Table 3 Baseline of hepatitis C virus carriers with 31–40 U/L aminotransferase who received antiviral therapy

	PLT ≥ 150 000/mL (group B-1)	PLT < 150 000/mL (group B-2)	*P*-value
No. patients	141	68	
Age	48.2 ± 11.9	57.9 ± 7.5	<0.001*
Sex (male/female)	80/61	37/31	0.751**
BMI (kg/m^2)	22.9 ± 3.1	22.7 ± 2.6	0.08*
HOMA-IR	3.0 ± 2.0	8.2 ± 9.5	0.8.8*
Genotype: 1/2/others	82/58/1	30/38/0	0.095**
Viral load: low/high	64/77	35/33	0.542**
Histology			
F stage (0/1/2/3/4)	17/91/31/2/0	4/30/26/6/2	<0.001**
Grade (0–1/2,3)	116/25	50/18	0.114**
Fatty change† 0–1/2–4	111/30	50/18	0.10**
Iron load‡ 0/1–4	67/12	30/7	0.762**
Ferritin (ng/mL)	114.4 ± 116.1	127.2 ± 167.8	0.869*
PLT count (/µL)	21.5 ± 4.9	12.2 ± 2.1	<0.001*
Hyaluronate (ng/mL)	46.9 ± 35.4	100.7 ± 0.98.1	<0.001*
Administration of IFN (weeks)	26.1 ± 11.9	27.7 ± 11.4	0.983*
Effects of therapy			
SVR/non-SVR	64/77	35/33	0.409**

**P*-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. In group B, there were significant differences in age ($P < 0.001$), distribution of F stage ($P < 0.001$), PLT count ($P < 0.001$), and hyaluronic acid ($P < 0.001$) between B-1 and B-2. Frequency of F2–4 was 23.4% in B-1and 50.0% in B-2, respectively. Values are expressed as mean ± SD.
BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance; IFN, interferon; PLT, platelet counts; SVR, sustained viral responders.

Demographic, clinical, and histological features of 129 HCV carriers with PNALT

The demographic and clinical features of the 129 HCV carriers with PNALT who were followed up for 7.2 years are shown in Table 4. Normal liver histology was noted in 17 patients, 102 showed minimal to mild CH, and 10 had moderate CH. Steatosis was seen in 7% and iron loading was noted in 12%.[18]

Of the 78 patients followed longer than 7 years (mean follow-up period; 10.4 ± 3.1 years), 11 (14%) had continuously normal ALT (G-1), 43 (55%) showed a transient elevation of ALT (G-2), and 24 (31%) changed to CH with continuously elevated ALT (G-3).

Thirty-nine patients received repeated liver biopsies (2–4 times). Of the 39 patients, six were in G-1, 17 were in G-2, and 16 were in G-3. The intervals between the first biopsy and the last biopsy in these three groups were 7.1, 7.8, and 7.2 years, respectively. The progression of the F stage was noted in two of six in G-1, six of 17 in G-2, and seven of 16 in G-3. The median rates of fibrosis progression per year for these three groups were 0.05, 0.05, and 0.08 fibrosis unit. HCC was not detected in any patients during the follow-up periods.

Guidelines for the antiviral therapy of HCV carriers with normal serum ALT focused on the inhibition of the development of HCC

Considering the risk of progression to liver cirrhosis and the development of HCC, as well as the expected efficacy and various side-effects of antiviral therapy, an algorithm is needed for the management of HCV carriers with normal serum ALT. The progression rate of liver fibrosis stage was 0.05/year in HCV carriers with PNALT. The annual incidence of HCC in CH-C patients has been reported to be 0.5% at stages F0–F1, 1–2% at stage F2, 3–5% at stage F3, and 7% at stage F4.[4]

In principle, follow up without antiviral treatment is recommended for HCV carriers with PNALT (ALT ≤30 U/L) and PLT counts ≥150 000/µL, particularly in older patients (i.e. >65 years old), because over 90% show normal or minimal liver damage with good prognoses. However, antiviral therapy is not contraindicated for such patients since roughly 40% are infected with HCV genotype 2,[18] which suggests a high rate of SVR to the therapy with PEG-IFN/Riba.

As for the indication of antiviral therapy for HCV carriers with normal serum ALT (≤40 U/L), the PLT

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Table 4 Characteristics of 129 HCV carriers with persistently normal ALT who received liver biopsy

	$n = 129$	Follow up over 5 years ($n = 78$)
Follow-up period (years)	7.2 ± 3.2	10.4 ± 3.1
Age (years)	48 (21–77)	45 (29–71)
Male ($n = 24$)	49.8 ± 16.4	42.3 ± 14.9
Female ($n = 105$)	47.2 ± 12.5	46.6 ± 11.6
Sex (male/female)	24/105	10/68
ALT (U/L)	8–30	9–30
Male ($n = 24$)	22.5 ± 5.7	21.1 ± 5.4
Female ($n = 105$)	21.6 ± 4.8	22.3 ± 5.1
PLT (×10^4/mL)	15–31	15–31
Ferritin (ng/mL)	5–225	5–225
Male ($n = 24$)	76.2 ± 53.5	84.6 ± 59.2
Female ($n = 105$)	60.0 ± 43.3	66.6 ± 52.5
HCV genotype	G1 ($n = 58$), G2 ($n = 45$) Mixed and unclassified ($n = 16$)	
BMI (kg/m^2)	16–27	16–27
Male	22.2 ± 1.7	21.9 ± 1.9
Female	21.3 ± 2.2	21.0 ± 2.4

Values are expressed as mean ± SD.
ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; PLT, platelet.

count is a good indicator for discriminating as to whether or not they have minimal to mild fibrosis or moderate to advanced fibrosis. Serum hyaluronate levels were significantly higher in HCV carriers with 31–40 U/L ALT having less than 150 000/μL PLT (Table 3). Advanced hepatic F stage, an elevated ALT level, old age (>65 years old), and sex (male) are important risk factors for the development of HCC.[6,18,30] We advocated an algorithm for such patients (Fig. 1) taking into consideration the risk of the progression to cirrhosis and the development of HCC. Therapy with PEG-IFN/Riba is the first-line treatment; therapy for 48 weeks is recommended for genotype 1 patients with high viral load and 12–24 weeks therapy for genotypes 2 and 1 with low viral load.

DISCUSSION

OUR PREVIOUS STUDY in 129 HCV carriers with PNALT demonstrated a predominance of females, higher frequency of genotype 2, minimal to mild liver histology, and very slow progression of hepatic fibrosis.[18] However, over 30% of these patients advanced to CH-C with elevated ALT levels during the 7-year follow up.

There are many reports concerning the natural course of liver fibrosis in CH-C patients, including those who are HCV carriers with normal serum ALT.[19,31–39] More than half of CH-C patients show progression of F stage from F1 to F2–4 within 10 years, and it was reported that the progression of liver fibrosis in HCV carriers with normal serum ALT was more rapid than was observed in the present study.[23] The main reason for the discrepancy between the report by Puoti *et al.*[23] and our results might be due to the definitions used for the normal range of serum ALT. In our previous study, the patients were HCV carriers with PNALT (ALT ≤ 30 U/L) and PLT counts ≥150 000/μL. On the other hand, the patients in the study by Puoti *et al.* had ALT levels ≤40 U/L, irrespective of PLT counts, in which cirrhotic patients might be included.[23] However, recent studies have demonstrated that normal ALT levels are less than 30 U/L[24] or 25 U/L in men[40] and less than 19 U/L[24] or 22 U/L in women.[40]

The present study demonstrated that the different distribution of hepatic F stage became remarkable when the A and B groups were divided into two subgroups according to their PLT counts. In HCV carriers with ALT levels ≤30 U/L, the frequency of stages F2–3 was 16.2% among those with PLT counts ≥150 000/μL; however, the frequency of stages F2–3 was 49.0% in those with PLT counts <150 000/μL. Conversely, in HCV carriers with ALT levels between 31 and 40 U/L, the frequency of stages F2–4 was 23.4% among those with PLT counts ≥150 000/μL and 50.0% in those with PLT counts <150 000/μL. The PLT count is a useful marker in dis-

