methods

Highly sensitive quantification of 7α-hydroxy-4-cholesten-3-one in human serum by LC-ESI-MS/MS

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Abstract **We describe a highly sensitive and specific method for the quantification of serum 7α-hydroxy-4-cholesten-3-one (C4), which has been used as a biomarker for bile acid biosynthesis. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). C4 was extracted from human serum (2–50 μl) by a salting-out procedure, derivatized into the picolinoyl ester (C4-7α-picolinate), and then purified using a disposable C_{18} cartridge. The resulting picolinoyl ester derivative of C4 was quantified by LC-MS/MS using the electrospray ionization mode. The detection limit of the C4 picolinoyl ester was found to be 100 fg (signal-to-noise ratio = 10), which was ~1,000 times more sensitive than the detection limit of C4 with a conventional HPLC-ultraviolet method. The relative standard deviations between sample preparations and between measurements by our method were calculated to be 5.7% and 3.9%, respectively, by one-way layout analysis. The recovery experiments were performed using serum spiked with 20.0–60.0 ng/ml C4 and were validated by a polynomial equation. The results showed that the estimated concentration with 95% confidence limit was 23.1 ± 2.8 ng/ml, which coincided completely with the observed $\overline{X}_0 \pm SD = 23.3 \pm 1.0$ ng/ml with a mean recovery of 93.4%. This method provides highly reliable and reproducible results for the quantification of C4, especially in small volumes of blood samples.**—Honda, A., K. Yamashita, M. Numazawa, T. Ikegami, M. Doy, Y. Matsuzaki, and H. Miyazaki. **Highly sensitive quantification of 7α-hydroxy-4-cholesten-3-one in human serum by LC-ESI-MS/MS.** *J. Lipid Res.* **2007.** 48: 458–464.

Supplementary key words bile acid synthesis • cholesterol 7α-hydroxylase • picolinic acid • liquid chromatography electrospray ionization-tandem mass spectrometry

The conversion of cholesterol to bile acids is a major reaction for the catabolism of cholesterol in the body (1). Cholesterol 7α-hydroxylase (CYP7A1) is the rate-limiting enzyme in one of the pathways of bile acid biosynthesis (2). Bile acid synthesis by this classic pathway accounts for <50% of total bile acid production in rats (3) and mice (4), whereas it represents a major route synthesizing >90% of total bile acids in humans (5). Therefore, the measurement of CYP7A1 activity is clinically useful for exploring the mechanisms of hypercholesterolemia (6) and evaluating the effects of hypocholesterolemic treatments (7, 8).

Because CYP7A1 is expressed solely in the liver, an invasive liver biopsy is necessary to measure the activity of this enzyme. However, a breakthrough of this problem was achieved in 1987 by Björkhem et al. (9). They quantified the plasma concentrations of 7α-hydroxycholesterol, the immediate product of CYP7A1, by gas chromatography-mass spectrometry with selected ion monitoring (GC-SIM) and reported that this measurement reflected hepatic CYP7A1 activities in humans.

In 1988, Axelson, Aly, and Sjövall (10) determined the plasma concentrations of 7α-hydroxy-4-cholesten-3-one (C4), a product of the next oxidative enzymatic reaction after CYP7A1, using HPLC with ultraviolet (UV) detection and found out that C4 is also a good marker for CYP7A1 activity in humans (11). It was subsequently reported that serum concentrations of 7α-hydroxycholesterol (12) and C4 (13) reflected not only CYP7A1 activities but also bile acid synthesis in humans.

We also developed new high-resolution GC-SIM methods for the quantification of these sterols in plasma. We found that there was a significant correlation between

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Abbreviations: C4, 7α-hydroxy-4-cholesten-3-one; CYP7A1, cholesterol 7α-hydroxylase; ESI, electrospray ionization; GC-SIM, gas chromatography-mass spectrometry with selected ion monitoring; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SRM, selected reaction monitoring; UV, ultraviolet.

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hepatic CYP7A1 activities and plasma levels of free but not esterified or total 7α-hydroxycholesterol (14) and that CYP7A1 activities correlated better with plasma levels of C4 than those of free 7α-hydroxycholesterol (15). In addition, we applied these methods to monitor the circadian rhythms of these sterol levels in human plasma. A zero-amplitude test (15) revealed that C4 was the most reliable plasma biomarker for hepatic CYP7A1 activity. Furthermore, our recent study demonstrated that plasma C4 relative to cholesterol is a better marker for hepatic CYP7A1 activity than the absolute concentration when hypercholesterolemia is present (16).

The aim of this study was to develop a simple, more sensitive and reliable method to quantify C4 in a small volume of human serum. For this purpose, we derivatized C4 into the picolinoyl ester (C4-7α-picolinate) and determined the concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled in electrospray ionization (ESI) mode.

