Table 1 Baseline demographics

	Overall $n = 94$	Skin toxicities			Hypertension		
		Presence $n = 58$	Absence $n = 36$	P	Presence $n = 23$	Absence $n = 71$	P
Age, years, median (range)	75 (50–87)	75 (50–87)	76 (51–84)	0.674	75 (54–87)	75 (50–86)	0.546
Sex, *n* (%)							
Male	77 (82)	46 (79)	31 (86)	0.408	18 (78)	59 (83)	0.602
Female	17 (18)	12 (21)	5 (14)		5 (22)	12 (17)	
ECOG PS, *n* (%)							
0–1	91 (97)	57 (98)	34 (94)	0.307	23 (100)	68 (96)	0.319
2–	3 (3)	1 (2)	2 (6)		0	3 (4)	
Cause of disease, *n* (%)							
HCV	62 (66)	33 (57)	29 (81)	0.019	17 (74)	45 (63)	0.357
HBV	10 (11)	9 (15)	1 (2)		1 (4)	9 (13)	
Others	22 (23)	16 (28)	6 (17)		5 (22)	17 (24)	
Child–Pugh class, *n* (%)							
A	78 (83)	53 (91)	25 (69)	0.006	18 (78)	60 (85)	0.491
B	16 (17)	5 (9)	11 (31)		5 (22)	11 (15)	
BCLC stage, *n* (%)							
A	2 (2)	2 (3)	0	0.117	1 (5)	1 (2)	0.229
B	33 (35)	16 (28)	17 (47)		10 (43)	23 (32)	
C	59 (63)	40 (69)	19 (53)		12 (52)	47 (66)	
Macroscopic vascular invasion, *n* (%)	29 (31)	19 (33)	10 (28)	0.613	7 (30)	22 (31)	0.961
Extrahepatic spread, *n* (%)	40 (43)	26 (45)	14 (39)	0.573	7 (30)	33 (46)	0.179
AFP, ng/mL, median (range)	131 ($0–2.4 \times 10^5$)	40 ($0–2.4 \times 10^5$)	753 ($0–9.0 \times 10^4$)	0.050	618 ($1.8–2.4 \times 10^5$)	91 ($0–1.1 \times 10^5$)	0.667
DCP, mAU/mL, median (range)	566 ($0–1.0 \times 10^6$)	378 ($0–1.6 \times 10^5$)	1932 ($0–1.0 \times 10^6$)	0.068	450 ($12–4.9 \times 10^4$)	639 ($0–1.0 \times 10^6$)	0.510
Previous therapy, *n* (%)	87 (93)	54 (93)	33 (92)	0.798	21 (91)	66 (93)	0.794
Surgical resection, *n* (%)	38 (40)	26 (45)	12 (33)	0.272	6 (26)	32 (45)	0.109
TACE, *n* (%)	74 (79)	47 (81)	27 (75)	0.489	18 (78)	56 (79)	0.951
Ablation therapy, *n* (%)	51 (54)	33 (57)	18 (50)	0.516	15 (65)	36 (51)	0.227
Hepatic arterial infusion chemotherapy, *n* (%)	38 (40)	22 (38)	16 (44)	0.534	7 (30)	31 (44)	0.264
Systemic chemotherapy, *n* (%)	17 (18)	13 (22)	4 (11)	0.169	3 (13)	14 (20)	0.472
Radiotherapy, *n* (%)	9 (10)	3 (5)	6 (17)	0.067	0	9 (13)	0.074

AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; DCP, des-γ-carboxy-prothrombin; ECOG PS, Eastern Cooperative Oncology Group Performance Status; HBV, hepatitis B virus; HCV, hepatitis C virus; TACE, transarterial chemoembolization.

Table 2 Summary of treatment efficacy

	Overall $n = 94$	Skin toxicities		P	Hypertension		P
		Presence $n = 58$	Absence $n = 36$		Presence $n = 23$	Absence $n = 71$	
Response rate, %	6	4	12	0.170	6	6	0.902
Disease-control rate, %	49	49	50	0.940	44	51	0.637
Time to progression, months, median	2.9	3.6	2.0	0.718	5.1	2.9	0.587
Overall survival, months, median	12.5	16.8	5.9	0.004	17.0	11.1	0.332

16 patients (17%) was B. The Child–Pugh score was 7 points in nine patients and 8 points in all others. All Child–Pugh class B patients were administrated sorafenib according to the patients' wishes.

Sixty-six patients (70%) began sorafenib monotherapy at 800 mg daily. The median total dose and relative dose intensity of sorafenib in overall patients was 39 200 mg and 72%, respectively. Eighty-two patients stopped treatment because of disease progression (61%), toxicity (37%) or refusal (2%).

Fifty-one patients died, and the surviving patients had a median follow up of 9.4 months. Table 2 shows a summary of sorafenib treatment efficacies. The median time to progression and survival time in overall patients were 2.9 and 12.5 months, respectively. Eighty-one patients were evaluable for the objective tumor response. The objective response rate and disease-control rate were 6% and 49%, respectively.

Adverse events and treatment effects

The overall incidence of treatment-related adverse events was 98% of patients. Table 3 shows the incidences of sorafenib-related adverse events that occurred in at least 5% of patients. Skin toxicities were observed

Table 3 Adverse events that occurred in at least 5% of the patients as defined by the Common Terminology Criteria for Adverse Events version 4.0

Adverse events	Any n (%)	Grade 1 n (%)	Grade 2 n (%)	Grade 3 n (%)	Grade 4 n (%)
Overall	92 (98)				
Skin toxicities	58 (62)				
Palmar-plantar erythrodysesthesia syndrome	52 (55)	17 (18)	25 (27)	10 (11)	–
Rash	9 (10)	6 (6)	2 (2)	1 (1)	0
Alopecia	7 (7)	5 (5)	2 (2)	–	–
Pruritus	2 (2)	1 (1)	1 (1)	0	–
Hypertension	23 (24)	1 (1)	17 (18)	5 (5)	0
Gastrointestinal disorders	44 (47)				
Diarrhea	31 (33)	13 (14)	14 (15)	4 (4)	0
Anorexia	19 (20)	11 (12)	5 (5)	3 (3)	0
Vomiting	4 (4)	2 (2)	2 (2)	0	0
Mucositis	3 (3)	3 (3)	0	0	0
Liver dysfunction	39 (41)				
AST or ALT increased	31 (33)	10 (11)	7 (7)	13 (14)	1 (1)
Bilirubin increased	10 (11)	2 (2)	2 (2)	5 (5)	1 (1)
Liver failure	6 (6)	–	–	3 (3)	3 (3)
Bleeding	9 (10)	2 (2)	3 (3)	4 (4)	0
Fever	7 (7)	5 (5)	2 (2)	0	0
Fatigue	7 (7)	3 (3)	2 (2)	2 (2)	–
Hoarseness	6 (6)	5 (5)	1 (1)	0	–

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

in 58 patients (62%). Among the skin toxicities, palmar-plantar erythrodysesthesia syndrome was the most common adverse event and was observed in 52 patients (55%). Hypertension was observed in 23 patients (24%). The patient baseline demographics with or without skin toxicities and hypertension are also shown in Table 1. There were significant differences in the cause of disease and the Child–Pugh class between the presence and absence of skin toxicities, while no differences were observed between the presence and absence of hypertension. The median total dose of sorafenib was 48 300 mg in the patients with skin toxicities, 23 800 mg in the patients without skin toxicities, 35 000 mg in the patients with hypertension and 39 200 mg in the patients without hypertension. There was a significant difference between the patients with and without skin toxicities ($P < 0.001$). On the other hand, the relative dose intensity in the patients with skin toxicities was lower than that in the patients without skin toxicities (median 69% and 90%, respectively; $P = 0.031$). The relative dose intensity in the patients with or without hypertension was comparable (median 73% and 71%, respectively; $P = 0.498$).

The patients with skin toxicities had a significantly longer survival than the patients without these toxicities (hazard ratio, 0.449; 95% confidence interval, 0.256–0.786; $P = 0.005$) (Fig. 1). In the patients with skin toxicities, the overall survival rate at 6 months was 79%. In the patients without skin toxicities, the overall survival rate at 6 months was 48%. The median survival time of patients with skin toxicities was 16.8 months and that of patients without skin toxicities was 5.9 months (Table 2). On the other hand, no statistically significant difference was noted among the patients stratified by sorafenib-related hypertension for survival (Table 2). According to the Cox proportional hazards model analysis, skin toxicities, Child–Pugh class A and lower serum α-fetoprotein level were significant, independent, good prognostic factors (Table 4). The median time to the first onset of skin toxicities was 21 days. There were no significant differences in antitumor responses or time to progression among the patients stratified by skin toxicities or hypertension related to sorafenib (Table 2).

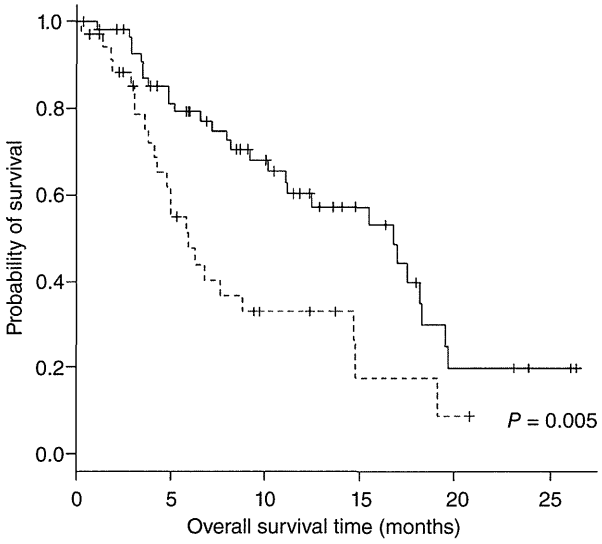

Figure 1 Kaplan–Meier analysis of overall survival stratified by skin toxicities. The patients with skin toxicities have significantly longer survival times than the patients without these toxicities ($P = 0.005$; log-rank test). (—), With skin toxicities; (---), without skin toxicities.

DISCUSSION

THE PRESENT STUDY shows that patients with sorafenib-related skin toxicities might be associated with a good survival prognosis in HCC. There have been some reports regarding the relationships between skin toxicities caused by molecular targeted agents and their antitumor effects. Among patients treated with epidermal growth factor receptor inhibitors, those with drug-induced rash had a better correlation with response and/or survival in colorectal cancer,[8,9] non-small cell lung cancer,[10] head and neck squamous cell cancer,[11,12] ovarian cancer[13] and pancreatic cancer.[14,15] Regarding treatment with sorafenib, there is also a report that HCC patients who developed early skin toxicities showed a significantly longer time to disease progression.[21] However, there were no correlations between skin toxicities related to sorafenib and the antitumor response or time to progression in our study. Although targeted agents have been shown to have significant survival advantages, it is sometimes difficult to assess the antitumor response because of modest tumor shrinkage. In the SHARP study[5] and the Asia–Pacific study,[6] the objective response rates according to RECIST[22] were 2% and 3.3%, respectively. However, 71% and 54% of patients had stable disease, respectively. The RECIST criteria were originally developed to assess responses to cytotoxic agents and may not be appropriate indicators of activity for targeted agents that are associated with prolonged stable disease.[23] Modified RECIST criteria that measure viable (enhancement in the arterial phase) lesions were proposed for targeted therapies or locoregional thera-

Table 4 Univariate and multivariate Cox proportional hazard models for overall survival

Variables	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
HCV	1.012	0.577–1.778	0.966			
Child–Pugh A	0.256	0.124–0.526	<0.001	0.399	0.176–0.906	0.028
BCLC stage A-B	0.784	0.443–1.388	0.404			
AFP <100 ng/mL	0.372	0.211–0.656	<0.001	0.504	0.260–0.977	0.042
DCP <500 mAU/mL	0.766	0.442–1.329	0.343			
Relative dose intensity >50%	0.808	0.426–1.530	0.512			
Skin toxicities	0.449	0.256–0.786	0.005	0.522	0.274–0.997	0.049
Hypertension	0.713	0.362–1.406	0.329			

95% CI, 95% confidence interval; AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; DCP, des-γ-carboxy-prothrombin; HCV, hepatitis C virus; HR, hazard ratio.

pies in HCC.[24] We also evaluated the antitumor response by the modified RECIST criteria, but found no links between sorafenib-related skin toxicities or hypertension and the antitumor response (data not shown). The development of an evaluation method for the antitumor response of sorafenib with regard to survival is necessary in the future.