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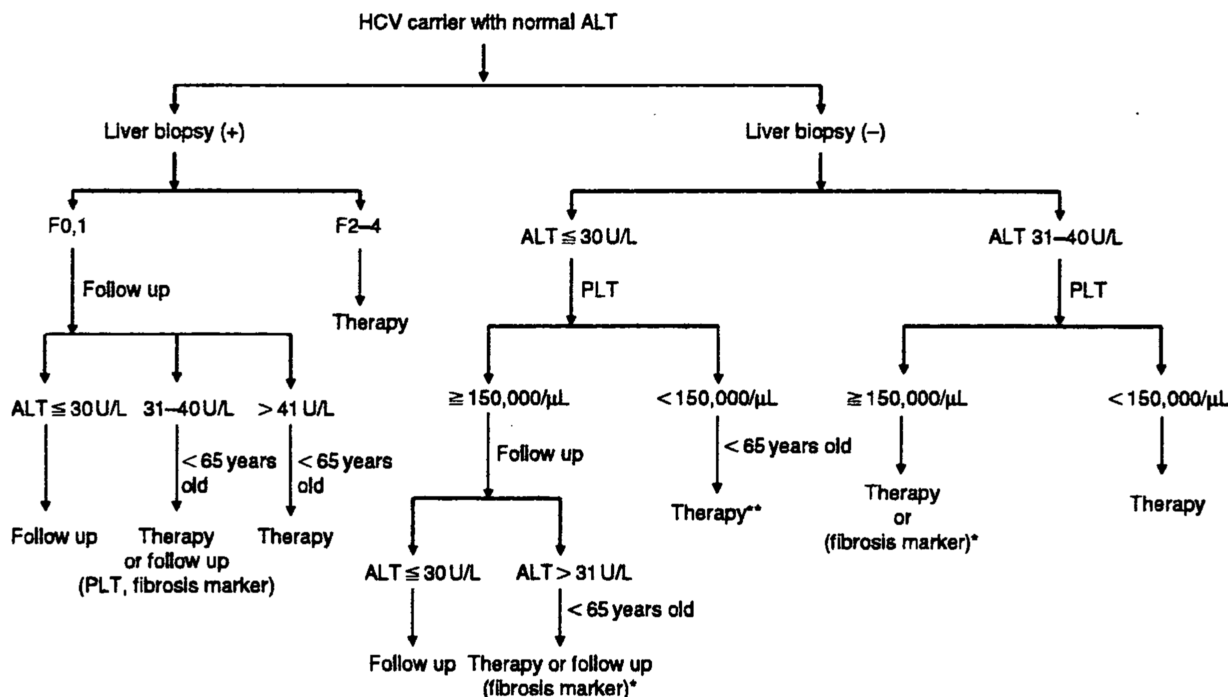

Figure 1 Algorithm for the management of hepatitis C virus (HCV) carriers with normal serum aminotransferase (ALT, ≤40 U/L) focused on the inhibition of the development of hepatocellular carcinoma. In patients who underwent liver biopsy, F0 and F1 patients younger than 65 years are candidates for antiviral therapy, especially those with genotype 2 after the elevation of serum ALT levels. In patients who did not undergo liver biopsy, ALT and platelet (PLT) levels are good indicators for determining candidates for antiviral therapy. Older patients (>65 years) and/or patients having uncontrolled hypertension, diabetes mellitus, or anemia should not be treated with pegylated interferon and ribavirin. Combination therapy with pegylated interferon and ribavirin for 48 weeks is recommended for patients with genotype 1 and high viral load, and 12–24 weeks therapy is suggested for patients with genotype 2 and genotype 1 with low viral load. *,**Serum fibrosis markers, such as hyaluronate, might be useful to decide whether patients are candidates for antiviral therapy or not.

criminating between stages F0–1 and F2–4 F in HCV carriers with normal serum ALT (≤40 U/L). In the present study, the mean PLT count in F2 and F3 patients was 16.9 ± 5.3 ($\times 10^4/\mu L$) and 15.9 ± 4.6 ($\times 10^4/\mu L$), respectively. The distribution of the F stage was not significantly different between patients with PLT counts $\geq 15 \times 10^4/\mu L$ versus $< 15 \times 10^4/\mu L$ and $\geq 17 \times 10^4/\mu L$ versus $< 17 \times 10^4/\mu L$.

The SVR rate for genotype 1 patients with high viral load treated with either IFN monotherapy or IFN/Riba were 12.5% and 37.7%, respectively. In genotype 2 patients with high viral load, the SVR rate in the present study was better than the data of Japanese CH-C patients with elevated ALT levels in our previous paper.[6] It was not reasonable to compare the SVR rates between HCV carriers with normal serum ALT and CH-C with elevated ALT in the present study, because the total dosage of IFN and the duration of treatment were significantly different.

The annual incidence of HCC is correlated with the progression of liver fibrosis, that is, the stage of liver disease.[2–4,6] Sustained low serum ALT levels are also associated with a lower incidence of HCC.[7,64,1] PEG-IFN/Riba therapy is expensive and induces various side-effects. The present results indicate that most HCV carriers with normal serum ALT (≤40 U/L) and PLT counts ≥150 000/μL have minimal to mild liver damage, indicating a low risk for the progression to cirrhosis and the development of HCC. This was more remarkable in patients with ALT levels ≤30 U/L and PLT counts ≥150 000/μL. However, nearly half of the patients with PLT count <150 000/μL have F2 or F3 F stages, indicating a certain risk for the progression to cirrhosis and the development of HCC. Fibrosis

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progression is associated with age, baseline and follow-up ALT levels, inflammatory activity and steatosis in the initial liver biopsy, and alcohol consumption.[47] The present results indicate that most HCV carriers with PNALT have a good prognosis and a low risk of developing HCC.

Liver biopsy is a useful procedure for identifying the stage of liver fibrosis; however, it is invasive and may sometimes cause complications.[43,44] The error rate of predicting the F stage with this procedure can be estimated to be as high as 20%.[45] Recently introduced biochemical markers, such as FibroTest,[46] and FibroScan,[47–49] are excellent procedures for identifying liver fibrosis stage in CH-C patients.[50] The combined use of FibroScan and FibroTest is useful for accurately estimating moderate to severe liver fibrosis in most patients with CH-C, but not in F0 and F1 patients.[51]

Recently, Alberti proposed an individualized management algorithm for HCV carriers with PNALT with or without liver biopsy in which HCV genotype, patient age, motivation to receive antiviral therapy, and factors influencing side-effects were included.[52] The algorithm using a combination of serum ALT levels and PLT counts in the present study is simple, but it is useful because it focuses mainly on the inhibition of the progression to cirrhosis and the development of HCC.

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Protein kinase C δ regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma

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Abstract

We have recently reported that attenuated phosphorylation of heat shock protein (HSP) 27 correlates with tumor progression in patients with hepatocellular carcinoma (HCC). In the present study, we investigated what kind of kinase regulates phosphorylation of HSP27 in human HCC-derived HuH7 cells. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol, direct activators of protein kinase C (PKC), markedly strengthened the phosphorylation of HSP27. Bisindorylmaleimide I, an inhibitor of PKC, suppressed the TPA-induced levels of HSP27 phosphorylation in addition to its basal levels. Knock down of PKCδ suppressed HSP27 phosphorylation, as well as p38 mitogen-activated protein kinase (MAPK) phosphorylation. SB203580, an inhibitor of p38 MAPK, suppressed the TPA-induced HSP27 phosphorylation. Our results strongly suggest that activation of PKCδ regulates the phosphorylation of HSP27 via p38 MAPK in human HCC.
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Keywords: PKCδ; HSP27; Phosphorylation; Protein kinase; Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is characterized by its high incidence in hepatitis virus-associated liver disease, reaching approximately 3% in hepatitis B virus-infected cirrhotic patients and 7% in hepatitis C virus-infected cirrhotic patients (Ikeda et al., 1993; Shiratori et al., 2001). Moreover, the incidence of post-therapeutic recurrence is approximately 20% to 25% a year in cirrhotic patients who have already undergone curative treatment of the primary HCC (Kumada et al., 1997; Koda et al., 2000). Thus, overall survival of patients with HCC is still unsatisfactory even after hepatectomy. Therefore, it is required to clarify the further exact mechanism of HCC carcinogenesis.

Heat shock proteins (HSP) are produced by cells exposed to biological stressors such as heat and chemicals (Shimada et al., 1998). HSPs are classified as high-molecular-weight HSPs, such as HSP70, HSP90, and HSP110, or low-molecular-weight HSPs, which have molecular masses from 10 to 30 kDa. High-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization, and translocation (Benjamin and McMillan, 1998). Though the functions of low-molecular-weight HSPs, such as HSP27 and αB-crystallin, are not as well-characterized as those of the high-molecular-weight HSPs, it is thought that they may also have chaperone functions (Benjamin and McMillan, 1998). It is recognized that HSP27 activity is regulated by post-translational modifications such as phosphorylation (Welch, 1985; Benjamin and McMillan, 1998). Mouse HSP27 is phosphorylated at two serine residues (Ser-15 and Ser-82), whereas human HSP27 is phosphorylated at three serine residues (Ser-15, Ser-78, and Ser-82) (Benjamin and McMillan, 1998). It was also reported that phosphorylated HSP27 is translocated from the cytosol to the nucleus in hippocampal progenitor cells, and prevents apoptosis (Geum et al., 2002). In a recent study (Yasuda et al., 2005), we have shown

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that the levels of phosphorylated HSP27 were correlated inversely with tumor stage by TNM classification in patients with HCC. It has been reported that HSP27 phosphorylation is catalyzed by the mitogen-activated protein kinase (MAPK) superfamily (p38 MAPK, phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p44/p42 MAPK) (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). In addition, it has been reported that p38 MAPK and p44/p42 MAPK are activated in HCC and contribute to the acceleration of the cell cycle (Ito et al., 1998; Iyoda et al., 2003).