MATERIALS AND METHODS

Chemicals

C4 and 7β-hydroxycholesterol were purchased from Steraloids (Wilton, NH). [25,26,26,26,27,27,27-$^2H_7$]7α-hydroxycholesterol was prepared as described previously (17). [$^2H_7$]C4 and 7β-hydroxy-4-cholesten-3-one were synthesized from [$^2H_7$]7α-hydroxycholesterol and 7β-hydroxycholesterol, respectively, with cholesterol oxidase (18). Picolinic acid and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and 4-dimethylaminopyridine and triethylamine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Additional reagents and solvents were of analytical grade.

Sample collection

Blood samples were collected from healthy human volunteers. After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration.

Sample preparation

C4 was extracted from serum by the salting-out method described previously (15). Fifty microliters of serum was diluted with 200 μl of distilled water in a microcentrifuge tube (1.5 ml; Eppendorf, Hamburg, Germany), and 1 ng of [$^2H_7$]C4 in 500 μl of acetonitrile was added as an internal standard. After the addition of ~100 mg of ammonium sulfate, the sample tube was vortexed for 1 min and centrifuged at 2,000 *g* for 5 min. The supernatant acetonitrile phase was collected and evaporated to dryness under nitrogen.

Derivatization to the picolinoyl ester was performed according to the method of Yamashita et al. (19) with minor modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), and tetrahydrofuran (1.5 ml). The freshly prepared reagent mixture (150 μl) and triethylamine (20 μl) were added to the extract, and the reaction mixture was allowed to stand at room temperature for 30 min. After the addition of 0.5 ml of 5% sodium bicarbonate, the mixture was applied to a Bond Elut C_{18} cartridge (100 mg; Varian, Harbor City, CA) preconditioned with 1 ml of tetrahydrofuran, 1 ml of methanol, and 2 ml of distilled water. The cartridge was washed with 1 ml of 5% sodium bicarbonate, 1 ml of distilled water, 2 ml of 5% HCl, 1 ml of distilled water again, and 2 ml of acetonitrile-water (1:1, v/v). The picolinoyl ester derivative of C4 was then eluted with 4 ml of acetonitrile-water (95:5, v/v) containing 0.1% acetic acid. After evaporation, the residue was redissolved in 50 μl of the same solvent, and an aliquot (1 μl) was injected into the LC-MS/MS system.

LC-MS/MS analysis

The LC-MS/MS system consisted of a TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a Hypersil GOLD column (150 × 2.1 mm, 3 μm; Thermo Fisher Scientific) at 40°C. The mobile phase consisted of acetonitrile-water (95:5, v/v) containing 0.1% acetic acid and was used at a flow rate of 200 μl/min. The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 35 p.s.i.; auxiliary gas (nitrogen) flow, 45 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; collision energy, 16 V; and ion polarity, positive.

Evaluation of the solid-phase purification process

The absolute recovery of C4-7α-picolinate from the Bond Elut C_{18} cartridge was determined by the addition of [$^2H_7$]C4-7α-picolinate (1 ng) to the cartridge with a derivatizing mixture, which was prepared from 50 μl of serum without adding [$^2H_7$]C4. After the purification steps, the picolinoyl ester of 7β-hydroxy-4-cholesten-3-one (1 ng) was added to the eluate as an internal standard, and both [$^2H_7$]C4-7α-picolinate and 7β-hydroxy-4-cholesten-3-one-7β-picolinate were quantified by LC-selected reaction monitoring (SRM).

To calculate the elimination rate of cholesterol by the Bond Elut C_{18} cartridge, unesterified cholesterol concentration in the first salting-out extraction was measured by colorimetric enzyme assay. The extract was then derivatized and purified by the cartridge, and the picolinoyl ester of [$^2H_7$]cholesterol (100 ng) was added to the final eluate as an internal standard. Cholesterol-3-picolinate and its $^2H_7$ variant were quantified by the LC-SIM method to measure the absolute amount of cholesterol-3-picolinate contaminant in the final eluate.

Statistics

Data are reported as means ± SD. Reproducibility was analyzed by one-way layout (JMP software; SAS Institute, Inc., Cary, NC). Recovery was analyzed using a polynomial equation (20). Linearity of the calibration curve and correlation between serum volume and the amount of C4 were analyzed by simple linear regression. The regression analysis was also used to calculate the estimated amount ± 95% confidence limit in the recovery study. For all analyses, significance was accepted at $P < 0.05$.

RESULTS

SRM

A typical ESI positive mass spectrum of the C4-7α-picolinate is shown in **Fig. 1A**. This picolinoyl ester deriv-