In our cohort, the patients without skin toxicities contained more Child–Pugh class B patients than did patients with skin toxicities. The median survival time was 15.5 months in Child–Pugh A patients and 4.8 months in Child–Pugh B patients. This poor prognosis of Child–Pugh B patients might have influenced the result of our study. However, it is thought that skin toxicities associated with sorafenib therapy have a good correlation with survival because skin toxicities were a statistically significant factor for survival along with Child–Pugh class according to the multivariate analysis (Table 4). The total dose of sorafenib was higher, but the relative dose intensity was lower in the patients with skin toxicities than in the patients without skin toxicities. This paradoxical result is attributed to a longer treatment duration (median, 114 and 37 days, respectively; $P < 0.001$) and higher frequency of dose reduction or interruption of sorafenib (79% and 47%, respectively; $P = 0.001$) in the patients with and without skin toxicities.

Sorafenib is known to cause frequently occurring skin toxicities that include palmar-plantar erythrodysesthesia syndrome, facial erythema (rash or desquamation), pruritus, dry skin and alopecia.[5,6,25] Palmar-plantar erythrodysesthesia syndrome is the most common dermatological toxicity, and histological analyses of this symptom show a thickened epidermis with hyperkeratosis, non-specific inflammatory dermal cell infiltrates and dilated dermal vessels.[25–27] Modifications in cytokeratin expression were observed after immunostaining with anti-cytokeratin antibodies, suggesting that sorafenib may affect keratinocyte differentiation. However, the pathogenesis of this syndrome caused by sorafenib has not been established because neither VEGF nor FLT-3 receptors are expressed in normal keratinocytes,[25] and immunostaining for c-kit and PDGFR showed no difference between areas of normal skin and the lesions of this syndrome.[28]

Hypertension is a common adverse event for inhibitors of angiogenesis, especially inhibitors of VEGFR signaling.[29,30] Arterial hypertension related to VEGFR inhibitors has good correlations with clinical outcomes in colorectal cancer,[16,17] pancreatic cancer[18] and renal cell cancer.[19] Although the mechanisms of the hypertension during antiangiogenic therapy have not been clarified, microvascular rarefaction may play an important role in the development of hypertension.[30] Regarding sorafenib therapy, there is a report that drug-induced hypertension can predict the clinical benefit in metastatic renal cell cancer.[31] Our study showed that there was no correlation between hypertension and clinical outcomes in patients with HCC. Further studies are needed to elucidate whether the alteration in blood pressure during sorafenib therapy is associated with an antitumor effect.

In conclusion, skin toxicities occur commonly at the early phase in HCC patients treated with sorafenib. Skin toxicities, mainly palmar-plantar erythrodysesthesia syndrome, can significantly affect the patient's quality of life, even though these toxicities are not usually life-threatening. Skin reactions could be a promising surrogate marker for the prognosis, and therefore early

identification and control of these reactions are critical for continuing sorafenib therapy.

REFERENCES

1 El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557–76.
2 McGlynn KA, Tsao L, Hsing AW, Devesa SS, Fraumeni JF Jr. International trends and patterns of primary liver cancer. *Int J Cancer* 2001; **94**: 290–96.
3 Llovet JM, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329–38.
4 Wilhelm SM, Carter C, Tang L *et al.* BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004; **64**: 7099–109.
5 Llovet JM, Ricci S, Mazzaferro V *et al.* Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008; **359**: 378–90.
6 Cheng AL, Kang YK, Chen Z *et al.* Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009; **10**: 25–34.
7 Morimoto M, Numata K, Kondo M *et al.* Higher discontinuation and lower survival rates are likely in elderly Japanese patients with advanced hepatocellular carcinoma receiving sorafenib. *Hepatol Res* 2011; **41**: 296–302.
8 Cunningham D, Humblet Y, Siena S *et al.* Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004; **351**: 337–45.
9 Van Cutsem E, Köhne CH, Hitre E *et al.* Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009; **360**: 1408–17.
10 Pérez-Soler R, Chachoua A, Hammond LA *et al.* Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. *J Clin Oncol* 2004; **22**: 3238–47.
11 Soulieres D, Senzer NN, Vokes EE, Hidalgo M, Agarwala SS, Siu LL. Multicenter phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. *J Clin Oncol* 2004; **22**: 77–85.
12 Herbst RS, Arquette M, Shin DM *et al.* Phase II multicenter study of the epidermal growth factor receptor antibody cetuximab and cisplatin for recurrent and refractory squamous cell carcinoma of the head and neck. *J Clin Oncol* 2005; **23**: 5578–87.
13 Gordon AN, Finkler N, Edwards RP *et al.* Efficacy and safety of erlotinib HCl, an epidermal growth factor receptor (HER1/EGFR) tyrosine kinase inhibitor, in patients with advanced ovarian carcinoma: results from a phase II multicenter study. *Int J Gynecol Cancer* 2005; **15**: 785–92.
14 Xiong HQ, Rosenberg A, LoBuglio A *et al.* Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II Trial. *J Clin Oncol* 2004; **22**: 2610–6.
15 Moore MJ, Goldstein D, Hamm J *et al.* Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960–6.
16 Scartozzi M, Galizia E, Chiorrini S *et al.* Arterial hypertension correlates with clinical outcome in colorectal cancer patients treated with first-line bevacizumab. *Ann Oncol* 2009; **20**: 227–30.
17 Österlund P, Soveri LM, Isoniemi H, Poussa T, Alanko T, Bono P. Hypertension and overall survival in metastatic colorectal cancer patients treated with bevacizumab-containing chemotherapy. *Br J Cancer* 2011; **104**: 599–604.
18 Spano JP, Chodkiewicz C, Maurel J *et al.* Efficacy of gemcitabine plus axitinib compared with gemcitabine alone in patients with advanced pancreatic cancer: an open-label randomised phase II study. *Lancet* 2008; **371**: 2101–8.
19 Bono P, Elfving H, Utriainen T *et al.* Hypertension and clinical benefit of bevacizumab in the treatment of advanced renal cell carcinoma. *Ann Oncol* 2009; **20**: 393–4.
20 Eisenhauer EA, Therasse P, Bogaerts J *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228–47.
21 Vincenzi B, Santini D, Russo A *et al.* Early skin toxicity as a predictive factor for tumor control in hepatocellular carcinoma patients treated with sorafenib. *Oncologist* 2010; **15**: 85–92.
22 Therasse P, Arbuck SG, Eisenhauer EA *et al.* New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000; **92**: 205–16.
23 Ratain MJ, Eckhardt SG. Phase II studies of modern drugs directed against new targets: if you are fazed, too, then resist RECIST. *J Clin Oncol* 2004; **22**: 14S. abstract.
24 Lencioni R, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis* 2010; **30**: 52–60.
25 Yang CH, Lin WC, Chuang CK *et al.* Hand-foot skin reaction in patients treated with sorafenib: a clinicopathological study of cutaneous manifestations due to multitargeted kinase inhibitor therapy. *Br J Dermatol* 2008; **158**: 592–6.
26 Autier J, Escudier B, Wechsler J, Spatz A, Robert C. Prospective study of the cutaneous adverse effects of sorafenib, a novel multikinase inhibitor. *Arch Dermatol* 2008; **144**: 886–92.
27 Robert C, Mateus C, Spatz A, Wechsler J, Escudier B. Dermatologic symptoms associated with the multikinase

inhibitor sorafenib. *J Am Acad Dermatol* 2009; **60**: 299–305.
28 Faivre S, Delbaldo C, Vera K *et al.* Safety, pharmacokinetic, and antitumor activity of SU11248, a novel oral multitarget tyrosine kinase inhibitor, in patients with cancer. *J Clin Oncol* 2006; **24**: 25–35.
29 Sica DA. Angiogenesis inhibitors and hypertension: an emerging issue. *J Clin Oncol* 2006; **24**: 1329–31.
30 Lévy BI. Blood pressure as a potential biomarker of the efficacy angiogenesis inhibitor. *Ann Oncol* 2009; **20**: 200–3.
31 Ravaud A, Sire M. Arterial hypertension and clinical benefit of sunitinib, sorafenib and bevacizumab in first and second-line treatment of metastatic renal cell cancer. *Ann Oncol* 2009; **20**: 966–7.

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 1668–1675

Journal of Nutritional Biochemistry

The role of PKC isoforms in the inhibition of NF-κB activation by vitamin K2 in human hepatocellular carcinoma cells☆

JingHe Xia[a], Sachiko Matsuhashi[a], Hiroshi Hamajima[a], Shinji Iwane[a], Hirokazu Takahashi[a], Yuichiro Eguchi[a], Toshihiko Mizuta[a], Kazuma Fujimoto[a], Shun'ichi Kuroda[b], Iwata Ozaki[a,c,*]

[a]*Department of Internal Medicine, Saga Medical School, Saga University 5-1-1 Nabeshima, Saga 849-8501, Japan*
[b]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8 601, Japan*
[c]*Health Administration Center, Saga Medical School, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan*

Received 16 June 2011; received in revised form 3 November 2011; accepted 30 November 2011

Abstract

Vitamin K (VK) has diverse protective effects against osteoporosis, atherosclerosis and carcinogenesis. We recently reported that menatetrenone, a VK2 analogue, suppressed nuclear factor (NF)-κB activation in human hepatoma cells. Although NF-κB is regulated by isoforms of protein kinase C (PKC), the involvement of PKCs in VK2-mediated NF-κB inhibition remains unknown. Therefore, the effects of VK2 on the activation and the kinase activity of each PKC isoform were investigated. The human hepatoma Huh7 cells were treated with PKC isoform-specific inhibitors and/or siRNAs against each PKC isoform with or without 12-*O*-tetradecanoylphorbol-13-acetate (TPA). VK2 inhibited the TPA-induced NF-κB activation in Huh7 cells. NF-κB activity was inhibited by the pan-PKC inhibitor Ro-31-8425, but not by the PKCα-specific inhibitor Gö6976. The knockdown of individual PKC isoforms including PKCα, δ and ε showed only marginal effects on the NF-κB activity. However, the knockdown of both PKCδ and PKCε, together with treatment with a PKCα-specific inhibitor, depressed the NF-κB activity. VK2 suppressed the PKCα kinase activity and the phosphorylation of PKCε after TPA treatment, but neither the activation nor the enzyme activity of PKCδ was affected. The knockdown of PKCε abolished the TPA-induced phosphorylation of PKD1, and the effects of PKD1 knockdown on NF-κB activation were similar to those of PKCε knockdown. Collectively, all of the PKCs, including α, δ and ε, and PKD1 are involved in the TPA-mediated activation of NF-κB. VK2 inhibited the NF-κB activation through the inhibition of PKCα and ε kinase activities, as well as subsequent inhibition of PKD1 activation.

Keywords: Vitamin K2; NF-κB; PKC; PKD1; Liver cancer

1. Introduction

Vitamin K (VK), an essential nutrient for the production of functional blood coagulation factors, has emerged as an important factor protecting against diverse diseases such as osteoporosis, atherosclerosis and several types of neoplasms, including hepatocellular carcinoma (HCC) [1–4]. The K vitamins can be divided into two groups: naturally produced VK1 (phylloquinone) and VK2 (menaquinone), and chemically synthesized VK3 (menadione). VK1 is contained in a wide range of plants; VK2 is of microbial origin and is widely distributed in leafy vegetables, eggs, cheese, meats and fermented soybeans.

Previously, several VK analogues have been reported to possess antitumor activity against hematological malignancies and solid tumors, such as HCC [3–7]. We and others have demonstrated that the administration of menatetrenone, a VK2 analogue, suppresses the recurrence of HCC after curative ablation therapy [8] or the development of HCC from cirrhotic livers [9] in the clinical setting. VK2 also suppresses the development and proliferation of HCC in *in vivo* animal models [10–12]. Recently, we have revealed that VK2 inhibits the growth of HCC cells by suppressing cyclin D1 expression through the inhibition of nuclear factor (NF)-κB activation [13], as well as via inhibition of the expression of matrix metalloproteinases (MMPs) that contain an NF-κB binding motif in their promoter region and are considered to be involved in both the invasion and metastasis of carcinoma cells [14,15].