Protein kinase C (PKC) is reportedly an upstream regulator of MAPK superfamily cascade (Noguchi et al., 1993; Tanaka et al., 2003; Tokuda et al., 2003). PKC is a Ser/Thr protein kinase family with multiple isoforms, its isoforms have been classified into three groups, classical PKC (α, β, γ), novel PKC (δ, ε, η, θ), and atypical PKC (ζ, ι/λ) (Saito et al., 2001). To date, these PKC isotypes are believed to play distinct regulatory roles. Regarding about the low-molecular-weight HSPs, PKC-dependent phosphorylation of low-molecular-weight HSPs by phorbol-esters has been previously described in Hela cells and MCF-7 cells (Arrigo, 1990; Faucher et al., 1993). In addition, it has been demonstrated that Ca^{2+}-independent PKCδ is superior in its ability to phosphorylate low-molecular-weight HSPs compared with a panel of other PKC isoforms in vitro (Maizels et al., 1998). Furthermore, the detection of phosphorylated low-molecular-weight HSPs in the rat corpora lutea of late pregnancy is reportedly associated with abundant and activated PKCδ (Maizels et al., 1998). However, the kinases that regulate phosphorylation of HSP27 in human HCC have not yet been clarified. In the present study, we investigated what kind of kinase regulates phosphorylation of HSP27 in human HCC. Our results strongly suggest that activation of PKCδ regulates the phosphorylation of HSP27 via p38 MAPK in human HCC.

Materials and methods

Materials

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Oleoyl-2-acetyl-glycerol (OAG) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). PD98059, bisindolylmaleimide I, and SB203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA). HSP27 antibodies, phospho-HSP27 (Ser-15) antibodies, and phospho-HSP27 (Ser-78) antibodies were purchased from Stressgen Biotechnologies. (Victoria, British Columbia, Canada). Phospho-HSP27 (Ser-82) was purchased from Biomol Research Laboratories. (Plymouth Meeting, PA). β-actin antibodies were purchased from Sigma. Phospho-Raf-1 antibodies, phospho-MEK1/2 antibodies, phospho-p44/p42 MAPK antibodies and p44/p42 MAP kinase antibodies, phospho-p38 MAPK antibodies, p38 MAPK antibodies, phospho-SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-PKC (pan) (βII Ser-660) antibodies, phospho-PKCδ (Thr-505) antibodies, PKCδ antibodies, and phospho-PKCθ (Thr-538) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). ECL Western blot detection system was purchased from Amersham Japan (Tokyo, Japan). The PKCδ siRNA (Silencer® Validated siRNA), PKCε siRNA (Silencer® Pre-designed siRNA) and non-specific control siRNA (Silencer® Negative control #1 siRNA) were obtained from Ambion, Inc. (Austin, TX). siLentFect was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Other materials and chemicals were obtained from commercial sources. TPA, PD98059, bisindolylmaleimide I, and SB203580 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for HSP27 phosphorylation.

Cell culture

Human HCC-derived HuH7, which were originated from well-differentiated HCC tissues, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HuH7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 90-mm diameter dishes in DMEM containing 10% FCS. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. When indicated, the cells were pre-treated with respective inhibitors and then stimulated with TPA or OAG for the indicated periods. Cell viability was estimated by the trypan blue dye exclusion method. Experiments were performed in triplicate.

Western blotting analysis

The cultured cells were washed twice with phosphate-buffered saline. The cultured cells were then lysed, homogenized and sonicated in lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 ×*g* for 10 min at 4 °C. The linear range of loading volume in Western blotting analysis was tested with serially diluted protein samples. Protein samples (10 μg) were loaded equally to SDS-polyacrylamide gel electrophoresis (PAGE) in respective experiments (except for total HSP27). For the detection of total HSP27, 2.5 μg of proteins were subjected to the each well of the gel. SDS-PAGE was performed by Laemmli (1970) in polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996). Band intensities visualized on X-ray film were determined by integrating the optical density over the band area (band volume) with NIH image software.

siRNA protocol

Transfection was performed according to the manufacturer's protocol (Bio-Rad). Briefly, 5 μl of siLentFect and finally 10 nM siRNA were diluted with FCS-free DMEM, pre-incubated at room temperature for 20 min and then added to the culture medium containing 10% FCS. Cells were incubated at 37 °C for 48 h with siRNA–siLentFect complexes and subsequently harvested for preparation of Western blotting analysis.

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean+S.E. of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Comparisons between phosphorylated levels of p44/p42 MAPK and HSP27 in HuH7 cells

It is recognized that HSP27 phosphorylation is catalyzed by the MAP kinase superfamily (p38 MAPK, SAPK/JNK, and p44/p42 MAPK) (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). It has been reported that p44/p42 MAPK is constantly activated in HCC (Ito et al., 1998). Therefore, we first examined the relationship between p44/p42 MAPK and HSP27 phosphorylation in HuH7 cells. The expression of HSP27 and its phosphorylated form (Ser-78 and Ser-82) were detectable in HuH7 cells (Fig. 1A). In addition, p44/p42 MAPK were constitutively phosphorylated in HuH7 cells (Fig. 1B). To elucidate whether p44/p42 MAPK is involved in the phosphorylation of HSP27 in HuH7 cells, we examined the effect of PD98059, a specific inhibitor of MEK1/2 (Alessi et al., 1995), on the phosphorylated levels of HSP27. Though PD98059 suppressed the phosphorylation of p44/p42 MAPK dose dependently in the range between 10 and 50 μM, the levels of HSP27 phosphorylation were not affected (Fig. 1C).

Effect of PKC activation on the HSP27 phosphorylation in HuH7 cells

It is well-recognized that PKC is an upstream regulator of Raf-1-MEK1/2-p44/p42 MAPK cascade (Noguchi et al., 1993). Thus, we investigated whether PKC is activated in HuH7 cells. PKC activity is controlled by three distinct phosphorylation events (specifically, the threonine 500 in the activation loop, the threonine 641 autophosphorylation site, and the serine 660 hydrophobic site at the carboxy terminus of PKCβII are phosphorylated in vivo) (Keranen et al., 1995). Since we have preliminary confirmed that phospho-PKC (pan) (βII Ser-660) antibodies can detect PKCα/β and PKCε by using the respective antibodies, we used phospho-PKC (pan) (βII Ser-660) antibodies to detect them. PKCα/β and PKCδ were markedly phosphorylated in HuH7 cells (Fig. 2A). To elucidate whether PKC is involved in the phosphorylation of HSP27 in these cells, we examined the effect of bisindolylmaleimide I, an inhibitor of classical PKC and novel PKC (Toullec et al., 1991), on the basal levels of HSP27 phosphorylation. Bisindolylmaleimide I decreased the phosphorylated levels of HSP27 dose dependently in the range between 10 and 50 μM (Fig. 2B). It is well-known that both classical and novel PKC are activated by phorbol-esters such as TPA (Nishizuka, 1991). We next investigated the effect of TPA (Nishizuka, 1991), a direct activator of PKC on the phosphorylated levels of HSP27 in HuH7 cells.

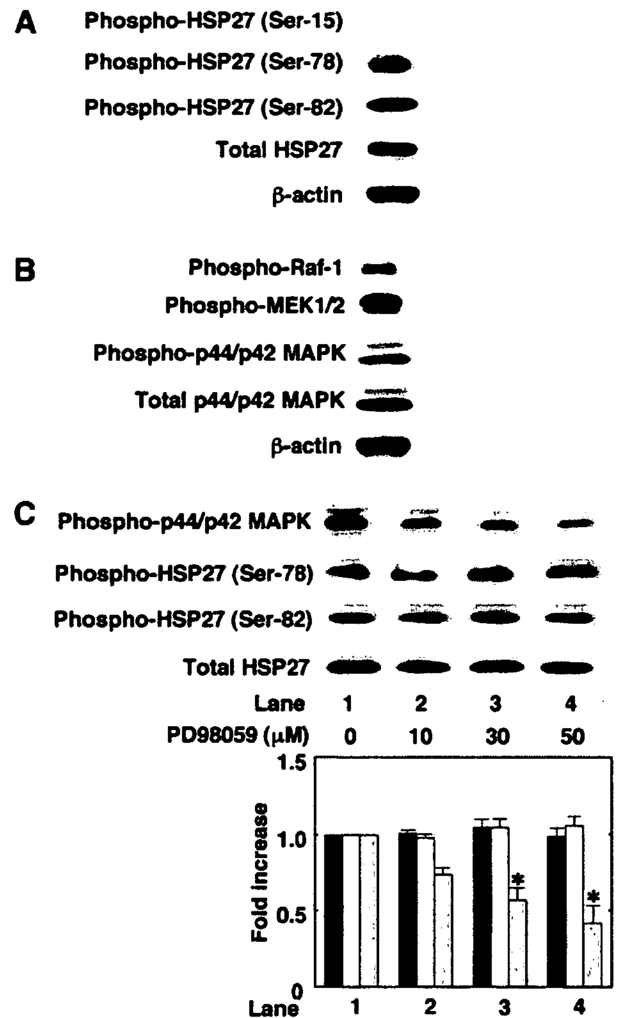

Fig. 1. The levels of HSP27 phosphorylation and the phosphorylated levels of Raf-1-MEK1/2-p44/p42 MAPK cascade, and effect of PD98059 on HSP27 phosphorylation in HuH7 cells. HuH7 were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82), total HSP27 and β-actin. (B) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-Raf-1, phospho-MEK1/2, phospho-p44/p42 MAPK, total p44/p42 MAPK and β-actin. (C) The cultured cells were pre-treated with various doses of PD98059 for 60 min, and then washed twice and collected. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-p44/p42 MAPK, phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27. The phosphorylated levels were normalized by the levels of total HSP27. The histogram shows the fold increase of levels of phospho-HSP27 (Ser-82) (black bars), phospho-HSP27 (Ser-78) (white bars) and phospho-p44/p42 MAPK (gray bars) in PD98059-treated cells versus those of PD98059-untreated cells. Each value represents the mean±S.E. of triplicate determinations from three independent experiments. Representative results from triplicate independent experiments with similar results are shown. $*p<0.05$, compared to the value of control.