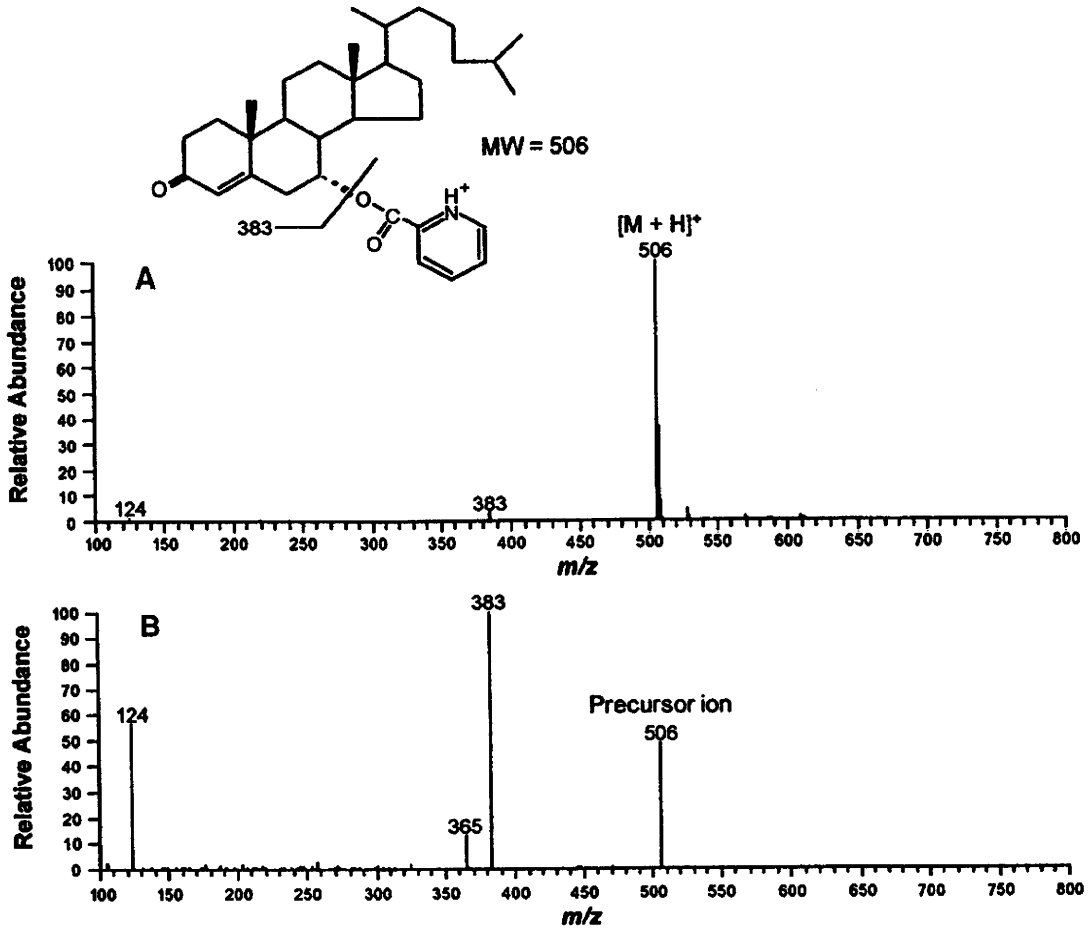

Fig. 1. Typical electrospray ionization (ESI) positive mass spectrum (A) and product ion mass spectrum using *m/z* 506 as a precursor ion (B) of the picolinoyl ester derivative of 7α-hydroxy-4-cholesten-3-one (C4). Mass spectrometric conditions were as follows: introducing solvent, acetonitrile-water (95:5, v/v) containing 0.1% acetic acid; flow rate, 200 μl/min; spray voltage, 1,000 V; collision energy, 16 V.

ative exhibited an $[M+H]^+$ ion at *m/z* 506 as the base peak. In the MS/MS spectrum, the $[M-C_6H_5O_2N]^+$ ion was observed at *m/z* 383 as the most prominent peak, as shown in Fig. 1B. The SRM was conducted using *m/z* 506 → *m/z* 383 for the C4-7α-picolinate and *m/z* 513 → *m/z* 390 for the $^2H_7$ variant.

Sensitivity of the present method

To determine the sensitivity of our LC-SRM method, the standard C4-7α-picolinate solution was diluted and injected into the LC-MS/MS system. As shown in **Fig. 2A**, the C4-7α-picolinate was easily detected to 100 fg, with a signal-to-noise ratio of 10, whereas conventional UV detection at 241 nm was barely able to detect 100 pg of C4 (Fig. 2B).

Absolute recovery of C4 and elimination rate of cholesterol in the solid-phase purification process

In this absolute recovery, $[^2H_7]$C4-7α-picolinate was used instead of the corresponding C4 derivative and was quantified by SRM using the picolinoyl ester of 7β-hydroxy-4-cholesten-3-one as an internal standard. Both picolinoyl esters of C4 and 7β-hydroxy-4-cholesten-3-one exhibited similar mass spectra, but the retention times were 4.66 and 5.00 min, respectively. Thus, the recovery of $[^2H_7]$C4-7α-picolinate was 104.0 ± 2.3% (n = 4).

The elimination rate of cholesterol by the Bond Elut C_{18} cartridge was calculated by quantifying cholesterol-3-picolinate contamination in the final eluate. The multiple-ion detector was focused on *m/z* 492 for cholesterol-3-picolinate and *m/z* 499 for $[^2H_7]$cholesterol-3-picolinate. Under the same HPLC conditions as for the determination of C4, the retention time of cholesterol-3-picolinate was ~11 min. The elimination rate of cholesterol by the cartridge was calculated as 98.6 ± 0.7% (n = 4).