Protein kinase C (PKC), one of the phospholipid-dependent serine/threonine kinase families, is subdivided into conventional PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKCs [16,17]. When cells are treated with activators, including tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cPKCs and nPKCs are activated and play pivotal and key regulatory roles in a number of cellular functions, such as cellular growth and migration, accompanied by changes in gene expression [16,17]. PKCs have been reported to participate in the activation of AP-1 and NF-κB and increase their subsequent gene expression [18–20]. We have shown that VK2 suppressed the expression of MMPs, along with the inhibition of TPA-

☆ Financial support: This study was supported, in part, by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to I.O. (P-03346) and to T.M. (No. 16590606).
* Corresponding author. Health Administration Center, Department of Internal Medicine, Saga Medical School, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan. Tel.: +81 952 3433215; fax: +81 952 34 2008.
E-mail address: ozaki@cc.saga-u.ac.jp (I. Ozaki).

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jnutbio.2011.11.010

induced activation of NF-κB [13,14], suggesting that VK2 might act as a PKC inhibitor. These results prompted us to clarify the role of PKCs in VK2-mediated inhibition of NF-κB activation. In this report, we have examined the role of each PKC isoform in the activation of NF-κB and have also evaluated the effects of VK2 on the activation of individual PKC isoforms and their kinase activities.

2. Materials and methods

2.1. Cell lines and reagents

Huh7 cells were obtained from the Japanese Cancer Research Resources Bank (Osaka, Japan). The cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. Menatetrenone, a VK2 analogue, was provided by Eisai Co. (Tokyo, Japan) and dissolved in ethanol. The PKC inhibitors Ro-31-8425 and Gö6976 were from Calbiochem (San Diego, CA, USA), and TPA was from Sigma-Aldrich. SiRNAs against the PKCα, δ and ε isoforms (HP validated, S100301308, S102660539, S100287784, respectively), PKD1 (S100301350) and Allstar negative control siRNA (S1027281) were from Qiagen (Heiden, Germany). Human PKCα plasmids, pHACE-PKCα-WT (wild type), -DN (dominant negative), -δNPS (constitutive active) and -CAT (constitutive active) were kindly denoted by Dr. Joe-Won Soh, Laboratory of Signal Transduction, Department of Chemistry, Inha University, Korea [21]. Anti-PKCα, δ, ε antibodies were obtained from Santa Cruz, CA, USA. Anti-phospho-PKCα (Thr497, Thr638), anti-phospho-PKCδ (Thr505, Ser643), anti-phospho-PKCε (Ser729), anti-phospho-PKD1 (Ser744), anti-PKD1 and anti-phospho-IκBα (Ser32/36) antibodies were the products of Cell Signaling Technology (Beverly, MA, USA). Anti-human β-actin antibody was purchased from Biomedical Technologies (Stoughton, MA).

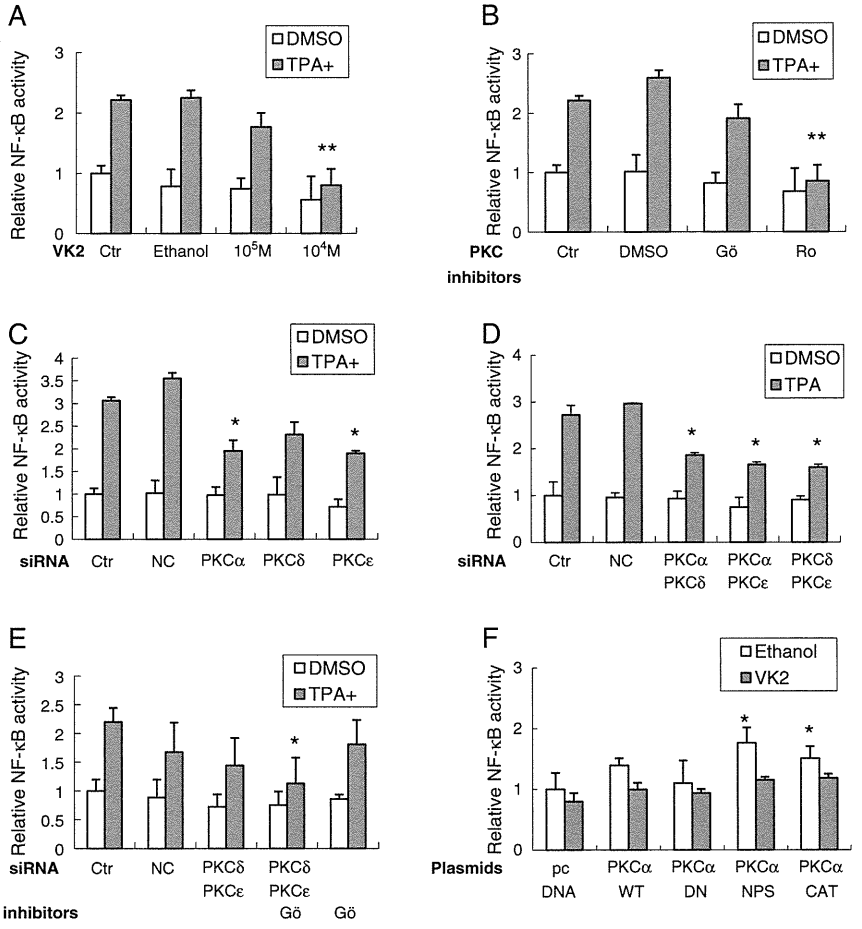

Fig. 1. Effect of VK2 and PKCs on the NF-κB activity in the NF-κB reporter gene transfectants. (A) VK2 suppressed the basal and TPA-induced NF-κB transcriptional activity in a dose-dependent manner in Huh7 cells. Huh7 NF-κB reporter gene transfectants in 48-well plates were cultured with or without the indicated concentrations of VK2 for 24 h, then treated with or without TPA(50 nM) for 3 h and subjected to luciferase assay as described in Materials and Methods. (B) PKC inhibitors showed inhibitory effect on NF-κB transcriptional activity in Huh7 cells. Huh7 transfectants were cultured in the presence or absence of PKC inhibitors as indicated in the figure for 24 h and subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (C) Individual siRNA-mediated knockdown of PKCs inhibited NF-κB transcriptional activity. Huh7 transfectants were transfected as described in Materials and Methods with siRNA of PKC isoforms as indicated in the figure, cultured for 24 h, and subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (D) Co-siRNA-mediated knockdown of PKCs significantly inhibited NF-κB transcriptional activity. Huh7 transfectants in 48-well plates were co-transfected with two siRNAs (10 nM for each) against two different PKCs or with negative control siRNA (20 nM), cultured for 24 h and subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (E) Combination of knockdown of PKCδ and ε and treatment with PKCα inhibitor Gö6976 suppressed the NF-κB activity to a similar level as the pan-PKC inhibitor did. Huh7 transfectants in 48-well plates were co-transfected with PKCδ and ε siRNA as described in panel (D). After culturing for 24 h, cells were treated with Gö for 24 h and then subjected to luciferase assay after treatments with or without TPA (50 nM) for 3 h. (F) Effect of various PKCα mutants on the NF-κB activity. Huh7 transfectants cultured in 48-well plates were transfected with PKCα mutant plasmids as described in Materials and Methods. At 24 h after transfection, 10^{-4} M of VK2 was added to the wells and cultured further for 24 h. Then, the cells were subjected to luciferase assay immediately. Columns, mean obtained from at least three independent experiments; bars, S.D.; *P<.05; **P<.01 (Student's t test); Ctr, no treatments; NC, negative siRNA; Gö, PKCα inhibitor Gö6976; Ro, pan-PKC inhibitor Ro-31-8425. PKCα WT, pHACE-PKCα wild type; PKCα DN, pHACE-PKCα dominant negative; PKCα NPS and PKCα CAT, pHACE-PKCα constitutive active form pcDNA, pcDNA3.1 vector as control.

2.2. Stable transfectants of the NF-κB reporter gene and the luciferase reporter gene assay

To obtain stable NF-κB reporter gene transfectants, pGL4.17NF-κB, a NF-κB reporter plasmid which was produced by conjugating the NF-κB promoter sequence of pNF-κB Luc (Clotech, CA, USA) with luceferase gene in pGL4.17[Luc2/Neo] vector (Promega, Madison, WI) or the vector alone (as a control) was introduced into Huh7 cells using Lipofectamine 2000 (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The transfected cells were treated with 500 ng/ml G418 for 2 weeks before selection. Clones expressing the NF-κB reporter gene were selected and subjected to further analysis. The NF-κB transcriptional activity was detected by a luciferase assay using the Single-Luciferase Reporter Assay System according to the method described by the supplier (Promega, Madison, WI, USA). Transfectant cells were seeded onto 48-well plates at 2×10^3 per well in DMEM with 10% FBS and incubated until 80% confluent at 37°C before use. After incubation with different concentrations of VK2 or PKC isoform-specific inhibitors for 24 h, 50 nM TPA/dimethyl sulfoxide (DMSO) in serum-free medium was added to the wells for 3h of further culture. Then, the cells were washed twice with phosphate-buffered saline (PBS) and carefully lysed in 1× passive lysis buffer (Promega, Madison, WI, USA). The cell extracts were immediately assayed for single luciferase activity using a Berthold Luminometer (MLR-100 Micro Lumino Reader, Corona Electric, Ibaragi, Japan). The amount of protein in cell extracts was determined using a Pierce660nm protein assay system (Thermo Scientific, USA). The luciferase activity was normalized by the protein amount.

2.3. SiRNA-mediated knockdown of PKCs and PKD1, and transfection of PKCα plasmids

The cells that were stably transfected with the NF-κB reporter gene were seeded onto 48-well plates at 2×10^3 cells per well in DMEM with 10% FBS and incubated until 80% confluent at 37°C. Next, the cells were replaced with new DMEM containing 10% FBS without antibiotics after being washed twice with DMEM, followed by the addition of 0.2 ml of OPTI-MEM Reduced Medium (Life Technologies, Rockville, MD, USA) containing the specific siRNA and Lipofectamine RNAiMAX (Life Technologies, Rockville, MD, USA) complex prepared according to the manufacturer's protocol. Lipofectamine 2000 (Life Technologies) was used for the transfection of PKCα mutant plasmids. After 24 or 48 h of incubation, the transfected cells were treated with TPA in DMEM without FBS for 3 h and subjected immediately to a single luciferase assay.

2.4. Western blotting

The protein expression of PKC isoforms was investigated by a Western blotting analysis. The cells that were cultured under various conditions were collected and lysed with sodium dodecyl sulfate (SDS) buffer [50 mM Tris (pH 6.8), 2.3% SDS and 1 mM phenylmethanesulfonyl fluoride]. The cell debris was eliminated by centrifugation at 12,000*g* for 10 min, and the supernatant was collected. After measuring the protein concentration with a protein assay kit (Bio-Rad, Hercules, CA, USA), a proper amount of protein was mixed with SDS sample buffer, separated by SDS–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked overnight at 4°C with 0.1% Tween 20 and 5% skim milk in PBS. The membranes were then incubated with the primary antibody for 1 h at room temperature or overnight at 4°C. The membranes were washed three times with 0.1% Tween 20 in PBS and stained with horseradish-peroxidase-conjugated secondary antibodies. All immunoblots were detected using an enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. For the detection of phospho-PKCs, Tris-buffered saline was used instead of PBS.