TPA significantly strengthened the phosphorylated levels of HSP27 in a time-dependent manner (Fig. 2C). The phosphorylated levels reached a peak at 60 min after the TPA-stimulation. TPA stimulated the phosphorylation of HSP27 dose dependently in the range between 0.01 and 0.1 μM, the maximum effect was

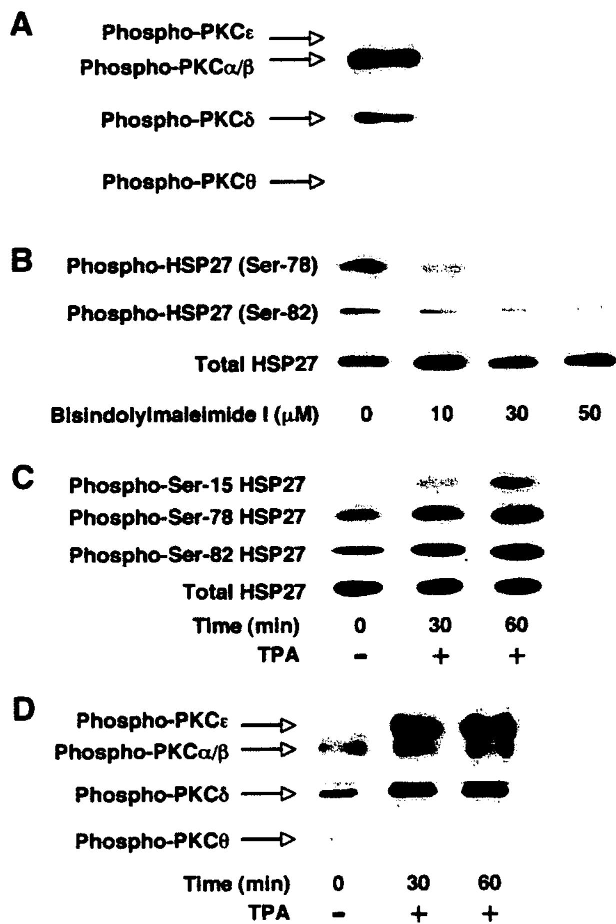

Fig. 2. Effect of bisindorylmaleimide I on HSP27 phosphorylation, and effects of TPA on HSP27 phosphorylation and PKC phosphorylation in HuH7 cells. HuH7 cells were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-PKC (pan) (βII Ser-660), phospho-PKCδ (Thr-505) and phospho-PKCθ (Thr-538). (B) The cultured cells were pre-treated with various doses of bisindorylmaleimide I for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27. (C, D) The cultured cells were stimulated with 0.1 μM TPA for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (C) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (D) phospho-PKC (pan) (βII Ser-660), phospho-PKCδ (Thr-505) and phospho-PKCθ (Thr-538). Representative results from triplicate independent experiments with similar results are shown.

observed at a dose of 0.1 μM (data not shown). In addition, TPA markedly enhanced the phosphorylation levels of novel PKC (δ, ε) in a time-dependent manner. On the contrary, the levels of both PKCα/β and PKCθ were not affected by TPA or were at least in part slightly enhanced by TPA (Fig. 2D).

Effects of bisindolylmaleimide I or PKC down-regulation on TPA-induced phosphorylation of HSP27 in HuH7 cells

We examined the effect of bisindolylmaleimide I on the TPA-induced phosphorylation of HSP27. Bisindolylmaleimide I (30 μM) suppressed the TPA-induced phosphorylation of HSP27 (Fig. 3A). TPA-induced novel PKC(δ, ε) phosphorylation was also suppressed by bisindolylmaleimide I (Fig. 3B).

It has been reported that treatment of TPA (0.1 μM) for 24 h down-regulates PKC (Blumberg, 1991). To clarify the role of PKC on the HSP27 phosphorylation in HuH7 cells, we examined the effect of TPA long term pre-treatment on the phosphorylation of HSP27. The effect of TPA on HSP27 phosphorylation was reduced in the PKC down-regulated cells compared with that in the cells without TPA treatment (Fig. 3C).

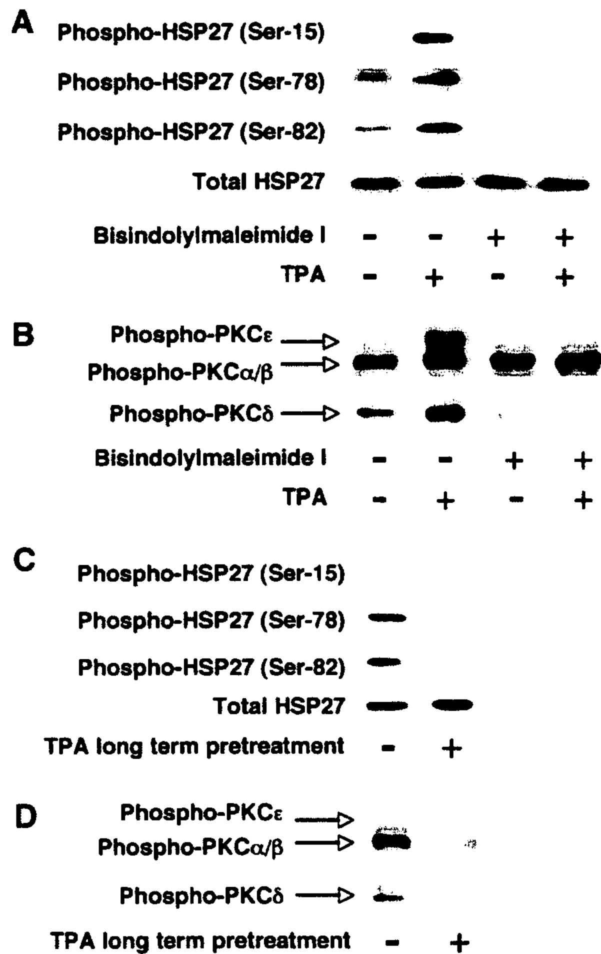

Fig. 3. Effects of bisindorylmaleimide I on TPA-induced HSP27 phosphorylation and PKC phosphorylation, and effect of PKC down-regulation on the phosphorylation of HSP27 in HuH7 cells. HuH7 cells were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A, B) The cultured cells were pre-treated with 30 μM of bisindorylmaleimide I or vehicle for 60 min and then, stimulated by 0.1 μM TPA or vehicle for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (A) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (B) phospho-PKC (pan) (βII Ser-660) and phospho-PKCδ (Thr-505). (C, D) The cultured cells were pre-treated with 0.1 μM TPA or vehicle for 24 h. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (C) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (D) phospho-PKC (pan) (βII Ser-660) and phospho-PKCδ (Thr-505). Representative results from triplicate independent experiments with similar results are shown.

We also found that TPA long term treatment down-regulated the phosphorylated levels of PKCα/β, PKCε and PKCδ compared with those in these cells without TPA (Fig. 3D).

Effect of OAG on the HSP27 phosphorylation in HuH7 cells

OAG, a synthetic diacylglycerol (DAG), which is generally recognized to be a physiological activator of PKC (Nishizuka, 1991; Schutze et al., 1991), strengthened the phosphorylated levels of HSP27 in a time-dependent manner (Fig. 4A) as well as TPA. The phosphorylated levels reached its peak at 10 min after the stimulation and decreased thereafter (Fig. 4A). We next examined the effect of bisindolylmaleimide I on OAG-induced levels of HSP27 phosphorylation. Bisindolylmaleimide I (30 μM) suppressed OAG-induced phosphorylated levels of HSP27 (Fig. 4B). In addition, OAG-induced phosphorylation of novel PKC(δ, ε) was also suppressed by bisindolylmaleimide I (Fig. 4C).

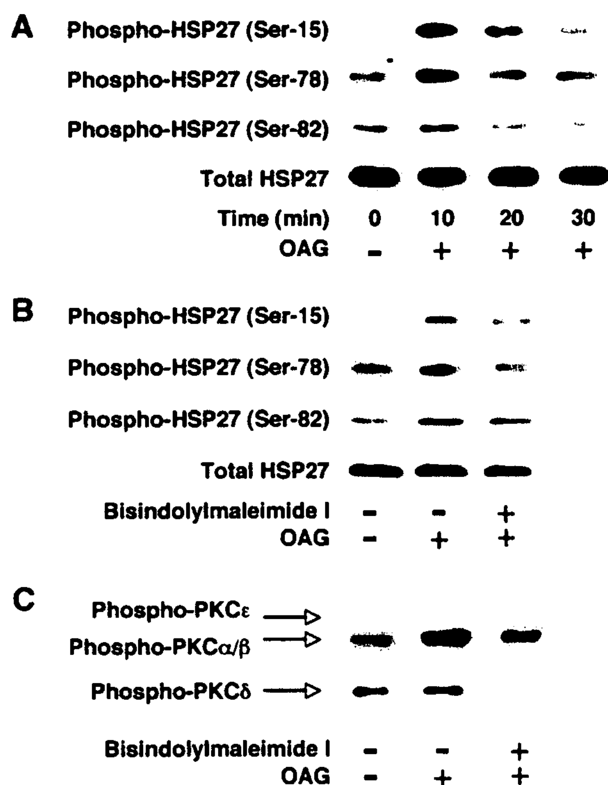

Fig. 4. Effect of OAG on the phosphorylation of HSP27, and effects of bisindorylmaleimide I on the OAG-induced HSP27 phosphorylation and PKC phosphorylation in HuH7 cells. HuH7 cells were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A) The cultured cells were stimulated with 100 μM OAG for the indicated periods. (B, C) The cultured cells were pre-treated with 30 μM of bisindorylmaleimide I or vehicle for 60 min, and then stimulated with 100 μM OAG or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (A, B) phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (C) phospho-PKC (pan) (βII Ser-660) and phospho-PKCδ (Thr-505). Representative results from triplicate independent experiments with similar results are shown.