Calibration curve

A calibration curve was established for C4 (**Fig. 3**). Different amounts (0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, and 4 ng) of authentic C4 were mixed with 1 ng of $[^2H_7]$C4, derivatized to the picolinoyl ester, and purified by the Bond Elut C_{18} cartridge, as described in Materials and Methods. The weight ratio of C4, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak area ratio of the C4-7α-picolinate to the $^2H_7$ variant, as measured by LC-SRM, was plotted

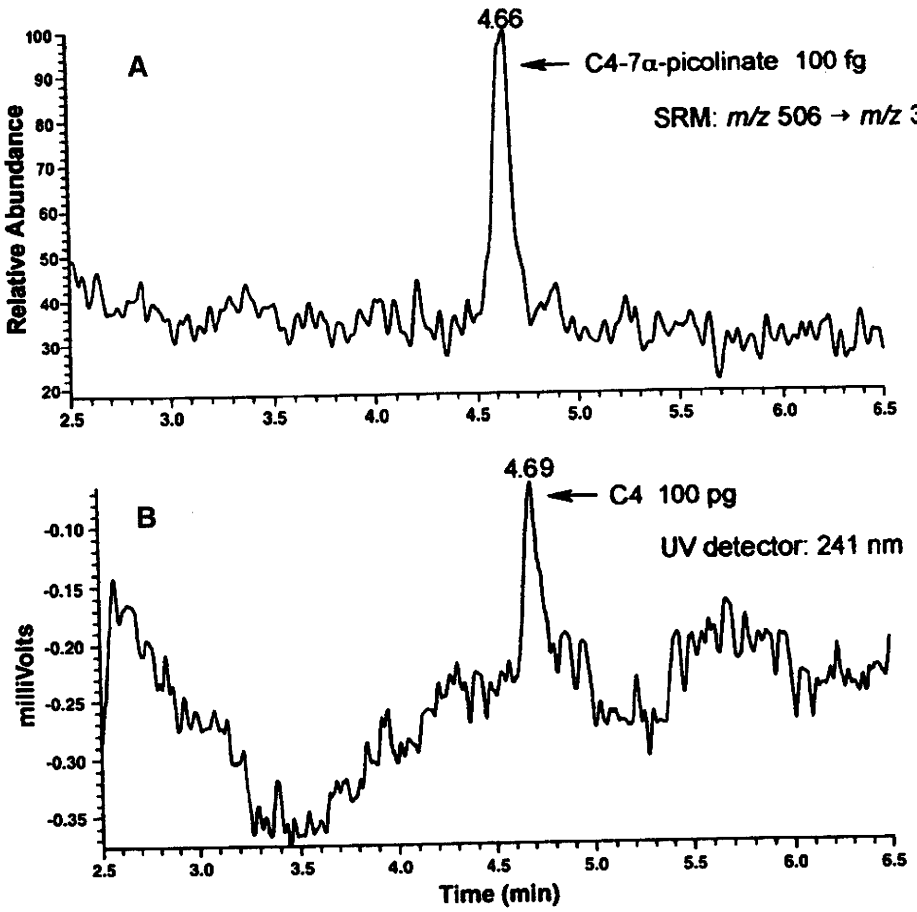

Fig. 2. Comparison of the detection limit of C4-7α-picolinate by selected reaction monitoring (SRM) at *m/z* 506 → *m/z* 383 (A) with that of C4 by ultraviolet (UV) detection at 241 nm (B). Authentic standard of C4-7α-picolinate (100 fg) or C4 (100 pg) was injected into the HPLC system.

on the ordinate. The linearity of the standard curve, as determined by simple linear regression, was excellent for weight ratios between 0.01 and 4.0 (n = 8; $r = 1.000$; $P < 0.0001$).

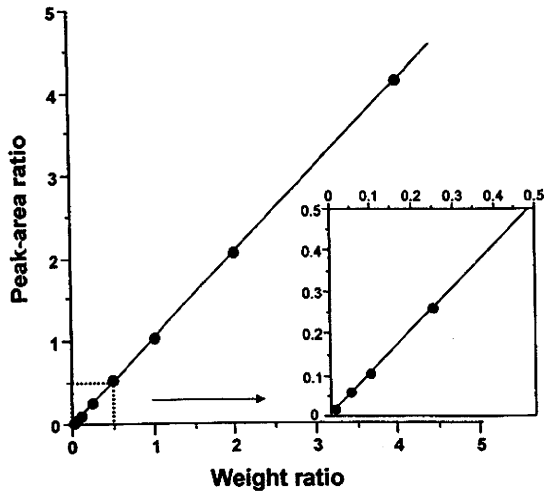

Fig. 3. Calibration curve for the weight ratio of C4 to the corresponding deuterated internal standard. Linearity was checked by simple linear regression, and the equation for the line of best fit was y = 1.035x + 0.008 (n = 8; $r = 1.000$; $P < 0.0001$).