2.5. Kinase assay

The PKC kinase assay was performed using the Promega Kit according to the manufacture's official protocol with minor modifications. Huh7 cells were plated at a

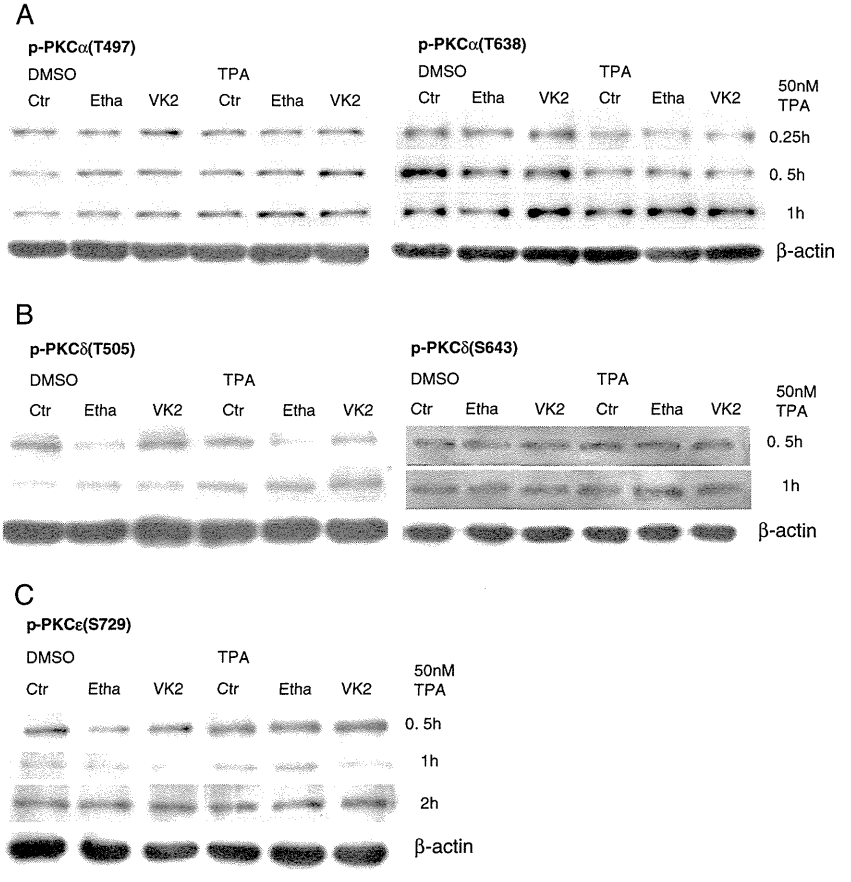

Fig. 2. VK2 effects on PKC activation in Huh7 cells. Huh7 cells were cultured in 3.5-cm dishes. After 24 h treatment with or without 10^{-4} M concentrations of VK2 or the VK2 solvent ethanol as a control, the cells were treated with 50 nM TPA or DMSO for the indicated time depending on the target protein. Cell extracts were analyzed by Western blotting using PKCs specific antibodies. Ctr, no treatments; Etha, ethanol.

density of 2×10^5 per 6-cm dish and were incubated at 37°C. After 72 h in culture, rat HA-tagged-PKC isoform plasmids [22] were introduced into the cells with Lipofectamine 2000 according to the manufacture's protocol. After 10^{-4} M VK2 was added, the cells were further cultured for 24 h, followed by 50 nM TPA treatment for 15 min in the case of PKCa and 1 h in the case of PKCd and PKCe. After cells were harvested with ice-cold PBS, they were sonicated in 1× cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin] in an ice-cold bath for 10 s. The lysates were centrifuged for 10 min at 14,000g at 4°C, and the supernatant was transferred to a new tube. The samples were then precipitated with 10–20 μl HA-tag agarose (MBL, Nagoya, Japan) with gentle rotating for 1 h at 4°C. Pellets were collected by centrifugation for 10 s at 2500g at 4°C and were washed three times with 500 μl of 1× washing cell lysis buffer, which was the same as the lysis buffer except that it contained 0.1% Triton X-100. The pellets were divided into five equal parts and subjected to the kinase assay according to the manufacture's protocol. One tube was subjected to a Western blotting analysis for the determination of the amount of PKC protein by determining the density of the PKC bands with the NIH ImageJ 1.41 software program (Bethesda, MD, USA).

2.6. Statistical analysis

Differences were analyzed using Student's *t* test, and $P<.05$ was considered to be significant. All experiments were done at least three times. The data are shown as the means±S.D.

3. Results

3.1. Effects of VK2 and PKCs on the NF-κB activity as determined by the NF-κB reporter gene transfectants

To investigate the mechanism responsible for the effects of VK2 on the inhibition of NF-κB transcriptional activity in Huh7 cells, a number of Huh 7 clones that were stably transfected with a NF-κB reporter gene were picked up successfully. Four of the clones, #4, #5, #8 and #14, were investigated, and similar results were observed for all four clones. As shown in Fig. 1A, TPA significantly stimulated NF-κB transcriptional activity in Huh7 cells, and VK2 abrogated the TPA-induced NF-κB transcriptional activity in a dose-dependent manner, in addition to weakly inhibiting the basal NF-κB transcriptional activity. The solvent used for VK2, ethanol, did not show any significant suppression, confirming that the TPA-induced activation of PKCs was involved in the NF-κB activation and that VK2 might inhibit NF-κB activation through the suppression of PKCs in Huh7 cells.

Various PKC isoforms, including PKCα, δ, ε, ι and ζ, are expressed in Hep3B hepatoma cells [23]. We confirmed by reverse transcriptase polymerase chain reaction that Huh7 cells also express these isoforms (data not shown). Of these PKCs, α (cPKC), δ and ε (nPKC) are activated by TPA. To determine which PKC isoform(s) was(were) involved in activating the NF-κB transcriptional activities, we first utilized specific inhibitors of PKCs, as shown in Fig. 1B. A pan-PKC inhibitor, Ro-31-8425 (100 nM), significantly inhibited TPA-induced NF-κB transcriptional activity. However, a PKCα inhibitor, Gö6976 (10 nM), only slightly suppressed the TPA-induced NF-κB activity. Next, we performed a siRNA-mediated PKC knockdown in the Huh7 cells. As shown in Fig. 1C and D, we observed that knockdown of individual PKC isoforms PKCα and ε significantly deceased the TPA-induced NF-κB luciferase activity, but knockdown of PKCδ only slightly decreased the activity (Fig. 1C). Furthermore, the simultaneous knockdown of two of these three PKC isoforms in the cells was still not sufficient to suppress the activity to the same level as that induced by the pan-PKC inhibitor or 10^{-4} M VK2 (Fig. 1D). These results suggested that all three of these PKC-isoforms, PKCα, δ and ε, may participate in the activation of NF-κB transcriptional activities.

To confirm this, we performed additional experiments combining siRNA-mediated knockdown of PKCs and treatment with a PKC inhibitor. At 24 h after co-transfection of cells with siRNAs against PKCδ and ε, 10 nM of the PKCα inhibitor (Gö6976) was added. After a further 24 h in culture, the cells were treated with DMSO/TPA for 3 h. When we used a combination of the knockdown of PKCδ and ε and Gö6976, we observed a similar level of suppression of the NF-κB activity as was observed for the pan-PKC inhibitor.

To further classify the PKCs involved, we employed various constitutively active forms of PKC enzymes. As shown in Fig. 1F, the constitutively active forms of PKCα, NPS and CAT, stimulated the activation of NF-κB in the absence of TPA more than the wild-type form (WT) did. In contrast, the dominant negative form (DN) showed no effect on the activation of NF-κB. Although constitutively active forms of PKCs δ and ε also were investigated, no significant changes were observed (data not shown). The results indicated that all of the TPA-induced PKC isoforms, α, δ and ε, are involved in the activation of NF-κB in Huh7 cells.

3.2. VK2 only partially change the phosphorylation levels of the PKC isoforms

To assess which PKC isoform(s) was (were) involved and whether the VK2 treatment affected its (their) activation or the activity itself, we performed a Western blotting analysis using various phosphorylation site-specific PKC antibodies. The phosphorylations of the activation loop (A-loop), turn motif (TM) and hydrophobic motif (HM) sites, which are thought to be involved in the activation of PKCs,

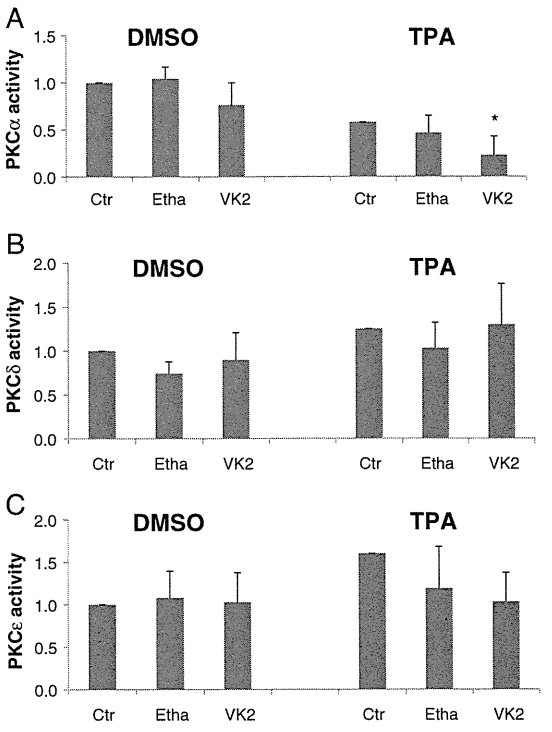

Fig. 3. Effects of VK2 on the enzyme activity of PKCs in Huh7 cells. PKC samples for enzyme assay were prepared as described in Materials and Methods, and PKC kinase activity of each sample was assayed at two different times of incubation when the activity linearly increased (usually at 15 and 30 min). (A) VK2 slightly suppressed PKCa activity at basal level (DMSO), while significantly at TPA-mediated level (TPA) in Huh7 cells. (B) VK2 inhibited PKCd activity at neither basal nor TPA-induced level. (C) VK2 did not suppress PKCe activity at both basal and TPA-induced levels. Columns are shown as relative enzyme activities when the activity obtained from the cells without any treatment is set at 1. The activity of each sample is a mean of data obtained from four independent cultures for (A), three independent cultures for (B), and four independent cultures for (C). Bars, S.D. * $P<.05$, compared with the activity of the enzyme obtained from the cells untreated with VK2 (Student *t* test). Ctr, control without treatment; Etha, treatment with the VK2 solvent ethanol; VK2, treatment with 10^{-4} M VK2.

were assayed. As shown in Fig. 2A, from 15 min to 1 h after treatment with TPA, after culturing with or without VK2, there were no clear effects on the phosphorylation of the Thr497 (A-loop) site. However, on the Thr638 (TM) phosphorylation site, after 15-min or 30-min treatments with 50 nM of TPA, PKCα showed significantly decreased phosphorylation in Huh7 cells, and these effects disappeared after 1 h of treatment. VK2 showed a limited effect on the Thr638 (TM) phosphorylation both at the basal level and in the TPA-treated state. As shown in Fig. 2B, TPA stimulated the phosphorylation of PKCδ at Thr505 (A-loop), but had a minimal effect on Ser643 (TM) after 1 h treatment. However, VK2 did not show any effect on either of the phosphorylation sites of PKCδ. TPA stimulated the phosphorylation of PKCε at Ser729 (HM) after 0.5 h and 1 h of treatments. At 1 h after treatment with TPA, this stimulation could be suppressed by VK2 (Fig. 2C). The expression levels of all PKCs were unchanged by the treatment (data not shown).

3.3. Effect of VK2 on PKC enzyme activity

Perhaps because of the limited phosphorylation site-specific antibodies for PKC that we used, we could not observe the inhibition of phosphorylation in any PKC induced by VK2 treatments. We next examined if the PKC enzyme activities were affected by VK2 treatment. HA-tagged PKC-plasmids were transiently introduced into Huh7 cells, then the HA-tagged PKCs were immunoprecipitated with an anti-HA antibody after VK2 and/or TPA treatments, and the phosphatidylserine- and diacylglycerol-stimulated PKC enzyme activity was determined *in vitro* by a kinase assay. TPA decreased the PKCα activity after 15 min of (Fig. 3A). The data may reflect the results of Western blot that the phosphorylation of the Thr638 was decreased with TPA treatment for 15 or 30 min (Fig. 2A). VK2 suppressed PKCα activity slightly at the basal level and significantly after TPA stimulation in Huh7 cells (Fig. 3A). VK2 suppression of PKCα activity was repeatedly observed in independent cultures, though the suppression levels were variable in the cultures. TPA stimulated the activities of PKCδ and ε after 1 h with 50 nM of TPA stimulation (Fig. 3B and C, respectively). Western blot analyses showed that the phosphorylation of PKCδ (Thr505) and PKCε (Ser729) was increased after 1-h treatment with TPA (Fig. 2B and C, respectively). VK2 did not show any inhibitory effects on the PKCδ activity at either the basal or TPA-induced level, as Fig. 3B showed. In PKCε, VK2 slightly suppressed the activity at the TPA-induced level, although it was not significant statistically (Fig. 3C). These results indicate that VK2 suppresses NF-κB activation through the inhibition of PKCα activity and also likely the activation of PKCε.