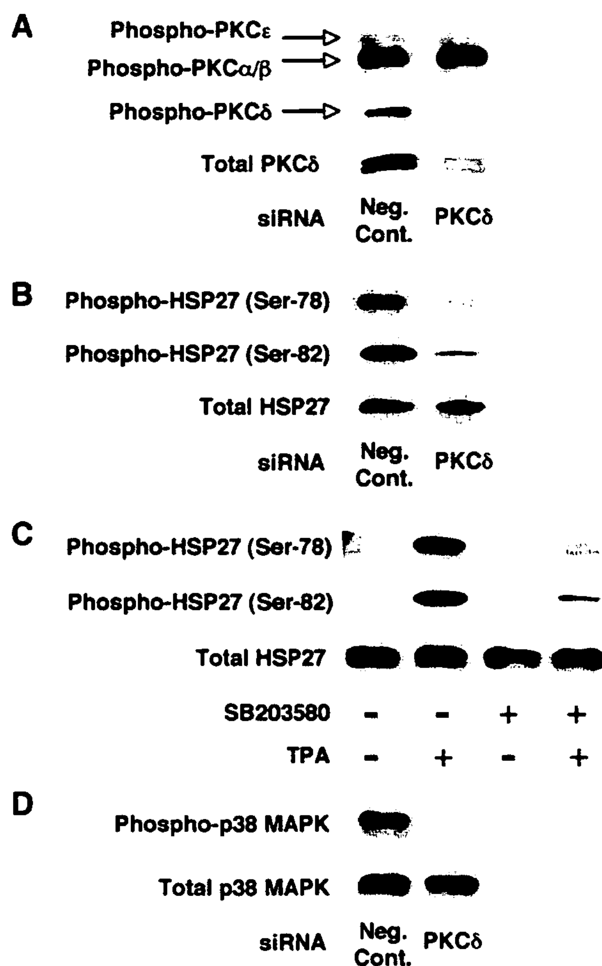

Fig. 5. Effect of gene silencing using PKCδ-siRNA-transfection into HuH7 cells and effect of SB203580 on the TPA-induced HSP27 phosphorylation in HuH7 cells. (A, B, D) HuH7 cells were cultured in DMEM containing 10% FCS for 24 h. After 24 h, the cells were incubated with 10 nM of PKCδ siRNA or negative control siRNA at 37 °C for 48 h in DMEM containing 10% FCS and subsequently harvested for preparation of Western blotting analysis. (C) HuH7 cells were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. The cultured cells were pre-treated with 30 μM of SB203580 or vehicle for 60 min and then, stimulated by 0.1 μM TPA or vehicle for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against (A) phospho-PKC (pan) (βII Ser-660), phospho-PKCδ (Thr-505) and total PKCδ, (B, C) phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27, and (D) phospho-p38 MAPK and total p38 MAPK. The total PKCδ antibody confirms silencing of PKCδ protein expression, and total HSP27 antibody is used to control for loading and specificity of PKCδ siRNA. Representative results from triplicate independent experiments with similar results are shown. Neg. Cont.: negative control.

Effect of gene silencing using PKCδ-siRNA- or PKCε-siRNA-transfection into HuH7 cells

Based on our findings, it is probable that phosphorylation of HSP27 is regulated by activation of novel PKC. To clarify which isoform of novel PKC acts in the phosphorylation of HSP27 in HuH7 cells, we examined the effect of gene silencing using PKCδ-siRNA-transfection into HuH7 cells. We found that PKCδ knock down selectively decreased expression of

PKCδ (Fig. 5A). In PKCδ-knocked down HuH7 cells, the phosphorylated levels of HSP27 were much reduced, while expression levels of total HSP27 were not changed (Fig. 5B). In addition, we examined the effect of gene silencing using PKCε-siRNA-transfection into HuH7 cells. However, both phosphorylated levels of HSP27 and expression levels of total HSP27 were not changed in PKCε-knocked down HuH7 cells (data not shown).

Effect of SB203580 on TPA-induced HSP27 phosphorylation

It is well-recognized that HSP27 is phosphorylated at serines 15, 78, and 82 by MAPKAP kinase 2 as a result of p38 MAPK pathway activation (Landry et al., 1992; Rouse et al., 1994). In contrast, a recent study showed that PKCδ directly binds to HSP27 and induces HSP27 phosphorylation (Lee et al., 2005). To determine whether p38 MAPK is involved in PKCδ-mediated HSP27 phosphorylation in HuH7 cells, we next investigated the effect of SB203580 (Cuenda et al., 1995), a specific inhibitor of p38 MAPK on TPA-induced HSP27 phosphorylation. SB203580 (30 μM) almost completely suppressed TPA-induced phosphorylation of HSP27 (Fig. 5C).

To clarify whether PKCδ exerts its effect at upstream of p38 MAPK activation, we examined the effect of gene silencing of PKCδ by siRNA into HuH7 cells. PKCδ knock down markedly suppressed phosphorylation of p38 MAPK in these cells (Fig. 5D).

Discussion

It has been shown that phosphorylation of HSP27 is mediated by the MAPK superfamily (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). Although p44/p42 MAPK is highly activated in HCC (Ito et al., 1998; Iyoda et al., 2003), we demonstrated that phosphorylation of HSP27 was not correlated with p44/p42 MAPK activity. It has been reported that PKC is an upstream regulator of the MAPK superfamily cascade (Noguchi et al., 1993; Tanaka et al., 2003; Tokuda et al., 2003). Therefore, we examined whether PKC regulates the HSP27 phosphorylation in HCC cells. As expected, we found that the inhibition of PKC with bisindorylmaleimide I, and PKC down-regulation suppressed the basal level of HSP27 phosphorylation in HuH7 cells consistently with the previous reports (Faucher et al., 1993). In addition, the activation of PKC induced by TPA or OAG markedly strengthened HSP27 phosphorylation in HuH7 cells. Although, bisindolylmaleimide I does not seem nearly as effective as an inhibitor of HSP27 phosphorylation prior to OAG (Fig. 4B), when compared to TPA (Fig. 3A), particularly at Ser-15 and Ser-82. TPA, a phorbol ester, is known to activate PKC in an irreversible manner (Nishizuka, 1986). On the other hand, OAG is a physiological activator of PKC (Nishizuka, 1991; Schutze et al., 1991). Therefore, the effect of OAG to induce HSP 27 (shown in Fig. 4B, lane 2) was weaker than that of TPA (shown in Fig. 3A, lane 2), leading to the relative up-regulation of the band (Fig. 4B, lane 3) which shows the inhibitory effect of bisindolylmaleimide I on the OAG-induced HSP27 phosphorylation. Collectively, these findings suggest that PKC might have a pivotal role in the HSP27 phosphorylation in human HCC.

The importance of PKC signaling in tumor cells is corroborated by investigations that characterized the roles of individual PKC isoforms in cell growth regulation and transformation (Hofmann, 1997; Mackay and Twelves, 2003). Since atypical PKC is insensitive to TPA (Nishizuka, 1991), our findings made us to speculate that classical PKC and novel PKC are the candidate for the regulator of HSP27 phosphorylation in HuH7 cells. In the present study, PKCδ and PKCε were phosphorylated by TPA-stimulation and suppressed with bisindorylmaleimide I. In contrast PKCα/β were constitutively activated in HuH7 cells, and were not affected or were at least in part slightly enhanced by both TPA or bisindorylmaleimide I. Furthermore, PKCδ knock down significantly suppressed HSP27 phosphorylation in HuH7 cells. Taking these findings into account, it is most likely that activation of PKCδ regulates the phosphorylation of HSP27 in human HCC.

It is well-recognized that the MAPK cascade, in particular p38 MAPK, phosphorylates HSP27 via MAPK-activated protein kinase-2 (MAPKAPK-2), one of the substrates of p38 MAPK (Landry et al., 1992; Rouse et al., 1994; Guay et al., 1997). While, it has recently been reported that PKCδ directly binds to and phosphorylates HSP27 (Lee et al., 2005). Therefore, we next investigated whether p38 MAPK is involved in PKCδ-mediated HSP27 phosphorylation in HuH7 cells. We showed here that PKCδ knock down resulted in the suppression of p38 MAPK phosphorylation. In addition, we found that SB203580 significantly reduced the levels of HSP27 phosphorylation. Therefore, it is most likely that PKCδ mainly regulates HSP27 phosphorylation at a point upstream of p38 MAPK in human HCC.

