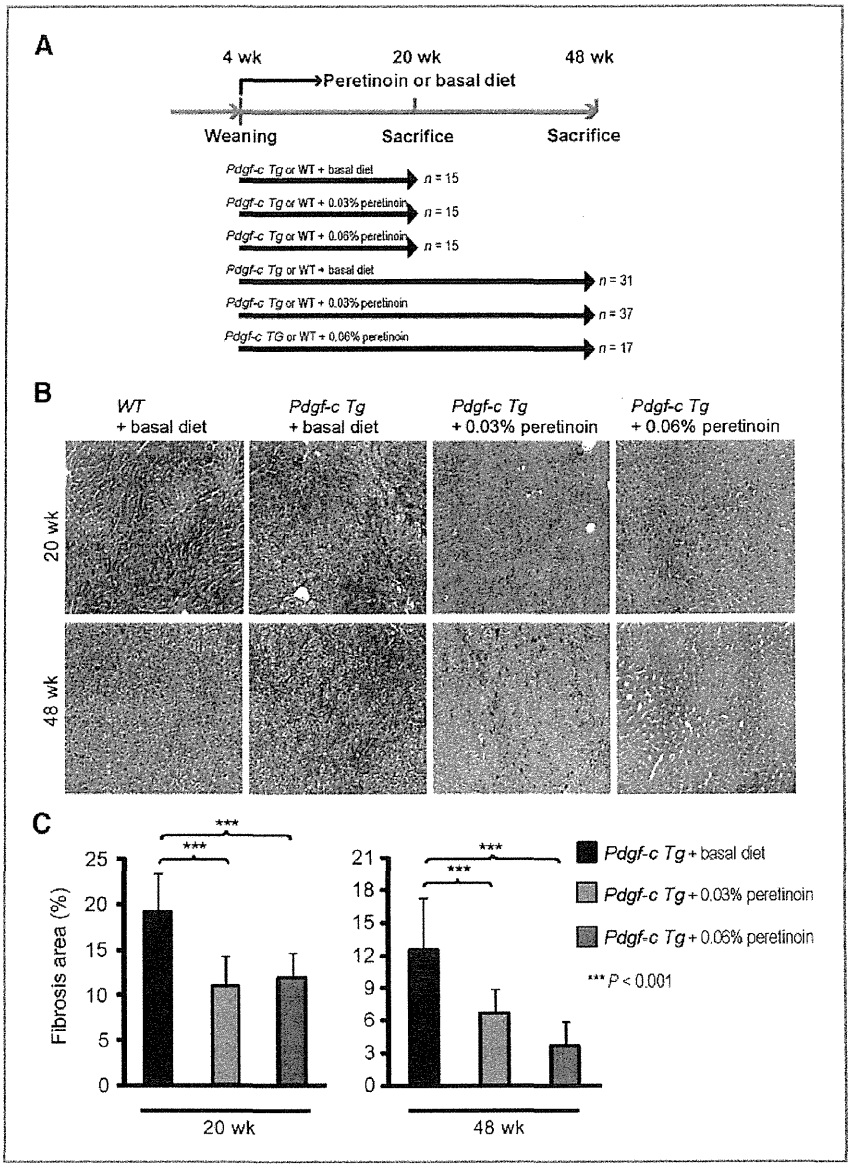

Figure 1. A, feeding schedule of *Pdgf-c Tg* and WT mice. After weaning, male mice were randomly divided into 3 groups: (i) *Pdgf-c Tg* or WT mice receiving basal diet, (ii) *Pdgf-c Tg* or WT mice receiving 0.03% peretinoin-containing diet, and (iii) *Pdgf-c Tg* or WT mice receiving 0.06% peretinoin-containing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

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 downregulated during the course of hepatic fibrosis and tumor development (Supplementary Fig. S4). 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 upregulated genes (clusters A and B) and restored the expression of downregulated genes (cluster C) at both weeks 20 and 48 (Supplementary Figs. S3 and S4).