3.4. PKD1 is involved in the VK2-mediated inhibition of NF-κB activation through PKCs

Since PKCδ and ε have been shown to be the upstream factors of PKD1 (formerly called PKCμ) [24–26], PKD1 might be involved in the regulation of VK2-mediated NF-κB inhibition. As shown in Fig 4A, TPA stimulated the phosphorylation of PKD1 at Ser744 in A-loop, and VK2 inhibited the TPA-induced phosphorylation. The phosphorylation of IκBα at Ser 32/36 that is critical for the activation of NF-κB also was inhibited by VK2 (Fig 4B). The pan-PKC inhibitor Ro-31-8425 inhibited the TPA-induced phosphorylation of both PKD1 and IκBα, while the PKCα inhibitor Gö6976 showed no effect on the phosphorylation of both proteins (Fig. 4B). These results suggest that PKCδ or/and PKCε is/are involved in the phosphorylation of both PKD1 and IκBα. To confirm this notion, siRNA-mediated knockdown of PKC isoforms was employed. As shown in Fig. 4C, knockdown of PKCδ

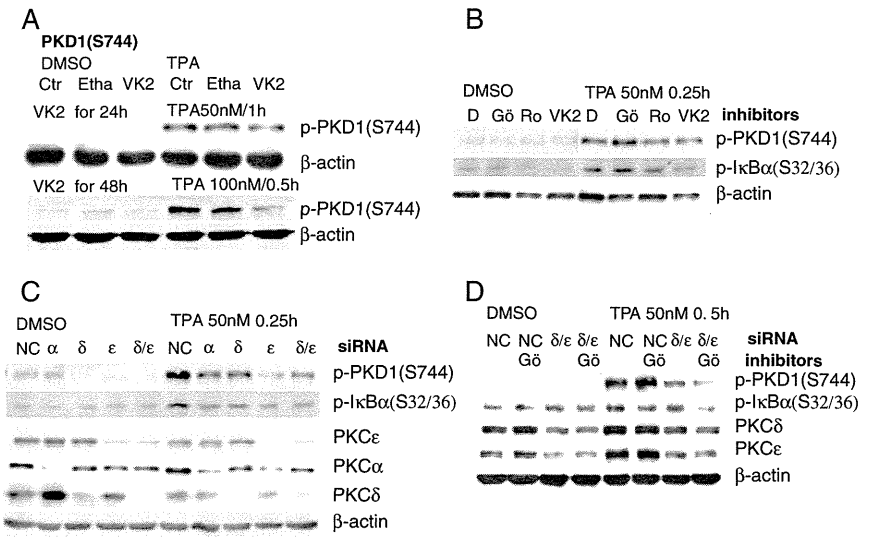

Fig. 4. VK2 suppressed the activation of PKD1, and PKCs are involved in the activation of PKD1 and IκBα in Huh7 cells. Activation of PKD1 and IκBα was assayed by Western blotting analysis using specific antibodies for p-PKD1 (S744) and p-IκBα (S32/36). (A) VK2 suppressed the activation of PKD1 in Huh7 cells. Cells were cultured in the presence of 10^{-4} M VK2 or the VK2 solvent ethanol as a control for 24 or 48 h and treated with or without TPA as indicated in the figure. (B) VK2 and pan-PKC inhibitor suppressed the phosphorylation of PKD1 and IκBα. Huh7 cells were cultured in the presence of the PKCα inhibitor Gö6976 (10 nM), pan-PKC inhibitor Ro-31-8425 (100 nM), VK2 (10^{-4} M) and DMSO as a control for 24 h. The cells were subjected to Western blot analysis after treatment with or without TPA. (C) Knockdown of PKCs inhibited the phosphorylation of PKD1 and IκBa. Huh7 cells cultured in 3.5 cm dishes were transfected with 20 nM siRNA of negative control (NC), PKCα (10 nM+10 nM NC), PKCδ (10 nM+10 nM NC), PKCε (10 nM+10 nM NC) and both PKCδ and PKCε (10 nM of each) as described in Materials and Methods. After culturing for 24 h, cells were treated with or without TPA and subjected to Western blot analysis. (D) Combination of knockdown of PKCδ and ε and PKCα inhibitor Gö6976 suppressed the activation of IκBα and PKD1 significantly. Huh7 cells were transfected with siRNA of negative control (NC), and both PKCδ and PKCε (10 nM of each) as described in panel (C). After 24 h, cells were treated with or without PKCα inhibitor Gö6976 (10 nM) for 1h and then induced with or without TPA for 0.5 h. NC, negative siRNA; α, PKCα siRNA; δ, PKCδ siRNA; ε, PKCε siRNA; Gö, PKCα inhibitor Gö6976; Ro, pan-PKC inhibitor Ro-31-8425. VK2, 10^{-4} M VK2.

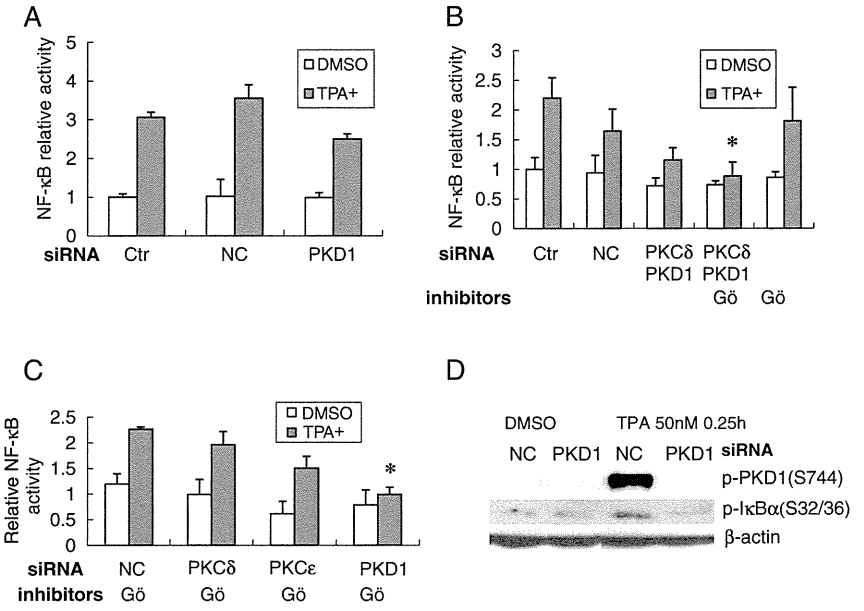

Fig. 5. PKD1 is involved in the TPA-induced activation of NF-κB. (A) Effect of PKD1 knockdown on NF-κB activity. Huh7 NF-κB reporter gene transfectant cells in 48-well plates were transfected with siRNA of negative control (NC) or PKD1. After culturing for 24 h, cells were treated with or without TPA (50 nM) for 3 h and subjected to luciferase assay. (B) Combination of knockdown of PKCδ, PKD1 and PKCα inhibitor Gö6976 suppressed the NF-κB activity to a similar level as pan-PKC inhibitor did. Huh7 NF-κB reporter gene transfectants cultured in 48-well plates were transfected with siRNA of negative control (NC) or PKCδ+PKD1 (10 nM of each). After culturing for 24 h, cells were treated with or without PKCα inhibitor Gö6976 (10 nM) for 24 h and subjected to luciferase assay after treatment with or without TPA. (C) Combination of knockdown of PKD1 and Gö6976 suppressed the NF-κB activity to a similar level as pan-PKC inhibitor did. Huh7 NF-κB reporter gene transfectants cultured in 48-well plates were transfected with 10 nM siRNA of negative control (NC), PKCα, PKCδ, PKCε and PKD1 and cultured for 24 h. Then, the cells were treated with or without PKCα inhibitor Gö6976 (10 nM) for 24 h and subjected to luciferase assay after with or without TPA induction. (D) At 24 h after transfection with 20 nM of PKD1 specific siRNA, phosphorylation of IκBα was suppressed effectively. Huh7 cells in 3.5-cm dishes were transfected with 20 nM siRNA of negative control (NC) or PKD1. After culturing for 24 h, cells were treated with or without TPA and subjected to Western blotting analysis with p-PKD1(S744) and p-IκBα (S32/36) specific antibodies. Bars, S.D.; *$P<.05$ (Student's t test); Ctr, no treatments; NC, negative siRNA; Gö, PKCα inhibitor Gö6976; Ro, pan-PKC inhibitor Ro-31-8425.

or/and PKCε decreased the phosphorylation of PKD1, but the knockdown of PKCα did not at the basal level. On TPA induction, however, the phosphorylation of both PKD1 and IκBα was decreased in all cases of knockdown of PKCα, PKCδ, PKCε and δ/ε compared with the control siRNA (Fig. 4C). On the knockdown of both PKCδ and ε, the phosphorylation levels of both PKD1 and IκBα were decreased compared to the levels of individually knocked down cells (Fig. 4C and D). When the cells simultaneously knocked down with PKCδ and ε siRNAs were treated with the PKCα inhibitor Gö6976, the TPA-induced phosphorylation of PKD1 and IκBα was further decreased (Fig. 4D). These results indicate that PKD1 signaling pathway might contribute to the IκBα phosphorylation that subsequently activates NF-κB.

To confirm the role of PKD1, siRNA-mediated knockdown of PKD1 was employed. The NF-κB reporter gene transfectants of Huh7 were transfected with PKD1 specific siRNA and subjected to luciferase assay. Knockdown of PKD1 only slightly decreased the TPA-induced NF-κB activity compared with the activity of negative control (Fig. 5A), while the TPA-induced NF-κB activity in PKD1 knockdown cells was significantly decreased by the treatment with the PKCα inhibitor Gö6976 (Fig. 5C and D). Furthermore, PKD1 knockdown decreased TPA-induced phosphorylation of IκBα (Fig. 5D). Altogether, the results indicate that PKD1 is involved in the TPA-induced activation of NF-κB, and all of the PKC isoforms, α, δ and ε, more or less contribute to the activation of PKD1.

4. Discussion

VKs have been revealed to have diverse effects, such as anticancer potential and antiosteoporotic effects, in addition to their essential roles in blood coagulation factors production [1,2]. Epidemiological studies have suggested a protective role for VKs against the development of atherosclerotic diseases [27]. Several mechanisms of VK2 action with regard to these effects have been reported. VKs have been shown to bind nuclear steroid xenobiotic receptor SXR (PXR) in bone-derived cells and to suppress osteoclastogenesis [28]. VKs also suppress the proliferation of several types of cancer cells [5–7]. In HCC cells, it has been reported that the activation of protein kinase A is involved in the growth suppression by VK [10,29]. We have revealed that VK2 inhibits the growth of HCC cells by suppressing cyclin D1 expression through the inhibition of NF-κB activation by suppressing IKK activity [13]. The suppression of NF-κB activation by VK was also reported in lipopolysaccharide-mediated macrophage activation [30] and in the VK-mediated suppression of osteoclastogenesis of bone cells through the RANK/RANKL pathway [31], suggesting that the suppression of NF-κB activation might play important roles in the activities of VK2.

NF-κB is a well-characterized transcription factor involved in the wide variety of important cellular functions such as the immune response, cell survival, apoptosis and carcinogenesis. Two distinct pathways have been described to activate NF-κB, and a number of factors are involved in the regulation of NF-κB activity (reviewed in Refs. [32,33]). PKCs are involved in the activation of NF-κB, and each isoform plays a different role in the regulation of NF-κB activation in various diseases [19,20,34,35]. Since VK2 inhibited the TPA-mediated activation of NF-κB, we hypothesized that VK2 might regulate PKC activation or enzyme activity. In the present study, we showed that all of the phospholipid-dependent PKCs, PKCα, δ and ε, which are expressed in Huh7 HCC cells participate to some degree in the activation of NF-κB. The simultaneous inhibition of three

PKCs, α, δ and ε, but not just two of them and treatment with a pan-PKC inhibitor resulted in the complete inhibition of TPA-induced NF-κB activation.