Recent evidence suggests that PKCδ acts as a pro-apoptotic, tumor-suppressive molecule (Hofmann, 1997; Mackay and Twelves, 2003; Steinberg, 2004). Unlike PKCβ (which stimulates growth) and PKCε (which acts as an oncogene when over-expressed in rat fibroblasts and promotes tumors in nude mice), PKCδ generally slows the proliferation, induces the cell cycle arrest, and/or enhances the differentiation of various undifferentiated cell lines (Steinberg, 2004). In addition, it has been reported that a PKCδ/p38 MAPK pathway mediates the pro-apoptotic effects in prostate cancer cells (Tanaka et al., 2003). Our present findings seem to be in accordance with these previous observations.

Although the role of phosphorylated HSP27 is not elucidated, it has been reported that p38 MAPK-mediated phosphorylation of HSP27 increases its association with IκB kinase complex to suppress TNF-mediated NF-κB activation (Park et al., 2003). In a previous study (Yasuda et al., 2005), we showed that attenuated phosphorylation of HSP27 correlates with the tumor progression in patients with HCC, i.e. the larger tumors exhibited lower levels of phosphorylated HSP27 than did the smaller tumors. In addition, it has been reported that the activation of p38 MAPK is inversely correlated with the tumor progression in patients with HCC (Iyoda et al., 2003). Based on these findings, it is speculated that PKCδ may prevent tumor progression through phosphorylation of HSP27. Further investigations would be required to clarify the detailed mechanism of

HSP27 phosphorylation and the role of phosphorylated HSP27 in human HCC.

In conclusion, our present results strongly suggest that PKCδ functions as an important regulator in the phosphorylation of HSP27 via p38 MAPK in human HCC.

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Review Article

Ursodeoxycholic acid: Mechanism of action and novel clinical applications

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Ursodeoxycholic acid (UDCA) is used in the treatment of cholestatic liver diseases, gallstone dissolution, and for patients with hepatitis C virus infection to ameliorate elevated alanine aminotransferase levels. The efficacy of UDCA treatment has been debated and the mechanisms of action in humans have still not defined. Suggested mechanisms include the improvement of bile acid transport and/or detoxification, cytoprotection, and anti-apoptotic effects. In this review, we summarize the proposed molecular mechanisms for the action of UDCA, especially in hepatocytes, and also discuss the putative future clinical usage of this unique drug.

Key words: bile acids, cholestatic liver diseases, colon cancer, non-alcoholic steatohepatitis, ursodeoxycholic acid

INTRODUCTION

BILE ACID SYNTHESIS from cholesterol is the primary pathway for cholesterol catabolism. Bile acids are amphipathic molecules that contain a sterol nucleus with hydroxyl groups and a side chain with a terminal carboxylic acid. Their amphipathic nature is essential for the solubilization of dietary lipids, which subsequently promotes lipid absorption in the digestive tract. The principal bile acid in humans are cholic acid (CA) and chenodeoxycholic acid (CDCA), which are primary bile acids, and deoxycholic acid (DCA) and lithocholic acid (LCA), which are secondary bile acids, and their glycine and taurine conjugates. Most bile acids are present in the enterohepatic circulation and are stored in the gallbladder. When a meal is ingested, bile acids flow into the duodenum and intestine, and are efficiently re-absorbed by passive diffusion and active transport in the terminal ileum and transported back to the liver via the portal vein. In the liver, the bile acids are taken up at the sinusoidal (basolateral) membrane and exported at the canalicular (apical) membrane of hepatocytes into bile canaliculus. Each bile acid molecule completes 4–12 cycles between the liver and intestine per day. Because of this efficient recirculation system, approximately only 5% of the bile acid pool is derived from de novo biosynthesis in the liver.

Ursodeoxycholic acid (UDCA) is a bile acid that is present in human bile at low concentrations, as only 3% of total bile acids. UDCA is a 7,-hydroxy epimer of the primary bile acid CDCA (Fig. 1), and can be isolated from the Chinese medicine Yutan, which is derived from the dried bile of adult Chinese black bears. UDCA has been used widely in clinical applications in the Western world since the mid 1980s, and has been shown to improve clinical and biochemical indices in a variety of biliary and liver diseases (Table 1). In the 1970s, the first prospective study of UCDA for the treatment of patients with gallbladder stones demonstrated gallstone dissolution,[1] and it is now recognized that UDCA dissolves gallstones by solubilizing cholesterol from the stone surface. UDCA also converts supersaturated bile to unsaturated bile,[2] and such desaturation enhances the transport capacity of bile for cholesterol.[2] Biliary desaturation by UDCA occurs through several mechanisms, most of which are not completely understood.[2] In addition, UDCA also has the unique property of promoting the formation of a liquid crystal mesophase of phospholipids and cholesterol.[3] Such liquid crystals can form even in the presence of bile supersaturated with cholesterol, which may account for the observation that UDCA can dissolve gallstones in the presence of supersaturated bile.

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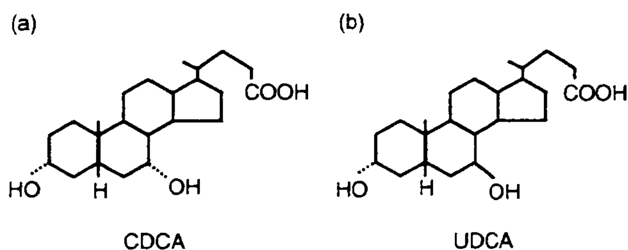

Figure 1 Chemical structure of (a) chenodeoxycholic acid (CDCA) and (b) ursodeoxycholic acid (UDCA). UDCA is a 7,-hydroxy epimer of the primary bile acid CDCA. Because of the different planar orientation of the 7-hydroxy group, UDCA is more hydrophilic than CDCA.

It has been claimed that UDCA reduces the risk of biliary pain, regardless of gallstone dissolution.[4] The proposed underlying mechanisms for this effect are impaired gallbladder motility with increased fasting and residual postprandial gallbladder volumes,[5] less cholesterol crystals,[6] or decreased mucin contents in bile[7] upon UDCA treatment. We analyzed the effect of UDCA (600 mg/dL) in a cohort of 527 uncomplicated gallstone patients who were followed for up to 18 years.[8] UDCA therapy was associated with a reduced risk of biliary pain in both symptomatic (62% *vs* 92% in untreated patients at 10 years, $P < 0.001$, relative risk: 0.19; 95% Cumulative Index (CI), 0.10–0.34) and asymptomatic patients (6% *vs* 12% in untreated patients at 10 years, $P = 0.037$, relative risk: 0.19; 95% CI, 0.04–0.91). The risk for conversion to cholecystectomy due to frequent attacks or cholecystitis was also reduced in UDCA-treated symptomatic patients (26% *vs* 88% in untreated patients at 10 years, $P < 0.001$, relative risk: 0.08; 95% CI, 0.03–0.22). These effects

Table 1 Current clinical use of ursodeoxycholic acid

Gallstone dissolution[1]
Prevention of biliary pain[8]
Adult cholestatic liver diseases
Primary biliary cirrhosis[11]
Primary sclerosing cholangitis[77]
Intrahepatic cholestasis of pregnancy[78]
Pediatric cholestatic liver diseases
Cystic fibrosis[79]
Progressive familial intrahepatic cholestasis[80]
Drug-induced cholestasis
Chronic viral hepatitis (ameliorate the elevation of alanine aminotransferase levels; no effect upon viral load)[31]

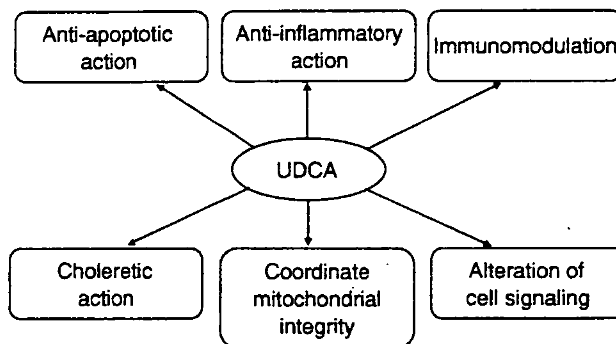

Figure 2 Putative mechanism of action of ursodeoxycholic acid (UDCA). UDCA exerts its action(s) in the liver through multiple mechanisms that are possibly interrelated with each other. Despite frequent novel discoveries of the molecular functions of bile acids, clear evidence for the mechanism of UDCA has yet to emerge.

were independent of stone dissolution. Although a recent placebo-controlled study suggested that UDCA does not reduce symptoms in highly symptomatic patients,[9] it still appears worthwhile to consider UDCA for patients with mild to moderate symptomatic gallstones.

Besides the evaluation of UDCA as a gallstone-dissolving agent, the beneficial effects of UDCA in patients with hepatic disorders were reported in the 1980s. UDCA improves clinical and biochemical serum parameters in a variety of cholestatic diseases,[10] and is now considered as the first-line treatment for patients with chronic cholestatic liver diseases, such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), intrahepatic cholestasis of pregnancy (ICP), and several less common adult and pediatric cholestatic conditions. For PBC, UDCA is the only drug approved by the Food and Drug Administration (FDA) for the treatment of the disease before liver transplantation. A combined analysis of the three largest randomized clinical trials of UDCA for PBC indicated that UDCA improves survival without liver transplantation.[11] However, the efficacy of UDCA treatment has been debated[12,13] and the mechanisms of action in humans are still not defined,[10] even 70 years after its initial isolation by a Japanese scientist. The suggested mechanisms of UDCA include improved bile acid transport and detoxification, cytoprotection, and anti-apoptotic effects (Fig. 2). In the present review, we focus on the unique character of UDCA by discussing what is known about these mechanisms, and we suggest possible novel clinical applications of UDCA.