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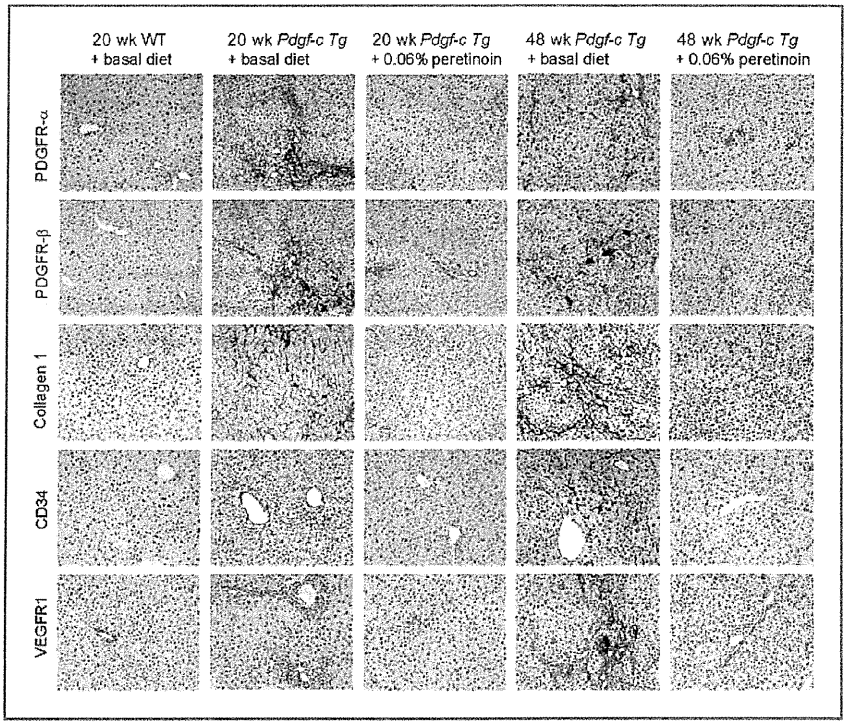

Figure 2. IHC staining of PDGFR-α, PDGFR-β, collagen 1, CD34, and VEGFR1 expression in *Pdgf-c Tg* or WT mouse livers fed a basal diet or 0.06% peretinoin.

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 downregulated and 100 genes were upregulated 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 (Supplementary Fig. S5) including interactions between representative growth factors, receptors (PDGFR and TGFβR), and transcriptional factors. Of these genes, the transcriptional factors Sp1 and Ap1 seem to be key regulators in the network (Supplementary Fig. S5).

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 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 and 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 (ref. 22; Supplementary Fig. S6A). Experimental conditions were optimized so that more than 90% of cells were variable at 20 μmol/L 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, whereas no obvious expression of PDGFR-α was observed in HepG2 cells and HUVECs (Supplementary Fig. S6A). 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 (Supplementary Fig. S6B). 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 2 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, whereas expression of phosphorylated c-Jun (p-c-Jun) was increased in Huh-7 cells (Supplementary Fig. S6C). Therefore, peretinoin seems 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