Interestingly, PKCε knockdown almost completely inhibited the TPA-induced phosphorylation of PKD1, a substrate of PKCε, indicating that PKCε stimulates NF-κB activity via PKD1 [35]. Knockdown of PKCα and PKCδ by siRNAs also inhibited the TPA-induced phosphorylation of PKD1, although the inhibition was partial (Fig. 4C), suggesting the involvement of PKCα and PKCδ in the phosphorylation of PKD1. The mechanism by which PKCα and PKCδ interact with PKD1 in Huh7 cells is not clear currently, but the regulation of PKD1 by PKCα [36] and PKCδ [26] was reported. The activity of PKCα and the phosphorylation of PKCε were inhibited by VK2 treatment, but PKCδ was not. Knockdown of PKCα decreased PKD1 phosphorylation, while the PKCα specific inhibitor showed no effect on the phosphorylation of PKD1. This might be due to the difference in mode and/or magnitude of inhibition between siRNA-mediated knockdown and pharmacological inhibition. Alternatively, the concentration of pharmacological inhibitor (10 nM of Gö6976) might not be enough to inhibit the phosphorylation of PKD1 because we used relatively low concentration of inhibitors to avoid the emergence of nonspecific kinase inhibitory action. PKD1 knockdown could not suppress completely the NF-κB activity to the same level as VK2 did, while the PKD1 knockdown with PKCα inhibitors reduced the NF-κB activity to the levels achieved by VK2. The results suggest that at least both PKCα- and PKCε/PKD1-mediated pathways are involved in the NF-κB activation by TPA, and the simultaneous suppression of both pathways might be required for enough suppression of TPA-induced NF-κB activation.

PKD1 was first identified as a PKC effector and was previously classified as PKCμ. However, PKD now constitutes a novel family of serine/threonine kinases and is classified as a subfamily of the calcium/calmodulin-dependent kinase superfamily. Recently, PKD has been shown to be dysregulated in several diseases, and it is regarded as a key regulator of diverse cellular functions such as cell growth, apoptosis, invasion and angiogenesis [37,38]. We have previously reported that VK2 suppressed the expression of MMPs, which are involved in the invasion and metastasis of cancer cells [15], in human HCC cell lines through the inhibition of NF-κB and/or the MAP kinase pathway [14]. Furthermore, VK has been shown to regulate angiogenesis through the modulation of VEGF [11]. PKD1 is also known to be involved in MMP expression [39,40] and VEGF-induced angiogenesis [36]. Endothelial cell proliferation, migration and angiogenesis by VEGF require PKD activity [41]. Therefore, VK2-mediated suppression of MMP expression and/or angiogenesis might involve the modulation of PKD activation.

The expression and distribution of PKCs are cell type and isoform specific. The PKCα, δ and ζ isoforms are found in all cells. The γ isoform is found only in neuronal cells, whereas the η and τ isoforms are predominantly expressed in epithelial and immune cells. The η, ε and λ isoforms are located in cells from various tissues. PKCs serve as the receptor for tumor-promoting phorbol esters, such as TPA, and the sustained activation of PKCs is associated with tumor promotion. Altered expression of PKCs has been linked with various types of malignancies, including HCC [42–44]. The activity of PKCs is regulated through three distinct conserved Ser/Thr phosphorylation sites: the A-loop, TM and HM [45,46]. In earlier literature, it was generally believed that phosphorylation at the A-loop was mediated by phosphoinositide-dependent kinase-1 (PDK1), which has been shown to be the upstream kinase for several members of the AGC family of kinases. Once phosphorylated at the A-loop, autophosphorylation at the TM and the HM occurred under the required conditions [47]. However, recent studies indicated that conventional PKCs can be phosphorylated at the TM and HM by the mammalian target of rapamycin complex 2 [48,49]. More recently, PKCδ, one of the novel PKCs, was found to be transphosphorylated at the A-loop by another novel PKC, PKCε, in place of PDK1 [50].

In conclusion, our data suggested that there are at least two pathways in PKC-mediated NF-κB activation in Huh7 cells, PKCα-NF-κB and PKCs-PKD1-NF-κB signaling pathways, and that VK2 might suppress the NF-κB activation via the inhibition of PKCα and PKCs (mainly PKCε)-PKD1 pathway. VKs have been shown to influence a wide variety of disease conditions, including bone health, cardiovascular diseases and neoplastic diseases, although the mechanisms by which VKs exert these diverse effects require further studies. NF-κB and PKCs/PKD are critical regulators for maintaining biological homeostasis; therefore, the understanding of the action of VKs involving NF-κB and PKCs/PKD will provide further information that can be useful for the prevention of a wide variety of diseases.

References

[1] Kaneki M, Hosoi T, Ouchi Y, Orimo H. Pleiotropic actions of vitamin K: protecter of bone health and beyond? Nutrition 2006;22:845–52.
[2] Kidd PM. Vitamins D and K as pleiotropic nutrients: clinical importance to the skeletal and cardiovascular systems and preliminary evidence for synergy. Altern Med Rev 2010;15:199–222.
[3] Tamori A, Habu D, Shiomi S, Kubo S, Nishiguchi S. Potential role of vitamin K(2) as a chemopreventive agent against hepatocellular carcinoma. Hepatol Res 2007;37: S303–7.
[4] Mamede AC, Tavares SD, Abrantes AM, Trindade J, Maia JM, Botelho MF. The role of vitamins in cancer: a review. Nutr Cancer 2011;63:479–94.
[5] Sakai I, Hashimoto S, Yoda M, Hida T, Ohsawa S, Nakajo S, et al. Novel role of vitamin K2: a potent inducer of differentiation of various human myeloid leukemia cell lines. Biochem Biophys Res Commun 1994;205:1305–10.
[6] Wu FYH, Liao WC, Chang HM. Comparison of antitumor activity of vitamins K1, K2, and K3 on human tumor cells by two (MTT and SRB) cell viability assays. Life Sci 1993;52:1797–804.
[7] Nishikawa Y, Carr BI, Wang M, Kars S, Finn F, Dowd P, et al. Growth inhibition of hepatoma cells induced by vitamin K and its analog. J Biol Chem 1995;270: 28304–10.
[8] Mizuta T, Ozaki I, Eguchi Y, Yasutake T, Kawazoe S, Fujimoto K, et al. The effect of menatetrenone, a vitamin K2 analog, on disease recurrence and survival in patients with hepatocellular carcinoma after curative treatment: a pilot study. Cancer 2006;106:867–72.
[9] Habu D, Shiomi S, Tamori A, Takeda T, Tanaka T, Kubo S, et al. Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. JAMA 2004;292:358–67.
[10] Otsuka M, Kato N, Shao RX, Hoshida Y, Ijichi H, Koike Y, et al. Vitamin K2 inhibits the growth and invasiveness of hepatocellular carcinoma cells via protein kinase A activation. Hepatology 2004;40:243–51.
[11] Yoshiji H, Kuriyama S, Noguchi R, Yoshii J, Ikenaka Y, Yanase K, et al. Combination of vitamin K2 and the angiotensin-converting enzyme inhibitor, perindopril, attenuates the liver enzyme-altered preneoplastic lesions in rats via angiogenesis suppression. J Hepatol 2005;42:687–93.
[12] Sakakima Y, Hayakawa A, Nagasaka T, Nakao A. Prevention of hepatocarcinogenesis with phosphatidylcholine and menaquinone-4: in vitro and in vivo experiments. J Hepatol 2007;47:83–92.
[13] Ozaki I, Zhang H, Mizuta T, Ide Y, Eguchi Y, Yasutake T, et al. Menatetrenone, a vitamin K2 analogue, inhibits hepatocellular carcinoma cell growth by suppressing cyclin D1 expression through inhibition of nuclear factor κB activation. Clin Cancer Res 2007;13:2236–45.
[14] Ide Y, Zhang H, Hamajima H, Kawaguchi Y, Eguchi Y, Mizuta T, et al. Inhibition of matrix metalloproteinase expression by menatetrenone, a vitamin K2 analogue. Oncol Rep 2009;22:599–604.
[15] Westermarck J, Kahari VM. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J 1999;13:781–92.
[16] Pearson RB, Kemp BE. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol 1991;200:62–81.
[17] Newton AC. Protein kinase C; structure, function and regulation. J Biol Chem 1995;270:28495–8.
[18] Reifel-Miller AE, Conarty DM, Valasek KM, Iversen PW, Burns DJ, Birch KA. Protein kinase C isozymes differentially regulate promoters containing PEA-3/12-*O*-tetradecanoyl-13-acetate response element motifs. J Biol Chem 1996;271: 21666–71.
[19] Das KC, White CW. Activation of NF-κB by antineoplastic agents. Role of protein kinase C. J Biol Chem 1997;272:14914–20.
[20] Catley MC, Cambridge LM, Nasuhara Y, Ito K, Chivers JE, Beaton A, et al. Inhibitors of protein kinase C (PKC) prevent activated transcription. Role of events downstream of NF-κB DNA binding. J Biol Chem 2004;279:18457–66.
[21] Soh JW, Weinstein IB. Roles of specific isoforms of protein kinase C in the transcriptional control of cyclin D1 and related genes. J Biol Chem 2003;278: 34709–16.

[22] Kuroda S, Tokunaga C, Kiyohara Y, Higuchi O, Konishi H, Mizuno K, et al. Protein-protein interaction of zinc finger LIM domains with protein kinase C. J Biol Chem 1996;271:31029–32.
[23] Takai S, Matsushima-Nishiwaki R, Tokuda H, Yasuda E, Toyoda H, Kaneoka Y, et al. Protein kinase C δ regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma. Life Sci 2007;81:585–91.
[24] Zugaza JL, Sinnett-Smith J, Van Lint J, Rozengurt E. Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. EMBO J 1996;15:6220–30.
[25] Berna MJ, Hoffmann KM, Tapia JA, Thill M, Pace A, Mantey SA, et al. CCK causes PKD1 activation in pancreatic acini by signaling through PKC-delta and PKC-independent pathways. Biochim Biophys Acta 2007;1773:483–501.
[26] Haworth RS, Roberts NA, Cuello F, Avkiran M. Regulation of protein kinase D activity in adult myocardium: novel counter-regulatory roles for protein kinase Cε and protein kinase A. J Mol Cell Cardiol 2007;43:686–95.
[27] Geleijnse JM, Vermeer C, Grobbee DE, Schurgers LJ, Knapen MHJ, van der Meer IM, et al. Dietary intake of menaquinone is associated with a reduced risk of coronary heart diseases: the Rotterdam study. J Nutr 2004;134:3100–5.
[28] Tabb MM, Sun A, Zhou C, Grun F, Errandi J, Romero K, et al. Vitamin K2 regulation of bone homeostasis is mediated by the steroid xenobiotic receptor SXR. J Biol Chem 2003;278:43919–27.
[29] Wei G, Wang M, Hyslop T, Wang Z, Carr BI. Vitamin K enhancement of sorafenib-mediated HCC cell growth inhibition in vitro and in vivo. Int J Cancer 2010;127: 2949–58.
[30] Ohsaki Y, Shirakawa H, Miura A, et al. Vitamin K suppresses the lipopolysaccha-ride-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor κB through the repression of IKKα/b phosphorylation. J Nutr Biochem 2010;21:1120–6.
[31] Takeuchi Y, Suzawa M, Fukumoto S, Fujita T. Vitamin K(2) inhibits adipogenesis, osteoclastogenesis, and ODF/RANK ligand expression in murine bone marrow cell cultures. Bone 2000;27:769–76.
[32] Shih VF, Tsui R, Caldwell A, Hoffmann A. A single NF-κB system for both canonical and non-canonical signaling. Cell Res 2011;21:86–102.
[33] Baud V, Karin M. Is NF-κB a good target for cancer therapy? Hopes and pitfalls. Nat Rev Drug Discov 2009;8:33–40.
[34] Moscat J, Diaz-Meco MT, Rennert P. NF-κB activation by protein kinase C isoforms and B-cell function. EMBO Rep 2003;4:31–6.
[35] Holden NS, Squires PE, Kaur M, Bland R, Jones CE, Newton R. Phorbol ester-stimulated NF-κB-dependent transcription: roles for isoforms of novel protein kinase C. Cell Signal 2008;20:1338–48.
[36] Wong C, Jin ZG. Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular endothelial growth factor. J Biol Chem 2005;280:33262–9.
[37] LaValle CR, George KM, Sharlow ER, Lazo JS, Wipf P, Wang QJ. Protein kinase D as a potential new target for cancer therapy. Biochim Biophys Acta 2010;1806: 183–92.
[38] Evans IM, Zachary IC. Protein kinase D in vascular biology and angiogenesis. IUMB Life 2011;63:258–63.
[39] Eiseler T, Doppler H, Yan IK, Goodison S, Stortz P. Protein kinase D1 regulates matrix metalloproteinases expression and inhibits breast cancer cell invasion. Breast Cancer Res 2009;11:R13.
[40] Lee JW, Kwak HJ, Lee JJ, Kim YN, Lee JW, Park MJ, et al. HSP27 regulates cell adhesion and invasion via modulation of focal adhesion kinase and MMP-2 expression. Eur J Cell Biol 2008;87:377–87.
[41] Qin L, Zeng H, Zhao D. Requirement of protein kinase D tyrosine phosphorylation for VEGF-A165-induced angiogenesis through its interaction and regulation of phospholipase Cg phosphorylation. J Biol Chem 2006;281:32550–8.
[42] Lee YS, Hong SI, Lee MJ, Kim MR, Jang JJ. Differential expression of protein kinase C isoforms in diethylnitrosamine-initiated rat liver. Cancer Lett 1998;126:17–22.
[43] Tsai JH, Hsieh YS, Kuo SJ, Chen ST, Yu SY, Huang CY, et al. Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma. Cancer Lett 2000;161:171–5.
[44] Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer 2007;7:281–94.
[45] Newton AC. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem J 2003;370:361–71.
[46] Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. EMBO J 2000;19:496–503.
[47] Behn-Krappa A, Newton AC. The hydrophobic phosphorylation motif of conventional protein kinase C is regulated by autophosphorylation. Curr Biol 1999;9:728–37.
[48] Ikenoue T, Inoki K, Yang Q, Zhou X, Guan KL. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signaling. EMBO J 2008;27: 1919–31.
[49] Facchinetti V, Ouyang W, Wei H, Soto N, Lazorchak A, Gould C, et al. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J 2008;27:1932–43.
[50] Basu A, Sridharan S, Persaud Sh. Regulation of protein kinase Cδ downregulation by protein kinase Cε and mammalian target of rapamycin complex 2. Cell Signal 2009;21:1680–5.