Representative LC-SRM

Figure 4 shows typical LC-SRM chromatograms of C4-7α-picolinate and the $^2H_7$ variant obtained from 50 μl of normal human serum. Both peaks corresponded to ~20 pg. The peak appearing at 5.46 min on this chromatogram (Fig. 4A) was identified as the picolinoyl ester derivative of 7-oxo-cholesterol by comparison with authentic 7-oxo-cholesterol-3-picolinate.

Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of our method using the same serum obtained from a normal human subject. Reproducibility was investigated by analyzing four samples in triplicate by LC-SRM (**Tables 1, 2**). The results were analyzed by one-way layout, in which the analytical errors were divided into two sources of sample preparation and LC-SRM measurement. The variances were considered to be attributable to the measurement, because the errors during sample preparation were negligible. The interassay coefficients of variation for the between- and within-sample variations were 5.7% and 3.9%, respectively.

For the recovery experiment (**Table 3**), known amounts of C4 (1.0–3.0 ng, 20.0–60.0 ng/ml) were spiked into 50 μl aliquots of the serum samples. After the clean-up

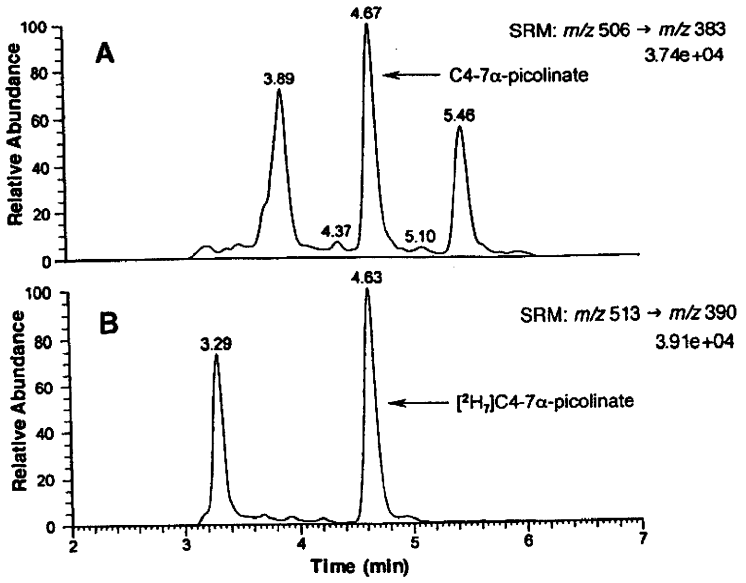

Fig. 4. Representative LC-SRM chromatograms of C4-7α-picolinate (A) and [$^2H_7$]C4-7α-picolinate (B) obtained from 50 μl of normal human serum. The peak of C4-7α-picolinate corresponds to ~20 pg (20 ng/ml).

procedure, LC-SRM was carried out in triplicate for each sample. The recoveries of the known spiked amounts of C4 ranged from 91.9% to 94.3%, with a mean of 93.4%. In addition, the amount of endogenous C4 found in unspiked 50 μl of serum was within the 95% confidence limit for the estimated amount of C4 calculated by linear regression analysis, which also constituted an index for the precision and accuracy of the method.

Application to a microscale assay

We studied the effect of reduced serum volume on the quantification of C4 amount. Different volumes (2, 5, 10, 25, and 50 μl) of normal human serum were diluted to 250 μl with distilled water, and extraction and quantification of C4 were conducted, as described in Materials and Methods. As shown in **Fig. 5**, proportionality was observed when the volume of serum ranged from 2 μl to at least 50 μl for the amount of C4.

DISCUSSION

HPLC with UV detection has been the most commonly used method for the determination of serum C4 concentrations (10, 11, 13, 21, 22). In HPLC-UV methods, a great deal of effort has been made to find appropriate internal standards and to develop superior clean-up procedures. In the latest method by Gälman et al. (22), 7β-hydroxy-4-cholesten-3-one was added as an internal standard, and a commercially available solid-phase extraction column (C_8) was used at a temperature of 64°C. One weak point of this method is that the sensitivity was not sufficient to quantify C4 in limited amounts of human serum. At least 1 ml of serum was needed for each assay, which is a considerably large volume for clinical blood chemistry tests.

We previously developed a more sensitive and specific method based on a stable isotope dilution technique using high-resolution GC-SIM (15). In this method, C4 was extracted from 200 μl of human plasma by a salting-out extraction, purified by a commercially available solid-phase extraction column (C_{18} and unbonded silica) at room temperature, and converted to the methyloxime-dimethylethylsilyl ether derivative before GC-SIM analysis. However, the sensitivity of this method was still not sufficient for the quantification of C4 in limited amounts of human serum, because the resulting derivatives exhibited two peaks of *syn* and *anti* isomers and their fragmentation patterns were not simple.

LC-MS or LC-MS/MS has come to be used more readily than GC-MS, because these methods do not always require

TABLE 1. Reproducibility in the quantification of C4 in human serum: analytical data

Sample	Individual Values			Mean ± SD
		ng/ml		
A	24.9	24.0	23.5	24.1 ± 0.7
B	22.7	23.5	24.7	23.6 ± 1.0
C	23.2	23.6	21.6	22.8 ± 1.1
D	21.7	22.4	23.3	22.5 ± 0.8
Mean ± SD				23.3 ± 1.0

C4, 7α-hydroxy-4-cholesten-3-one. C4 was quantified in 50 μl of normal human serum.