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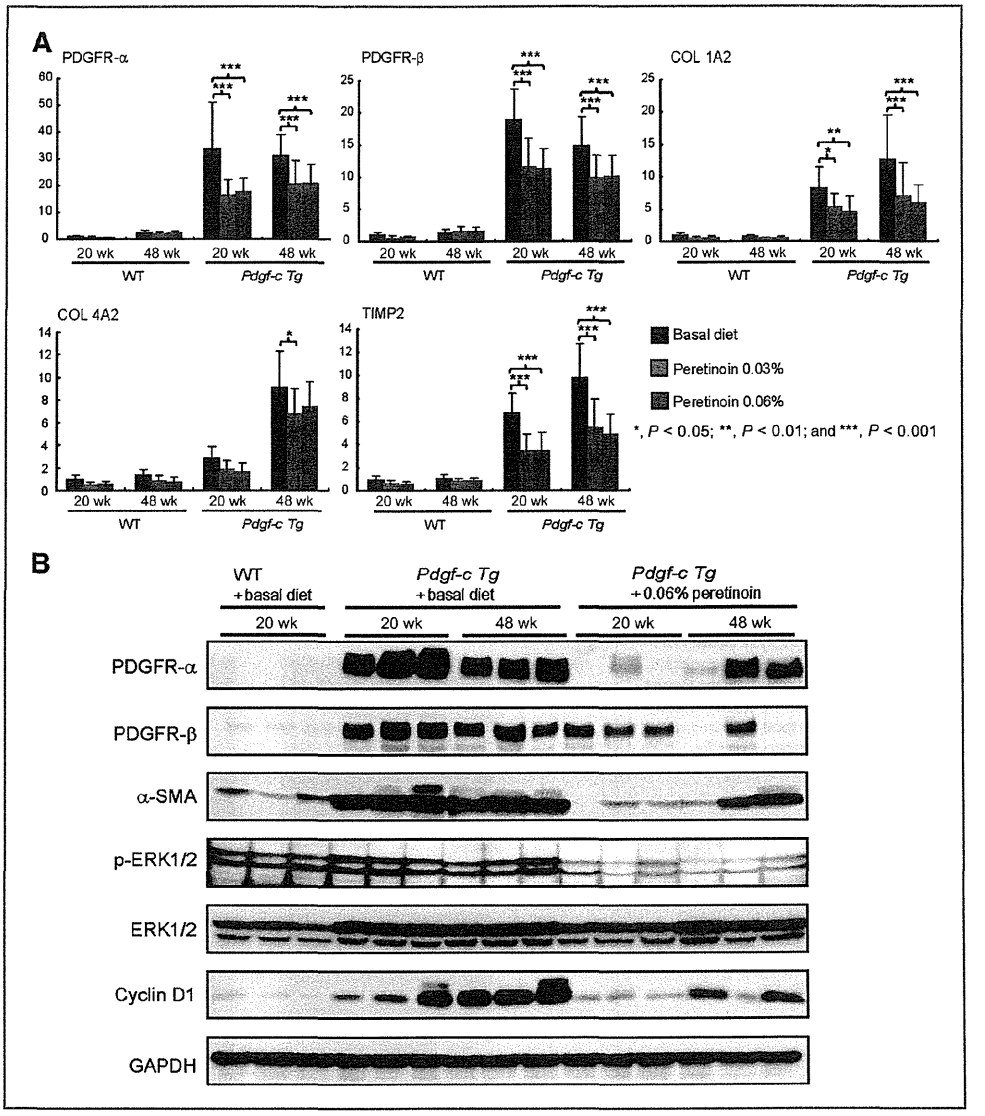

Figure 3. A, RTD-PCR analysis of PDGFR-α, PDGFR-β, collagen (COL) 1a2, collagen 4 a2, and TIMP2 expression in *Pdgf-c Tg* ($n = 5$) or WT mouse livers ($n = 15$). B, Western blotting of PDGFR-α, PDGFR-β, α-SMA, p-ERK, ERK, cyclin D1, and GAPDH expression in PDGF-C Tg or WT mouse livers fed a basal diet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

livers at weeks 20 and 48 revealed overexpression 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 Supplementary Fig. S1A). This expression was significantly repressed by peretinoin as determined by the densitometric area (Supplemental Fig. S1B). RTD-PCR results revealed significant upregulation of VEGFR1 (Flt-1) in *Pdgf-c Tg* mice compared with WT mice at both weeks 20 and 48, whereas the expression of VEGFR2 (Flk-1) and Tie2 was only upregulated at week 48. The expression of these genes was significantly repressed by peretinoin (Fig. 6A). Western blotting confirmed the upregulation 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 upregulated 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 upregulated in *Pdgf-c Tg* mice at week 48 but significantly repressed by peretinoin (Fig. 6C and D). Thus, peretinoin