Immunochemical Localization of Protein Kinase Cα in the Biopsies of Human Hepatocellular Carcinoma

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Abstract

The purpose of this study was to elucidate the protein kinase C (PKC) α distribution in human hepatocellular carcinoma (HCC). The histoimmunopathologic technique was used to determine the localization and expression of PKCα in HCC biopsies. The HCC tissues were classified as cytosolic type (PKCα deposited in the cytoplasm in > 50% of cells) and membranous type for the remaining ones. There was a significant association of the membranous type with non-hepatitis C virus (HCV) infected patients. Moreover, the expression of PKCα in this type was significantly higher in HCC cells than that in the adjacent non-tumor liver cells. The result indicated that PKCα may play an important role in carcinogenesis of HCC patients with HBV infection and/or non-HCV infection.

Key Words: hepatitis C virus, hepatocellular carcinoma, protein kinase C alpha

Introduction

Protein kinase C (PKC) is a lipid-regulated and calcium-dependent protein kinase playing an important role in the control of cell growth and differentiation (31). It has 10 isoforms with different distributions, structures, and functions in various tissues (28, 32). PKC isoforms have been found to display variable expression profiles during cancer progression depending on the particular cancer type (23). PKCα has found to be overexpressed in high grade urinary bladder, prostate, and endometrial cancers (12, 24, 25, 36). In contrast, downregulation of PKCα expression was observed in breast, colon, and basal cell cancers (14, 22, 29). The expression of PKCβ has been reported to be upregulated in colon and prostate cancers (14, 24) and downregulated in bladder cancer (12). However, PKCι, PKCε and PKCλ decreased in pancreatic cancers (11) and PKCη increased in renal cancer (4). PKCι has been suggested

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to be a negative prognostic factor in ovarian carcinoma (37) and PKCγ as a positive prognostic factor in B-cell lymphoma (19). These studies provide a specific role of PKC isotype in different kinds of malignant tumors. However, little is known about the specific role of isoforms during liver carcinogenesis.

Recent studies have found that PKCα is highly expressed in human HCC (35, 40) and in human poor differentiated HCC cells (39). In human HCC cells, the elevated-PKCα is regulated by two transcriptional factors Elk-1 and MZF-1 (15, 17) and essential for cell migration and invasion by mediating through p38 mitogen-activated protein kinase signaling pathway (16).

Moreover, PKCα activation and translocation from cytosol to membrane or nucleus with an expression of increasing proliferation potentiation in response to a low phorbol 12-myristate 13-acetate (PMA) concentration (10 nM) in Hep3B cells are also observed in our previous study (18). The translocation of PKC from the cytosol to the membrane allows the enzyme to have access to several membrane protein substrates, many of which are involved in the signal transduction of factors that influence mitogenesis. PKC translocation has often been described as part of the pleiotropic response to mitogens and tumor promoters (5, 30).

To further determine the distribution of the PKCα in human HCC, we employed histoimmunopathologic techniques to investigate the localization of PKCα in HCC biopsies.

Materials and Methods

Specimen Collection

Liver biopsies were obtained from 19 patients in the Division of Hepatogastroenterology, Department of Internal Medicine, Changhua Christian Hospital. These patients were suspected to have a tumor in the liver based on clinical history, physical examination, the presence of α-fetoprotein, and ultrasonography. An informed consent was obtained from each patient before the liver puncture. After collection, the specimens were immediately fixed in 4% formalin. Histopathological grading was then determined according to the cell differentiation (well, moderately, or poorly differentiated). The remaining portion of each specimen was employed for the analysis of tumor markers by immunohistochemistry. In addition, hepatitis B virus surface antigen (HBs-Ag) and anti-HCV were also detected. The tumor size was measured by ultrasonography, and the tumor-node-metastasis (TNM) staging was determined by histopathology, ultrasonography, computerized axial tomography, and angiography.

Preparation of Polyclonal Anti-PKCα Antibodies

PKCα peptide (657-672) was obtained from Dr. T. H. Liao, Institute of Biochemistry, College of Medicine, National Taiwan University, Taiwan. After coupling to carbonic anhydrase (Sigma, St. Louis, MO, USA) using glutaraldehyde (Sigma), the PKCα peptide preparation was immunized to male New Zealand white rabbits according to a protocol described by Wetsel *et al.* (38). Serum samples were obtained from the rabbits since day 42 after immunization. Antibodies were purified by affinity chromatography on a column bearing the PKCα peptide.