TABLE 2. Reproducibility in the quantification of C4 in human serum: ANOVA

Source	*S*	*f*	*V*	F_0	Relative SD
					%
Sample preparation	5.23	3	1.74	2.08	5.7
Error (selected reaction monitoring)	6.72	8	0.84		3.9
Total	11.95	11			
			$F(3,8,0.05)$ = 4.07		

S, residual sum of squares; *f*, number of degrees of freedom; f_1, $f_{\text{sample preparation}}$; f_2, f_{error}; *V*, unbiased variance; F_0, observed value following *F* distribution variance ratio ($V_{\text{sample preparation}}/V_{\text{error}}$); $F(f_1, f_2, \alpha)$, density function of *F* distribution with f_1 and f_2 degrees of freedom.

TABLE 3. Recovery of C4 from human serum

Sample (X_0 + na) (n = 0,1,2,3)	Amount Added	Amount Found			Recovery[a] (mean ± SD)	Estimated Amount ±95% Confidence Limit[b]
		ng/ml			%	*ng/ml*
X_0	0	$\bar{X}_0$ ± SD = 23.3 ± 0.1[c]				23.1 ± 2.8
X_0 + a	20.0	43.1	44.2	43.4		
X_0 + a	20.0	41.0	40.1	40.9	94.3 ± 8.3	
X_0 + 2a	40.0	60.6	59.1	62.6		
X_0 + 2a	40.0	62.5	58.3	57.0	91.9 ± 5.7	
X_0 + 3a	60.0	82.7	78.1	76.6		
X_0 + 3a	60.0	81.1	78.1	81.3	94.0 ± 4.0	

Known amounts of C4 were spiked into 50 μl of normal human serum before sample preparation.
[a]Recovery (%) = (amount found − $\bar{X}_0$)/amount added × 100.
[b]The estimated amount was calculated by linear regression analysis.
[c]This value was obtained from Table 1.

a derivatization step. However, it is also true that the introduction of charged moieties enhances the ionization efficiency of neutral steroids in ESI and atmospheric pressure chemical ionization processes. A permanently charged *N*-methylpyridyl group was introduced into the hydroxyl group of cholesterol (23) and 5α-dihydrotestosterone (24, 25), whereas a permanently charged quaternary pyridinium moiety was introduced to the carbonyl group of oxosteroids with Girard P reagent (26–28) or 2-hydrazino-1-methylpyridine (29). Very recently, Yamashita et al. (19) developed a new picolinoyl derivatization that is a simple and versatile method suitable for the sensitive and specific quantification of hydroxysteroids by positive ESI-LC-MS/MS. Interestingly, because the picolinoyl group is not charged permanently, even in the case of estradiol with two hydroxyl groups, a single charged ion was observed in the positive ESI mass spectrum. Therefore, picolinoyl ester may be a suitable derivative not only for monohydroxy steroids but also for hydroxycholesterols with plural hydroxyl groups.

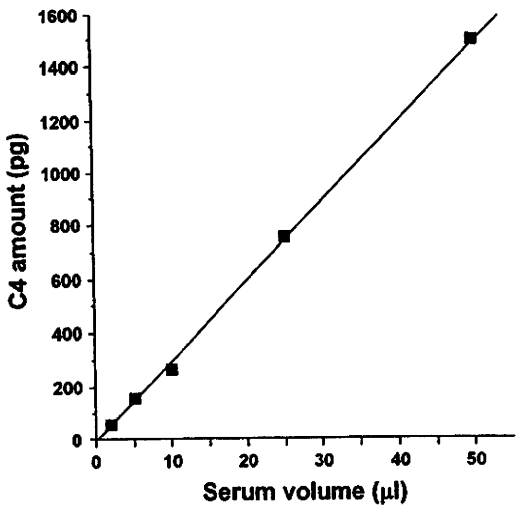

Fig. 5. Effects of reduced serum volume on quantification of the amount of C4. Linearity was checked by simple linear regression, and the equation for the line of best fit was y = 30.23x − 7.83 (n = 5; r = 0.999; $P < 0.0001$).

Then, we applied this derivatization to quantify C4 in a very small volume of human serum by LC-SRM. The picolinoyl ester of C4 exhibited $[M+H]^+$ as a single ion in ESI mode. The detection limit of C4 (as the C4 picolinoyl ester) in SRM mode was 100 fg, which was ~1,000 and 30 times more sensitive than that by the HPLC-UV and high-resolution GC-SIM methods, respectively. The detection limit of 100 fg corresponds to 0.1 ng/ml in 50 μl of serum, whereas the range of serum C4 concentrations has been reported to be 3–40 ng/ml (median, 12 ng/ml) in healthy subjects (10). Consequently, even when a sufficient volume of serum was not available, C4 concentrations could be determined by our method using a few microliters of serum. However, we recommend that 50 μl of serum be used for routine assay because some subjects treated with chenodeoxycholic acid (11) and patients with CYP7A1 deficiency (6) appear to exhibit unusually low serum concentrations of C4.