肝臓　53巻10号　591-601（2012）

＜原　著＞

肝炎ウイルス検査受診率向上には医療者による個別の意義の説明が有効である

堀江　弘子[1)]　江口有一郎[2)*]　中村　隆典[4)]　水田　敏彦[3)]　桑代　卓也[1)]
岩本　英里[1)]　古賀さやか[1)]　田代　貴也[1)]　冨永智香子[1)]　黒木　茂高[1)]
小野　尚文[1)]　木下　淳[4)]　本多　義昭[4)]　駒田富佐夫[4)]　尾崎　岩太[5)]
安西　慶三[3)]　藤本　一眞[3)]　江口　尚久[1)]

要旨：佐賀県は肝がん粗死亡率全国一位が続いている．肝炎ウイルス検査の受診率向上は肝がん対策の最重要課題と捉え，当健診センターでは，検査の受検案内を積極的に行い，2年間で総受診者の約8割が受検した．今回，更なる受検率の向上のために，対象者に検査を「受ける意思」と「受けない理由」の要因を明らかにするアンケート及び共分散分析を行った（総数447人，検査の受診者：A群373人，不受診者：B群74人）．A群の受検要因に，「佐賀県の肝がんの死亡率が高い」との情報提供と「慢性肝炎の早期治療での意義」の説明，「スタッフから検査案内」の検査意義の説明等が有効と判った．B群の不受検の理由には，「自分は大丈夫だろう」との認識と「治療に伴う経済的な負担」等が見出され，検査意義の理解不足や治療に伴う経済的な負担が推測された．ウイルス感染の意味と肝がん予防の健康教育，治療費助成制度の説明等は受検率の向上に寄与すると示唆された．

索引用語：　肝炎ウイルス検査　　肝炎検診　　受検勧奨　　職域健診　　個別健診

緒　言

当健診センターが位置する佐賀県はC型肝炎高罹患地区であり，肝硬変・肝がんの死亡率が高い．平成21年の統計[1)]によれば肝硬変・肝がんによる死亡率は，人口10万に当たり45.7と全国で，11年連続1位という状況であり，その発生原因の90～95%はウイルス性肝炎，特にC型肝炎であることが明らかにされてきた．治療の基本は「いかに肝炎ウイルス感染者を早期に発見し，定期的な管理とインターフェロン等の抗ウイルス剤をはじめとする数々の治療を施行すること」[2)]である．

佐賀県では平成20年4月から「佐賀県肝がん対策緊急総合対策事業」として「佐賀県肝炎ウイルス検査事業」と「ウイルス性肝炎治療費助成制度」を実施している．この事業により20歳以上の佐賀県民は佐賀県肝疾患検診医療提供体制登録医療機関で肝炎ウイルス検査を無料で受検できるシステムが構築され，行政による広報や県内の登録医療機関での検査案内等で県民への情報提供がなされている．しかし厚生労働省のホームページの「平成23年度肝炎検査受検状況実態把握事業　事業成果報告書」[3)]にも，国民調査で肝炎ウイルスの具体的な症状や治療方法について認知している割合は，B型肝炎で11.8%，C型肝炎で13.5%と低い状況であり，肝炎ウイルス検査の受検率は全国的にみても低い．

当健診センターでも肝炎ウイルス検査を自ら希望した人は，平成19年度は14人（1%）と極めて低かった．そこで，我々は，平成20年4月からは肝炎ウイルス検査事業の開始年度でもあり，当健診センターとしても新たな肝炎ウイルス保有者の発見のために，健診の機会が肝炎ウイルス検査の受検の契機となるように検査

1）医療法人ロコメディカル江口病院
2）佐賀大学医学部肝疾患医療支援学
3）佐賀大学医学部内科学
4）姫路獨協大学薬学部
5）佐賀大学保健管理センター
*Corresponding author: eguchiyu@cc.saga-u.ac.jp
＜受付日2012年8月1日＞＜採択日2012年9月9日＞

案内をより積極的に一貫して取り組んだ．

その検査案内は，行政の広報の案内に加え「佐賀県は肝がんの死亡率が全国1位であること，その状況の改善のための対策としては，その持続感染の有無を知るために肝炎ウイルス検査を1回は受けることであり，もしその検査で陽性であれば精密検査へと進み，更に医療機関において適応を検討のうえ，早期に抗ウイルス剤等による治療を受けることが肝がんの予防に繋がる」という肝炎ウイルス検査を受ける意義と抗ウイルス剤による治療の意義等に関する基本的な健康情報[4]を，プライバシーを十分配慮しつつ，個別に情報提供を行うものであった．この介入をすることで平成21年度には健診の総受診者1695人のうち肝炎ウイルス検査の既受検者は1429人（84%）に至った．しかし一方，検査案内をしても肝炎ウイルス検査に同意が得られない人が266人（16%）いた．

そこで今回は更に，「肝炎ウイルス検査を受ける意思」と共に「肝炎ウイルス検査を受けない理由」の素因の検証をすることで検査不受診者の受検率の向上を図る目的の為に以下の検討を行った．

対象及び方法

1. 対象

当健診センターは小城市（人口4.6万人）に位置し，当健診センターを利用の企業（年間健診総数のべ2000人）はほとんどが小城市近郊からである．また職域健診と特定健診（市町村の個別健診）の割合は9：1であり，職域健診の全体の約4.5割は協会けんぽの生活習慣病予防健診の受診者である．

今回の検討は平成21年度の健診者で肝炎ウイルス検査の案内をし，且つアンケート調査に協力が得られた553人のうちの有効回答が得られた447人を対象とした．対象者は脂肪肝やアルコール性肝障害の既往歴を持つ者は含めたが，問診にて肝疾患で医療機関受診中の者は除外した．また肝炎ウイルス検査の制度は，協会けんぽの生活習慣病予防健診の対象者には協会けんぽが導入している肝炎ウイルス検査の受検を勧奨した．それ以外は佐賀県肝炎ウイルス検査事業の受検を勧奨した．

2. 方法

肝炎ウイルス検査受診者（A群）と不受診者（B群）として，それぞれの自記式の質問紙（受診者群には16項目，不受診者群には9項目）を設定し健診当日に回答を求めた（Table 1，Table 2）．統計処理は，アンケートの質問に対する回答は，自由回答法による5段階順位回答，すなわち，「1：非常に考える」，「2：かなり考える」，「3：少し考える」，「4：あまり考えない」および「5：まったく考えない」の5件法で回答を求めた．

A群のアンケート回答のうち，全16項目の質問のうち，回答傾向が偏っていることを示す天井効果（平均＋SD＞5）が認められた質問は，質問②「家族（血縁）に肝疾患（肝がん，肝硬変）がいるので受ける」では，「4：あまり考えない」と「5：まったく考えない」に回答した合計は71.3%，質問③「普段から飲酒の習慣があり，肝臓を気にしているので受ける」では61.4%，質問⑨「過去に手術や輸血を受けたことがあるため受ける」では81.2%，質問⑩「針治療やピアス・刺青をしているので受ける」では88.2%，質問⑪「過去に薬物の注射をしたことがあるので受ける」では90.3%，質問⑫「仕事が医療従事者で，血液での感染が気にかかるので受ける」では83.9%であった．そこで，これらの質問を除いた10項目の質問の回答を用いて解析した．なお，フロア効果（平均－SD＜1）は認められなかった．

B群のアンケート回答も同じく，全9項目の質問のうち，質問⑥「検査を受けたいが検査の結果が市町村に通知されるので受けない」では，「4：あまり考えない」と「5：まったく考えない」と回答した合計は78.4%と天井効果（平均＋SD＞5）が認められたため，この質問を除く8項目の質問の回答を用いて解析した．なお，フロア効果（平均－SD＜1）は認められなかった．

3. 統計解析

共分散構造分析にはAMOS（Ver. 18J）を用いた．なお，共分散構造分析によるモデル適合性は，GFI（Goodness of Fit Index：適合度指標）とAGFI（Adjusted Goodness of Fit Index：修正適合度指標）の値が0.90以上であれば有意と評価した．ただし，GFIに比べてAGFIが著しく低下するモデルでは不適合と評価した．また，CFI（Comparative Fit Index）は0.95以上，RMSEA（Root Mean Square Error of Approximation）が0.05以下であればモデルに適合と評価した．さらに，複数のモデルのうちどれが良いかを選択する際には，AIC（Akaike's Information Criterion；赤池情報量基準）が最小のモデルを選択した．また，有意差は$P<0.05$とした．まず，すべての関連性を網羅した種々のパス図により検証的に分析を行った．

Table 1 The questionnaire for subjects who received HBV/HCV screening test

あなたが肝炎ウイルス検査を受けるかどうか決めるとき，
以下の事柄はどのくらいのきっかけや要因と考えられますか？
すべての項目について，今現在の気持ちをお答えください．