Immunohistochemistry

The formalin-fixed specimens were dehydrated through graded alcohol and embedded in paraffin. Sections (3 μm) were prepared from the paraffin-embedded specimens and then deparaffinized in xylene and rehydrated through an alcohol series. The sections were then incubated with 3% H_2O_2 for 5 min. After washing with phosphate buffer saline (PBS), the sections were heated to boiling in EDTA solution (1 mM EDTA, 0.1% NP-40; pH 8.0) for 5 min in a microwave oven. This non-competitive inhibition procedure was repeated once after an interruption of 10 min.

After cooling for 20 min, the sections were washed three times in PBS for 5 min and then incubated in PBS with 3% normal bovine serum for 25 min. The sections were washed with PBS and incubated with the purified polyclonal antibodies to PKCα (10 ng/ml PBS plus 0.2% BSA) at room temperature for 1 h. After washing three times in PBS for 5 min, the sections were incubated with biotinylate-labeled goat anti-rabbit IgG (Sigma) at room temperature for 30 min. The sections were then washed with PBS and incubated with ABC regent (Avidin/Biotin kit, Vector Laboratories, Inc., Burlingame, CA, USA) conjugated with peroxidase at room temperature for 30 min. PKCα antigen staining was visualized by adding 3,3′-diaminobenzidine substrate (Sigma). The reaction was terminated by rinsing the sections in distilled water.

The sections were counterstained with Gill's hematoxylin V (Mute pure Chemicals Ltd., Tokyo, Japan), dehydrated through graded alcohol, and cleared with xylene before mounting with Malinol (Mute Pure Chemicals Ltd., Tokyo, Japan). Immunoreactivity was examined by the BX40 system microscope (Olympus, Tokyo, Japan) with a CCD DPII Camera (Olympus, Tokyo, Japan). Images were analyzed by the Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD, USA). Two sections were prepared from each specimen. The measurements were obtained from the four quadrants in each section. Corresponding

Table 1. Clinical characteristics of patients with hepatocellular carcinoma

Patient	Sex	Age (years)	HBs-Ag	Anti-HCV	TNM staging	Tumor size (cm)	Histopathological grading of cancer tissue	Survival found	Follow up (months)
1	M	49	+	–	T4	11.7	Moderately-differentiated	No	0.03
2	M	57	–	+	T2	4.3	Moderately-differentiated	Yes	0.23
3	M	60	–	+	T4	2.5	Well-differentiated	Yes	9.3
4	M	73	–	+	T1	1.9	Moderately-differentiated	Yes	0.67
5	F	57	+	+	T4	3.3	Moderately differentiated	No	4.2
6	M	70	–	+	T3	5.5	Well-differentiated	Yes	12.17
7	M	72	–	+	T2	2.1	Poorly-differentiated	Yes	11
8	M	81	–	+	T3	8.4	Moderately-differentiated	Yes	3.9
9	M	70	+	–	T4	11.6	Moderately-differentiated	Yes	2.57
10	M	65	+	–	T3	7.2	Well-differentiated	Yes	9.5
11	M	69	–	+	T2	1	Well-differentiated	Yes	19.37
12	M	48	+	–	T3	6	Moderately-differentiated	No	0.77
13	M	64	+	–	T4	13.4	Moderately-differentiated	Yes	10.7
14	M	39	+	–	T3	21.8	Well-differentiated	No	3.53
15	M	33	+	–	T3	3.4	Poorly-differentiated	No	1.07
16	M	79	–	–	T3	3.2	Moderately-differentiated	No	4.5
17	M	64	–	–	T2	6.4	Well-differentiated	Yes	11.73
18	M	65	–	+	T3	4.7	Moderately-differentiated	Yes	14.53
19	M	58	–	+	T3	8.3	Well-differentiated	No	7.4

nonspecific binding of section was performed by parallel incubation with the antibody preneutralized with antigenic peptide. No immnoreactivity was revealed (data not shown). The tissues were classified as cytosolic type for those with PKCα deposited in the cytoplasm in more than 50% of the cells and the remaining ones were defined as membranous type.