The derivatization and purification steps in this method are very simple. After the previously described salting-out extraction procedure (15), hydroxysteroids, including C4 and cholesterol, were derivatized to the picolinoyl esters within 30 min at room temperature. The C4-7α-picolinate was completely recovered from a commercially available C_{18} column with >98% elimination of cholesterol-3-picolinate. However, the amount of cholesterol-3-picolinate contaminant in the final eluate was still 300 times greater than that of C4-7α-picolinate. Therefore, the next injection into the LC-MS/MS system was not performed until cholesterol-3-picolinate had eluted completely from the column (~12 min), whereas the retention time of C4-7α-picolinate was ~4.7 min.

$[^2H_7]$C4 was added to serum as an ideal internal standard for quantification by SRM. Although the object of our study was to quantify a very small amount of C4, the specificity and reproducibility of this method were highly satisfactory.

In summary, a very sensitive and specific method for the quantification of C4 in human serum was developed. Derivatization of C4 into the picolinoyl ester made it possible to be quantified by LC-ESI-MS/MS with excellent sensitivity. Recovery and reproducibility experiments verified that this method provided analytical results with high reliability and reproducibility.

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REFERENCES

1. Everson, G. T. 1992. Bile acid metabolism and its role in human cholesterol balance. *Semin. Liver Dis.* **12:** 420–428.
2. Vlahcevic, Z. R., W. M. Pandak, D. M. Heuman, and P. B. Hylemon. 1992. Function and regulation of hydroxylases involved in the bile acid biosynthesis pathways. *Semin. Liver Dis.* **12:** 403–419.
3. Vlahcevic, Z. R., R. T. Stravitz, D. M. Heuman, P. B. Hylemon, and W. M. Pandak. 1997. Quantitative estimations of the contribution of different bile acid pathways to total bile acid synthesis in the rat. *Gastroenterology.* **113:** 1949–1957.
4. Schwarz, M., E. G. Lund, K. D. R. Setchell, H. J. Kayden, J. E. Zerwekh, I. Björkhem, J. Herz, and D. W. Russell. 1996. Disruption of cholesterol 7α-hydroxylase gene in mice. *J. Biol. Chem.* **271:** 18024–18031.
5. Duane, W. C., and N. B. Javitt. 1999. 27-Hydroxycholesterol: production rates in normal human subjects. *J. Lipid Res.* **40:** 1194–1199.
6. Pullinger, C. R., C. Eng, G. Salen, S. Shefer, A. K. Batta, S. K. Erickson, A. Verhagen, C. R. Rivera, S. J. Mulvihill, M. J. Malloy, et al. 2002. Human cholesterol 7α-hydroxylase (CYP7A1) deficiency has a hypercholesterolemic phenotype. *J. Clin. Invest.* **110:** 109–117.
7. Root, C., C. D. Smith, S. S. Sundseth, H. M. Pink, J. G. Wilson, and M. C. Lewis. 2002. Ileal bile acid transporter inhibition, CYP7A1 induction, and antilipemic action of 264W94. *J. Lipid Res.* **43:** 1320–1330.
8. Urizar, N. L., A. B. Liverman, D. T. Dodds, F. V. Silva, P. Ordentlich, Y. Yan, F. J. Gonzalez, R. A. Heyman, D. J. Mangelsdorf, and D. D. Moore. 2002. A natural product that lowers cholesterol as an antagonist ligand for FXR. *Science.* **296:** 1703–1706.
9. Björkhem, I., E. Reihnér, B. Angelin, S. Ewerth, J-E. Åkerlund, and K. Einarsson. 1987. On the possible use of the serum level of 7α-hydroxycholesterol as a marker for increased activity of the cholesterol 7α-hydroxylase in humans. *J. Lipid Res.* **28:** 889–894.
10. Axelson, M., A. Aly, and J. Sjövall. 1988. Levels of 7α-hydroxy-4-cholesten-3-one in plasma reflect rates of bile acid synthesis in man. *FEBS Lett.* **239:** 324–328.
11. Axelson, M., I. Björkhem, E. Reihnsér, and K. Einarsson. 1991. The plasma level of 7α-hydroxy-4-cholesten-3-one reflects the activity of hepatic cholesterol 7α-hydroxylase in man. *FEBS Lett.* **284:** 216–218.
12. Hahn, C., C. Reichel, and K. von Bergmann. 1995. Serum concentration of 7α-hydroxycholesterol as an indicator of bile acid synthesis in humans. *J. Lipid Res.* **36:** 2059–2066.
13. Sauter, G., F. Berr, U. Beuers, S. Fischer, and G. Paumgartner. 1996. Serum concentrations of 7α-hydroxy-4-cholesten-3-one reflect bile acid synthesis in humans. *Hepatology.* **24:** 123–126.