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PROPOSED MOLECULAR MECHANISMS FOR THE ACTION OF UDCA

Choleretic effect of UDCA

IN PATIENTS WITH cholestasis, hydrophobic bile acids accumulate in hepatocytes and can cause cell damage, apoptosis, and necrosis. In experimental cholestasis, UDCA stimulates biliary secretion of bile acids, bilirubin glucuronides, glutathione conjugates, and other organic anions. In the rodent liver, UDCA counteracts cholestasis induced by hydrophobic bile acids by transcriptional and post-transcriptional mechanisms.[14] In patients with PBC and primary PSC, UDCA stimulates biliary secretion of bile acids,[15] and during long-term treatment, decreases the elevated serum levels of bilirubin and CDCA.[10] Therefore, UDCA might exert beneficial effects in cholestatic disease; in part, by stimulating the elimination of toxic compounds from hepatocytes. The secretion capacity of hepatocytes is determined by the number and activity of transporter proteins in the basolateral membrane, and crucially, the canalicular membrane. The expression of transporter proteins is regulated at the transcriptional and post-transcriptional levels,[14] since UDCA stimulates the expression of transporters required for biliary secretion in the liver of humans[16,17] and in experimental models[17,18] (Fig. 3), and stimulates the targeting and insertion of transporters into the hepatocyte canalicular membrane in experimental animal models[19–21] and isolated cells.[22]

In non-cholestatic humans given UDCA before undergoing wedge liver biopsy during cholecystectomy, the levels of mRNA encoding numerous transporter proteins, including ABCB11 (BSEP), ABCC2 (MRP2), ABCC3 (MRP3), ABCC4 (MRP4), and ABCB4 (MDR3), remained unaffected, whereas the levels of the Bile Salt Export Pump (BSEP), multidrug resistance-associated protein (MDR3), and multidrug resistance (MRP4) proteins were elevated,[17] suggesting that the regulation of the protein expression by UDCA is post-transcriptional. In the cholestatic rat liver, tauroursodeoxycholic acid (TUDCA) significantly increases the amount of transporter proteins Mrp2 and Bsep, which are present in the hepatocyte canalicular membrane and thereby stimulate biliary excretion of potentially toxic compounds.[19,21] Experimental evidence indicates that TUDCA activates a complex network of signals, which in turn stimulate hepatobiliary vesicular exocytosis and the insertion of transporter proteins into the canalicular membrane of hepatocytes.[10,23] The signals modulated by UDCA include those regulating intracellular Ca^{2+}, protein

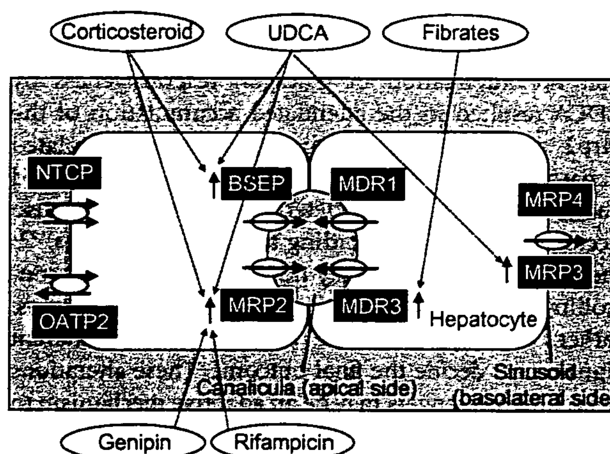

Figure 3 Effect of ursodeoxycholic acid (UDCA) on hepatic transporter expression. UDCA stimulates canalicular Mrp2 and Bsep expression, but also induces basolateral Mrp3, which facilitates alterative efflux of bile acids and other organic anions into the systemic circulation in mice models.[76] In humans, elevated levels of BSEP, MDR3, and MRP4 by UDCA administration was reported.[17] Proposed action point of other drugs used treat cholestatic liver diseases (steroids, fibrates, genipin, and rifampicin) are also demonstrated.

kinase C (PKC) isoforms, and the cAMP level; these second messengers are thought to modulate the trafficking and insertion of transporters in hepatocytes. TUDCA selectively induces translocation of conventional (c)PKC-α, the calcium-sensitive isoform of PKC, a mediator of regulated exocytosis, to hepatocellular membranes and activates membrane-bound PKC; thus, it is speculated that TUDCA enhances the secretion of bile acids in part through a Ca^{2+}-dependent and PKC-dependent mechanism. However, different PKC isoforms have now been shown to be involved in TUDCA-induced Bsep targeting to the canalicular domain in HepG2 cells transfected with the sodium-dependent bile acid transport protein Ntcp.[22]

Cyclic AMP is also reported to be a stimulator of the sorting of Mrp2 protein to the apical domain of hepatocytes.[24] In addition, cAMP stimulates the translocation of Ntcp to the plasma membrane through the activation of the protein kinase B signaling pathway.[25] Bile acids, including UDCA, directly inhibit glucagon-induced cAMP formation through a PKC-dependent mechanism,[26] and we have demonstrated that ligation of the bile duct decreases the efficacy of glucagon-stimulated cAMP synthesis by 40–50% without changing its potency in the Syrian hamster.[27] This decreased hormone responsiveness to bile acid is caused, at least

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in part, by the desensitization of the glucagon receptor through PKC-induced phosphorylation.[28] We confirmed that the attenuation of glucagon responsiveness by CDCA resulted in the attenuated accumulation of the Mrp2 protein in the canalicular domain of isolated hepatocyte couplets (Ikegami T., 2003, unpublished data), but it is yet to be determined whether this also occurs with UDCA. According to the above reports,[19,21] the alteration in cAMP may not be the only factor controlling the behavior of hepatic transporter proteins; rather, a complex balance between several different signals may decide the final outcome. These alterations in second messengers may be an adaptive mechanism to block the additional uptake of bile acids and to enhance the secretion of potentially toxic intracellular bile acids. However, the exact effects on signaling pathways remain unknown when UDCA is administered to humans.

Cytoprotection and anti-apoptotic action of UDCA

The amphipathicity of bile acids contributes to lipid solubilization, but a higher concentration of bile acids beyond the physiological level often results in disruption of the plasma membrane phospholipid bilayer. In cholestatic diseases, the potent cytotoxic effects of bile acids on hepatocytes and biliary epithelial cells have been discussed. Individual bile acids differ in hydrophobicity.[29] LCA is the most hydrophobic among the major human bile acids, and UDCA is the most hydrophilic. Thus, the composition of bile acids may affect the pathophysiological condition. For instance, an increased concentration of CDCA in the intestinal lumen causes intestinal epithelial cell injury, which often results in the appearance of diarrhea. Partly for this reason, the clinical use of CDCA was abandoned approximately two decades ago. In contrast, UDCA is thought to have a less cytotoxic profile, probably due to its relatively hydrophilic character. Thus, the replacement of hydrophobic bile acids with UDCA may attenuate the damage to hepatocytes and biliary cells, and currently UDCA is administered to patients with chronic viral hepatitis to ameliorate biochemical changes, with the importance of the replacement efficiency of UDCA suggested in this setting. In a multicenter trial in 57 patients with chronic hepatitis due to hepatitis C virus infection (CH-C), Takano *et al.* determined the serum bile acid composition in UDCA-treated patients and found a dose-dependent increase of the UDCA fraction in the serum, a significant correlation between a decrease in serum alanine transferase (ALT), and a decrease in the hydrophobicity index of serum bile acids.[30] This finding was reconfirmed by a recent large-scale, multicenter, double-blinded trial.[31] In this study, the authors concluded that a UDCA dose of 600 mg/day was optimal to decrease serum ALT and aspartate aminotransferase levels in CH-C patients without serious adverse effects, although the long-term effects of UDCA administration on prognosis, hepatocarcinogenesis in particular, remain unclear.

Anti-apoptotic activity of UDCA has been suggested mainly from *in vitro* experiments. In contrast, hydrophobic bile acids have apoptotic effects through the induction of cell death via ligand-independent, death receptor pathways involving the Fas receptor[32,33] and also through classic mitochondrial pathways of apoptosis.[34] Therefore, the final injurious effects of bile acids depend on the balance between insult and protection. There is strong evidence to suggest that the cytoprotective mechanism of UDCA and its conjugates results from the inhibition of apoptosis in hepatic cells by preventing mitochondrial depolarization and reducing the production of reactive oxygen species.[34] UDCA prevents apoptosis by modulating mitochondrial membrane perturbation, opening of the Permeability Transition Pore (PTP), Bax translocation, cytochrome *c* release, and subsequent caspase activation and poly(ADP-ribose) polymerase cleavage.[35] In addition, apoptosis can be inhibited not only by blocking pro-apoptotic pathways, but also by eliciting survival signals through the cAMP, Akt, Nuclear Factor (NF)-κB, mitogen-activated kinase (MAPK), and Phosphoinositide-3 kinase (PI3K)-mediated pathways.[36–38] Tauroursodeoxycholic acid (TUDCA) rapidly inhibits Glycoursodeoxycholic acid (GCDCA)-induced apoptosis in rat hepatocytes independently of the inhibition of caspase 8, the activation of NF-κB, and transcriptional mechanisms.[39] Recently, it has been suggested that the glucocorticoid receptor (GR) plays an important role in the nuclear translocation of UDCA for reducing apoptosis.[40] However, since the anti-apoptotic effects of UDCA have been demonstrated, mainly in experimental models, the relevance of hepatocyte apoptosis in cholestatic liver diseases and the importance of anti-apoptotic mechanisms in the beneficial effects of UDCA remain unclear.