Statistical Analysis

Levels of PKCα were represented by the percentages of the measurements to the average of the adjacent non-cancerous in the biopsies and expressed as means ± standard error. Differences between groups were analyzed by the unpaired Student's *t* test. Categorical variables were analyzed by the Fisher exact test. Survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. $P < 0.05$ was considered to be statistically significant.

Results

Clinical Characteristics

The 19 patients included 18 males and 1 female and were aged from 33 to 81 years (61.7 ± 3.0 years). Seven patients had HBs-Ag alone, eight patients had Anti-HCV alone, and one patient had both HBs-Ag and Anti-HCV. Only two patients were negative for HBs-Ag and Anti-HCV. TNM staging identified 1 T1, 4 T2, 9 T3, and 5 T4 lesions. The tumor size was between 1 cm and 21.8 cm (mean: 6.7 cm). Histopathologic analysis of the tumors revealed 7 with well-differentiated cells, 10 with moderately-differentiated cells, and 2 with poorly-differentiated cells. Most (12/19) of the patients were found to have survived during the follow-up (Table 1).

Immunohistological Findings

The expression of PKCα was assessed by immunohistochemistry in the 19 HCC specimens. In the tumor with membranous type of PKCα (Fig. 1A), a membranous location of PKCα was also seen in the adjacent non-tumor tissues (Fig. 1B). PKCα showed a significantly increased staining intensity in the tumor (129 ± 9; patient no. 9~19; $P < 0.01$) than that in the adjacent normal hepatic tissue (84 ± 8). However, the adjacent non-tumor tissues in the tumor with cytosolic type (Fig. 1C) of PKCα had the membranous location (Fig. 1D) of PKCα. The staining intensity in the tumor (134 ± 20; patient no 1~8; $P < 0.05$) also showed a significant increase than that in the adjacent normal hepatic tissue (89 ± 12).

PKCα Type and Clinical Characteristics

There was a significant association between the

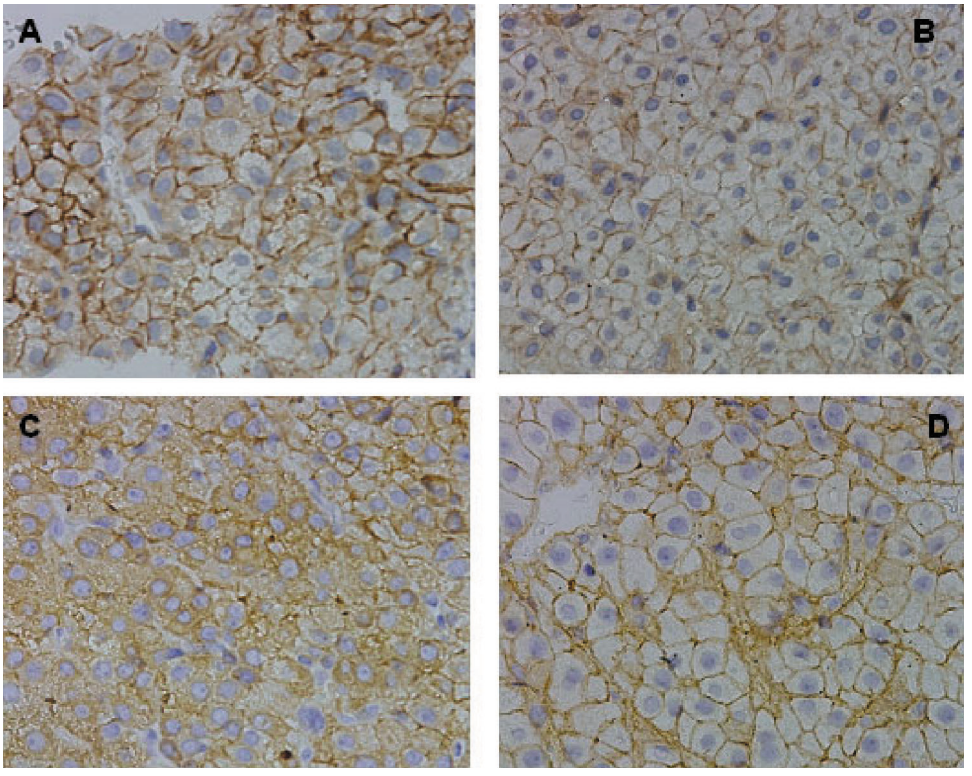

Fig. 1. Immunohistochemical analyses of protein kinase Cα (PKCα) in the cancerous (A and C) and adjacent normal hepatic tissue (B and D) of two patients with hepatocellular carcinoma (magnification, ×400). A and B show membrane types in both cancerous and normal tissues (patient No. 17). Although the cancerous tissue shows cytosolic PKCα type (C), the normal tissue is of the membranous type (patient No. 7).