		非常に考える	かなり考える	少し考える	あまり考えない	まったく考えない
①	健診で肝障害の指摘があるので，この検査はその精密検査の1つであると考える．	1	2	3	4	5
②	家族（血縁）に肝疾患（肝がん，肝硬変）がいるので受ける．	1	2	3	4	5
③	普段から飲酒の習慣があり，肝臓を気にしているので受ける．	1	2	3	4	5
④	慢性肝炎の原因は90%が肝炎ウイルス感染であるため早期発見に有効と考える．	1	2	3	4	5
⑤	慢性肝炎を早期に治療することは，肝がん・肝硬変の予防になるので受ける．	1	2	3	4	5
⑥	肝炎ウイルスに感染しても自覚症状は出ないため，検査を受けないとわからないので受ける．	1	2	3	4	5
⑦	佐賀県は，C型肝炎が原因で，肝がん・肝硬変で死亡する頻度が高いことから受ける．	1	2	3	4	5
⑧	今までに歯科治療を受けているため，感染が気になるので受ける．	1	2	3	4	5
⑨	過去に手術や輸血を受けたことがあるため受ける．	1	2	3	4	5
⑩	はり治療やピアス・刺青をしているので受ける．	1	2	3	4	5
⑪	過去に薬物の注射をしたことがあるので受ける．	1	2	3	4	5
⑫	仕事が医療従事者で，血液の感染が気にかかるので受ける．	1	2	3	4	5
⑬	今回健診で，肝炎ウイルス検査の案内があったので受ける．	1	2	3	4	5
⑭	今回健診で，健診施設のスタッフから肝炎ウイルス検査の声かけがあったので受ける．	1	2	3	4	5
⑮	検査の料金がかからないので受ける．	1	2	3	4	5
⑯	血液検査が健診項目にあるので，同時にできるので受ける．	1	2	3	4	5

このアンケートにより得られた結果は，統計データとして疫学的な調査・研究に利用したりすることはありますが，個人のデータを公表することはありません．

上記のことを了承した上で，アンケートに回答することに同意します．

氏名：＿＿＿＿＿＿＿＿

（自署してください）

Table 2 The questionnaire for subjects who did not received HBV/HCV screening test

あなたはなぜ，肝炎検査を希望しないのですか？
以下の項目について，今現在の気持ちをお答えください．

①	過去に肝炎ウイルス検査を受けており，共に陰性であった．	1．はい　　2．いいえ
	※『はい』の方は，どこで調べましたか？	1．献血 2．職場の健診 3．住民健診

上記の①で『2．いいえ』と答えた方にお尋ねします． 以下の項目は，検査を希望しないきっかけや要因として，どれくらい考えますか？ 右の5段階で最も近いものに○をつけてください．		非常に考える	かなり考える	少し考える	あまり考えない	まったく考えない
②	自分は検査をしなくても大丈夫だろうと思うので，受けない．	1	2	3	4	5
③	検査の必要性をまだ理解していないので，受けない．	1	2	3	4	5
④	検査について，何も関心がないので，受けない．	1	2	3	4	5
⑤	検査を受けて，異常の有無に関わらず，治療を考えてないので，受けない．	1	2	3	4	5
⑥	検査を受けたいが，検査の結果が市町村に通知されるので，受けない．	1	2	3	4	5
⑦	検査を受けたいが，検査の結果で異常が出た場合に，家族や周囲のことを考え，負担になるので，受けない．	1	2	3	4	5
⑧	検査を受けたいが，検査の結果で異常が出た場合に，仕事の都合で時間が取れず治療ができないので，受けない．	1	2	3	4	5
⑨	検査を受けたいが，検査の結果で異常が出た場合に，経済的な負担から治療ができないので，受けない．	1	2	3	4	5
⑩	検査を受けたいが，検査の結果で異常が出た場合に，治療の方法や副作用が心配なので，受けない．	1	2	3	4	5

すでにC型肝炎と診断されている方にお尋ねします．		
⑪	医療機関で治療及び経過観察を定期的に受けている．	1．はい　　2．いいえ
⑫	治療していたが，仕事で時間の都合が悪く，中断している．	1．はい　　2．いいえ
⑬	治療していたが，治療費が経済的に掛かりすぎて，中断している．	1．はい　　2．いいえ

すでにB型肝炎と診断されている方にお尋ねします．		
⑭	医療機関で治療及び経過観察を定期的に受けている．	1．はい　　2．いいえ
⑮	治療していたが，仕事で時間の都合が悪く，中断している．	1．はい　　2．いいえ
⑯	治療していたが，治療費が経済的に掛かりすぎて，中断している	1．はい　　2．いいえ

このアンケートにより得られた結果は，統計データとして疫学的な調査・研究に利用したりすることはありますが，個人のデータを公表することはありません．
上記のことを了承した上で，アンケートに回答することに同意します．

氏名：＿＿＿＿＿＿＿＿＿＿
（自署してください）

Table 3 Clinical characteristics of all subjects

	総数 (n＝447名)	A群 (n＝373名)	B群 (n＝74名)	P値
年齢（才）	41.7±12.5	41.3±12.6	43.9±11.3	0.2139
体重（kg）	63.5±12.3	63.6±12.6	63.3±10.4	0.7712
BMI	22.9±3.4	22.9±3.5	22.7±2.9	0.9979
腹囲（cm）	83±9.2	83.4±9.5	81.0±7.6	0.3437
収縮期血圧（mmHg）	125.7±17.9	125.3±17.7	127.7±18.6	0.5471
拡張期血圧（mmHg）	74±11.8	73.9±11.9	74.4±10.9	0.975
中性脂肪（mg/dl）	129.3±151.3	130.4±156.7	123.5±121.7	0.5216
LDL-C（mg/dl）	120.1±34.0	119.9±34.2	121.0±33.2	0.776
HDL-C（mg/dl）	60.7±15.9	60.8±15.7	60.4±16.7	0.6479
AST（IU/L）	22.6±10.2	22.9±10.6	20.8±7.7	0.3139
ALT（IU/L）	24.6±19.2	25.5±20.6	20.2±8.2	0.0495
血糖（mg/dl）	102.1±21.0	101.2±18.9	106.8±29.0	0.4351

Table 4 Clinical characteristics of male subjects

	総数	A群 (n＝321名)	B群 (n＝253名)	P値
年齢（才）	42.1±12.1	41.6±12.3	43.9±11.1	0.8367
体重（kg）	67.4±11.2	68.2±11.4	64.4±10.0	0.1866
BMI	23.3±3.4	23.4±3.5	22.7±3.0	0.5245
腹囲（cm）	84.3±8.6	85.2±8.7	81.6±7.6	0.261
収縮期血圧（mmHg）	127.6±17.6	127.4±17.4	128.4±18.6	0.2172
拡張期血圧（mmHg）	74.9±12.1	74.9±12.4	74.7±10.9	0.6989
中性脂肪（mg/dl）	148.6±172.8	154.0±183.2	128.8±126.3	0.4934
LDL-C（mg/dl）	57.6±14.5	123.5±35.5	121.9±34.0	0.4702
HDL-C（mg/dl）	123.3±35.1	57.0±13.9	59.9±16.5	0.3456
AST（IU/L）	24.1±11.2	24.9±11.9	21.2±7.6	0.2128
ALT（IU/L）	27.9±21.1	29.9±23.0	20.6±8.0	0.0318
血糖（mg/dl）	105.2±23.5	104.4±21.4	108.1±30.1	0.5243

4. 倫理的配慮

対象者には問診で，検査内容と健診データ及び問診票の活用について文書によるインフォームドコンセントを得た．また本研究は，当院および佐賀大学倫理委員会の承認を得ている．

結　果

有効回答者総数は447人(男性321人，女性126人)で，年齢は平均41.7±12.5歳，A群の対象者は373人(男性253人，女性120人)，B群の対象者は74人(男性68人，女性6人)であった(Table 3)．A群の対象者の全てが肝炎ウイルス検査結果はHCV抗体及びHBV共に（陰性）であった．また，男性においては，A群に比較してB群が有意にALTは低値であった($P<0.05$)(Table 4)．女性ではA群とB群の背景は，B群が有意に高齢であった（$P<0.05$)(Table 5)．

1) 肝炎ウイルス検査をうける理由

A群の受診者アンケートの回答のうち，天井効果(平均＋SD＞5)が認められ質問を除いた10項目の質問について，パス図(Fig. 1)に示すような3つ潜在変数を有するモデル，すなわち，質問①，⑦および⑧の測定変数と潜在変数を(『感染の恐れ』)，質問④，⑤及び⑥の測定変数と潜在変数を(『検査と疾患の関連性』)，質問⑬，⑭，⑮及び⑯の測定変数と潜在変数を(『検査を受ける理由』)，として解析した．

A群のアンケートで測定変数における潜在変数の影

Table 5 Clinical characteristics of female subjects

	総数 (n＝126名)	A群 (n＝120名)	B群 (n＝6名)	P値
年齢（才）	40.9±13.5	40.8±13.5	44.0±14.8	0.013
体重（kg）	53.7±8.9	53.8±9.0	51.4±6.3	0.3185
BMI	21.9±3.4	21.9±3.5	21.8±1.8	0.4798
腹囲（cm）	79.3±9.7	79.5±9.9	75.6±4.8	0.3616
収縮期血圧（mmHg）	120.8±17.7	120.8±17.7	120.5±18.7	0.0206
拡張期血圧（mmHg）	71.6±10.6	71.5±10.6	71.8±10.3	0.0514
中性脂肪（mg/dl）	79.9±42.0	80.5±42.9	70.0±19.9	0.6012
LDL-C（mg/dl）	68.6±16.5	112.0±30.0	112.5±24.8	0.2494
HDL-C（mg/dl）	112.1±29.7	68.8±16.4	65.2±19.2	0.7702
AST（IU/L）	18.7±5.5	18.8±5.3	17.5±9.4	0.6678
ALT（IU/L）	16.3±8.8	16.2±8.7	17.2±10.2	0.9623
血糖（mg/dl）	94.6±9.2	94.6±9.4	93.8±3.9	0.5941

響力は，F1：『感染の恐れ』という潜在変数では，「佐賀県は，C型肝炎が原因で，肝硬変・肝がんで死亡する頻度が高いことから受ける」の標準化係数（パス係数）は0.81であり，「健診で肝障害の指摘があるので，この検査はその精密検査の1つであると考える」や「今までに歯科治療を受けているため，感染が気になるので受ける」等と比較して大きく，この『感染の恐れ』のモデルの中で特に関連性が高いことが明らかとなった（Table 6）．F2：『検査と疾患の関連性』という潜在変数では，3つの標準化係数は共に0.82以上であった．特に「慢性肝炎を早期に治療することは，肝がん・肝硬変の予防になるので受ける」の標準化係数は0.91と最も高いパス係数が推定され，大きな影響力を有していることが明らかとなった．F3：『検査を受ける理由』という潜在変数では，「検査の料金がかからないので受ける」のパス係数は0.46と小さく，その影響力は低いと考えられた．しかしその一方，「今回の健診で健診施設のスタッフから肝炎ウイルス検査の声掛けがあったので受ける」のパス係数は0.84と大きく，最も関連性が高いことが明らかとなり，『検査を受ける理由』としての重要性が高いものと考えられた．

2）肝炎ウイルス検査を受けない理由

B群の不受診者のアンケートの結果では全9項目の質問のうち，天井効果（平均＋SD＞5）が認められた質問⑥を除いた．そこで，パス図（Fig. 2）に示すような2つ潜在変数を有するモデル，すなわち，質問②～⑤の測定変数と潜在変数を（『検査の必要性』）並びに，質問⑦～⑩の測定変数と潜在変数を（『検査結果と治療との関連』）として解析した．

肝炎検査をなぜ受けないかについての回答結果は，F1：『検査の必要性』という潜在変数では，4つのパス係数はともに0.77以上と高く推定され，特に「自分は検査をしなくても大丈夫だろうと思うので，受けない」の標準化係数は0.89と最も高いパス係数が推定され，この『検査の必要性』のモデルの中で最も関連性があることが明らかとなった（Table 7）．F2：『検査結果と治療との関連』という潜在変数では，4つのパス係数はともに0.75以上と推定され，特に「検査を受けたいが，検査の結果で異常が出た場合に，経済的な負担から治療できない」の標準化係数は0.97と最も高いパス係数が推定され，極めて大きな影響力を有していることが明らかになった．

考　察

今回の検討から「佐賀県ではC型肝炎が原因で肝硬変・肝がんで死亡する頻度が高いことから肝炎ウイルス検査を受ける」という疫学特異性の情報提供及び「慢性肝炎を早期に治療することは，肝硬変・肝がんの予防になるので受ける」という早期発見，早期治療の概念の啓発が肝炎検査を受けるきっかけに大きな影響力を有する要因として明らかになった．また「今回健診で，健診施設のスタッフから肝炎ウイルス検査の声掛けがあったので受ける」という受検者の受検意識が明らかになったように，健診の前に行った肝炎ウイルス検査の意義説明が，検査意義理解の増進と受検率の向上に寄与したと考えられた．一方，歯科治療と感染，