Interestingly, UDCA may also have pro-apoptotic actions, based on the following observations: UDCA potentiates photodamage in leukemia cells;[41] UDCA does not protect against apoptosis induced by hydrophobic bile acids in several colonic cancer cell lines;[42] and UDCA induces apoptosis in hepatocytes when the MAPK and PI3K pathways are inhibited.[43] We have also reported that UDCA potentiates apoptosis induced by a

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DNA topoisomerase I inhibitor in colonic and hepatic cancer cell lines.[44] Therefore, UDCA may act differentially on death and survival pathways, depending on the cell type, physiological conditions, and/or stimulus. Thus, further clarification of UDCA action in terms of anti- or pro-apoptotic effects is required to foster a better understanding of the associated mechanisms and to widen the clinical usage of UDCA.

Immunomodulatory effects of UDCA

The GR is considered to be a potential target of UDCA in immune-mediated cholestatic liver diseases, such as PBC. The modulation of cell-mediated immunity by UDCA has been observed,[45] and UDCA has been shown to activate the GR in rat hepatocytes in a ligand-independent fashion, and to suppress interferon-γ-induced Major Histocompatibility Complex (MHC) class II expression in a GR-dependent fashion.[46] In contrast to the binding of dexamethasone (DEX), the binding of UDCA to the rat hepatocyte GR is non-specific.[47] We have also reported that the expression level of type IIA phospholipase A_2 (PLA_2IIA) is downregulated by UDCA via ligand-independent GR activation.[48] Glucocorticoids are known to inhibit PLA_2IIA expression, and there is a putative binding site for the GR located in the −208 to −203 region of the human PLA_2IIA promoter. UDCA may interact with the GR in a different manner to that of the classic receptor–ligand interaction seen with DEX. Our findings that UDCA has an additive inhibitory effect on PLA_2IIA expression above that of DEX and that RU486 (a prototype GR antagonist) can reverse the effect of DEX, but not that of UDCA, are compatible with this hypothesis. Furthermore, the siRNA suppression of the GR levels suggests that the GR is required for the inhibitory action of UDCA.

These findings provide an explanation of the action of UDCA in PBC, which has been considered to be an autoimmune disease. However, further studies are needed to determine if the effects of UDCA on the GR are unique among physiological bile acids and if these effects are secondary to the choleretic effect of UDCA.

EMERGING CONCEPTS FOR UDCA USE

CURRENTLY, THE USE of UDCA in Japan is limited to the treatment of chronic cholestatic diseases (PBC, ICP, and cholestatic drug-induced liver injury), gallstone dissolution or prevention of colic pain, and chronic hepatitis under the regulation of the national health insurance system. However, several other possible applications have been suggested and two of these are described below.

Colon cancer chemoprevention and UDCA

An effect of UDCA on colorectal cancer (CRC) chemoprevention has been shown in preclinical studies.[49–54] UDCA inhibits the proliferation of colon cancer cell lines *in vitro*, and significantly decreases the size and number of colon tumors induced by N-methylnitrosourea[53,54] or azoxymethane[49–51] in rats. In this respect, UDCA has also been shown to be superior to piroxicam, an established chemopreventive agent for colon cancer.[49,50] The chemopreventive mechanism of UDCA in colon cancer has been associated with the reduction of DCA in stools, since DCA has been implicated in the pathogenesis of colorectal cancer through the disruption of the balance between colorectal crypt cell proliferation, differentiation, and apoptosis.[55,56] Secondary bile acids appear to modify intracellular signaling and gene expression,[52,57,58] and DCA in particular appears to stimulate signaling through at least two different pathways that regulate the activity of activator protein-1.[57] Thus, the reduction of fecal DCA in UDCA therapy observed in animal studies may explain the chemopreventive action of UDCA,[59–61] but contradictory results have been obtained in clinical studies. Furthermore, several reports claim that the biological activity of UDCA itself is opposite to that of DCA.[62,63] For instance, UDCA suppresses many of the signaling pathways activated by DCA, such as the MAPK pathway.[62,63] In addition, the modulation by UDCA of changes in PKC isoforms induced by carcinogens[64–66] and changes in arachidonic acid metabolism[51] have been discussed as potential chemopreventive mechanisms. Therefore, UDCA may prevent colon cancer development by the replacement of DCA and through its own molecular actions.

The chemopreventive role of UDCA in CRC has been examined in patients with ulcerative colitis (UC). UDCA treatment has been associated with decreased recurrence rates of colorectal adenomas in patients with a history of PBC after a median intervention period of 45.6 months,[67] and with a lower prevalence of colonic neoplasia in patients with UC and PSC after median treatment periods of 50.4[68] and 42 months,[69] respectively, in a small cohort study. In addition, a recent phase III trial demonstrated a statistically significant 39% reduction in the recurrence of adenomas with high-grade dysplasia ($P = 0.03$), but failed to show a significant difference in total colorectal adenoma

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recurrence between UDCA and placebo.[70] The authors concluded that UDCA may work at a later point of colorectal carcinogenesis because of its significant suppressive effect on the recurrence of highly dysplastic adenomas, rather than that of lower-risk adenomas.[70] Collectively, the chemopreventive action of UDCA in colon cancer carcinogenesis has been shown in several clinical studies, but further longer-term studies are required for the clarification of the appropriate population for UDCA supplementation.

Non-alcoholic steatohepatitis and UDCA

Non-alcoholic steatohepatitis (NASH) is a common liver disease that is estimated to affect nearly 1% of the population. The pathophysiology is not clearly understood, but insulin resistance and oxidative stress appear to be involved. Currently, there is no approved therapy for NASH. The treatment of associated conditions, such as obesity, diabetes mellitus, and hyperlipidemia is frequently attempted, but is seldom effective in reversing NASH.[71] UDCA may be a therapeutic option for NASH, because of its multiple hepatoprotective activities in patients with a wide range of chronic liver diseases. Following an open-label pilot study, a large-scale, placebo-controlled trial aimed at determining the efficacy of UDCA for NASH was conducted.[72] However, in this randomized study of 166 patients over 2 years, the administration of UDCA at a dose of 13–15 mg/kg/dL was not better than placebo.[72] A review published by the Cochrane Library in 2007 concluded that there are insufficient data to support or refute the use of UDCA for patients with NASH, since only one[72] of four randomized clinical trials identified was considered to be a low-bias risk trial, and no significant differences were found regarding mortality, improvement of liver function, and the radiological and histological response.

Recent studies have shown that bile acids are also signaling molecules, in addition to their roles in dietary lipid absorption and cholesterol homeostasis. Bile acids inhibit diet-induced obesity and prevent the development of insulin resistance,[73] with several different hypotheses proposed to explain these actions. The direct ability of bile acids to modify the gene expression associated with lipid homeostasis by activating the farnesoid X receptor-á (FXRα), a nuclear hormone receptor, may partly explain these effects.[74] Furthermore, Watanabe *et al.* recently showed that administration of CA to mice increases energy expenditure in brown adipose tissue, thereby preventing obesity and insulin resistance.[75] This effect of bile acids is dependent on the induction of the cAMP-dependent thyroid hormone activating enzyme type 2 iodothyronine deiodinase (D2), since it is lost in mice with targeted disruption of D2. In addition, the effect is independent of FXRα, and instead is mediated by increased cAMP production that stems from the binding of bile acids with TGR5. TGR5 is a novel G-Protein coupled receptor (GPCR) that responds to bile acids by inducing receptor internalization, activation of MAPK pathways, and cAMP production. Taken together, these results suggest that bile acid-controlled signaling pathways are promising targets in the treatment of common metabolic diseases, such as NASH or non-alcoholic fatty liver disease (NAFLD). However, the administration of bile acids, such as CA, that can act as ligands for these receptors may improve lipid catabolism, but also exert a cytotoxic effect. UDCA, a less cytotoxic bile acid, may be useful as an alternative option, but since UDCA has less specific binding to FXRα and TGR5, the modulation of these signaling pathways by UDCA may be relatively weak. The replacement of a more potent bile acid by UDCA may even attenuate the effect of the initial bile acid and result in disease progression. Clinical trials of UDCA in metabolic diseases have not shown a worsened outcome, but currently it appears that a beneficial effect of UDCA in patients with NAFLD or NASH is unlikely.

FUTURE PERSPECTIVES

A VARIETY OF studies have shown beneficial effects of UDCA in hepatobiliary disorders. In clinical practice, UDCA has a defined role in treating patients with cholestatic liver diseases. The safety of the clinical use of UDCA is widely accepted, although the efficacy of UDCA for several clinical conditions is still under debate. Despite frequent novel discoveries of the molecular functions of bile acids, clear evidence for the mechanism of UDCA has yet to emerge. However, further basic research into bile acids may provide this answer and suggest more efficient ways to use this classical compound as a new drug.

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