type of PKCα and the presence of anti-HCV ($P < 0.05$) and more HCV carriers had the cytosolic type than the membranous type (Table 2). However, no significant associations were found between PKCα type and age, the presence of HBs-Ag, tumor size, TNM staging, and histopathological grading ($P > 0.05$). The survival rate was also not significantly different between the cytosolic type and the membranous type ($P = 0.7214$).

Discussion

Comparing the images obtained from HCC and adjacent normal hepatic tissue, we found that PKCα had a membranous location in the normal tissue (Fig. 1). Moreover, we also found a significant association of the membranous type with non-HCV infection, and the cytosolic type with HCV infection.

Chronic hepatitis due to HCV is a well-documented major risk factor of HCC (10). HCV is an RNA virus with a genome size of about 10 kb encoding a number of structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, and NS5B) proteins (6, 9). The HCV core protein has the strongest influence on NF-κB-, AP-1-, and SRE-associated pathways (20). Activation of these signaling pathways by HCV core proteins has been reported to play an important role in the progression of liver injury, cirrhosis, and human HCC (9, 34). It has been reported that NS3 of HCV may inhibit the nuclear transport and the enzymatic activity of the catalytic subunit of PKA. These viral nonstructural proteins may also inhibit TPA-induced redistribution of PKCα or PKCβ (3). These changes may lead to the impairment in the functions of these kinases. Hence the cytosolic type of PKCα in the specimens with positive anti-HCV may reveal in a few of the patients with stage III-IV cancers.

Chronic hepatitis due to HBV is also a well-documented major risk factor of HCC (10). HBV is a partially double-stranded DNA virus with a circular genome of 3.2 kb encoding two structural proteins (core protein and surface protein) and two nonstructural proteins (HBx and polymerase) involving in viral replication (13). HBx may activate signaling pathways associated with activator protein-1 (AP-1), activator protein-2 (AP-2), nuclear factor-kappa B (NF-κB), and serum response element (SRE) (1). Activation of these signaling pathways by HBx proteins has also been reported to play an important role in the progression of liver injury, cirrhosis, and HCC (2, 8, 26).

Table 2. Association between the types of PKCα location and the clinical characteristics of patients with hepatocellular carcinoma

Clinical characteristics	Types of PKCα location		$P^{\dagger}$
	Membrane	Cytosole	
Age			
≤ 60 years	4	4	NS
> 60 years	7	4	
HBs-Ag			
Positive	6	2	NS
Negative	5	6	
Anti-HCV			
Positive	3	7	< 0.05
Negative	8	1	
Tumor size			
≤ 3 cm	1	3	NS
> 3 cm	10	5	
TNM-staging			
T1 and T2	2	3	NS
T3 and T4	9	5	
Histopathological grading			
Well-differentiated	5	2	NS
Moderately- or poorly-differentiated	6	6	

†Statistical analyses were performed by the Fisher exact test. $P < 0.05$ was considered significant. NS, not significant.

HBx activates PKC, probably *via* increasing the endogenous PKC activator sn-1,2-diacylglycerol (DAG) (27). An increase in the endogenous PKC activator sn-1,2-diacylglycerol and the subsequent activation of PKC induces the activation of the transcription factor AP-1 (Jun-Fos) (21). Since HBx is more active than HBsAg in HCC (7, 33), it may be an important factor for PKC activation in the progressive malignancy of HCC. Based on the results of this study, although no significant association between the PKCα membranous type and the anti-HBsAg positive (Table 2), was found many of the patients with HCC of PKCα membranous type were positive for HBsAg. Moreover, PKCα of this type showed a significantly increased staining intensity in the tumor than that in the adjacent normal hepatic tissue, and was present in many of the patients with stage III-IV cancers, suggesting that the membranous location of the PKCα in HCC patients may be highly activated, and that PKCα may play an important role in carcinogenesis of HCC patients with HBV infection and/or non-HCV infection, but further studies on the phosphorylation and activation of PKCα are required.

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