14. Yoshida, T., A. Honda, N. Tanaka, Y. Matsuzaki, B-F. He, T. Osuga, N. Kobayashi, K. Ozawa, and H. Miyazaki. 1993. Simultaneous determination of mevalonate and 7α-hydroxycholesterol in human plasma by gas chromatography-mass spectrometry as indices of cholesterol and bile acid biosynthesis. *J. Chromatogr.* **613:** 185–193.
15. Yoshida, T., A. Honda, N. Tanaka, Y. Matsuzaki, J. Shoda, B-F. He, T. Osuga, and H. Miyazaki. 1994. Determination of 7α-hydroxy-4-cholesten-3-one level in plasma using isotope-dilution mass spectrometry and monitoring its circadian rhythm in human as an index of bile acid biosynthesis. *J. Chromatogr.* **655:** 179–187.
16. Honda, A., T. Yoshida, G. Xu, Y. Matsuzaki, S. Fukushima, N. Tanaka, M. Doy, S. Shefer, and G. Salen. 2004. Significance of plasma 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol concentrations as markers for hepatic bile acid synthesis in cholesterol-fed rabbits. *Metabolism.* **53:** 42–48.
17. Honda, A., J. Shoda, N. Tanaka, Y. Matsuzaki, T. Osuga, N. Shigematsu, M. Tohma, and H. Miyazaki. 1991. Simultaneous assay of the activities of two key enzymes in cholesterol metabolism by gas chromatography-mass spectrometry. *J. Chromatogr.* **565:** 53–66.
18. Shimasue, A. 1974. The action of cholesterol:oxygen oxidoreductase on cholest-5-ene-3β,7α-diol and the enzymatic preparation of the labeled cholest-4-en-7α-ol-3-one. *Hiroshima J. Med. Sci.* **23:** 265–272.
19. Yamashita, K., S. Kobayashi, S. Tsukamoto, and M. Numazawa. 2006. Synthesis of pyridine-carboxylate derivatives of hydroxysteroids for liquid chromatography-electrospray ionization-mass spectrometry. *Steroids.* Epub ahead of print. November 30, 2006; doi: 10.1016/j. steroids.2006.10.005.
20. Taguchi, G. 1986. Introduction to Quality Engineering—Designing Quality into Products and Process. Asian Productivity Organization, Tokyo, Japan.
21. Pettersson, L., and C. G. Eriksson. 1994. Reversed-phase high-performance liquid chromatographic determination of 7α-hydroxy-4-cholesten-3-one in human serum. *J. Chromatogr.* **657:** 31–36.
22. Gälman, C., I. Arvidsson, B. Angelin, and M. Rudling. 2003. Monitoring hepatic cholesterol 7α-hydroxylase activity by assay of the stable bile acid intermediate 7α-hydroxy-4-cholesten-3-one in peripheral blood. *J. Lipid Res.* **44:** 859–865.
23. Quirke, J. M. E., C. L. Adams, and G. J. Van Berkel. 1994. Chemical derivatization for electrospray ionization mass spectrometry. I. Alkyl halides, alcohols, phenols, thiols, and amines. *Anal. Chem.* **66:** 1302–1315.
24. Nakagawa, Y., and Y. Hashimoto. 2002. Polar derivatization of 5α-dihydrotestosterone and sensitive analysis by semimicro-LC/ESI-MS. *J. Mass Spectrom. Soc. Jpn.* **50:** 330–336.
25. Nishiyama, T., Y. Hashimoto, and K. Takahashi. 2004. The influence of androgen deprivation therapy on dihydrotestosterone levels in the prostatic tissue of patients with prostate cancer. *Clin. Cancer Res.* **10:** 7121–7126.
26. Lai, C. C., C. H. Tsai, F. J. Tsai, C. C. Lee, and W. D. Lin. 2001. Rapid monitoring assay of congenital adrenal hyperplasia with microbore high-performance liquid chromatography/electrospray ionization tandem mass spectrometry from dried blood spots. *Rapid Commun. Mass Spectrom.* **15:** 2145–2151.
27. Griffiths, W. J., S. Liu, G. Alvelius, and J. Sjovall. 2003. Derivatisation for the characterisation of neutral oxosteroids by electrospray and matrix-assisted laser desorption/ionisation tandem mass spectrometry: the Girard P derivative. *Rapid Commun. Mass Spectrom.* **17:** 924–935.
28. Griffiths, W. J., Y. Wang, G. Alvelius, S. Liu, K. Bodin, and J. Sjövall. 2006. Analysis of oxysterols by electrospray tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **17:** 341–362.
29. Higashi, T., A. Yamauchi, and K. Shimada. 2005. 2-Hydrazino-1-methylpyridine: a highly sensitive derivatization reagent for oxosteroids in liquid chromatography-electrospray ionization-mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **825:** 214–222.

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Liver Fibrosis: Possible Involvement of EMT

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Key Words
Hepatic fibrosis · Epithelial-mesenchymal transition · Hepatocellular carcinoma · Hepatic stellate cell · Portal myofibroblast · Integrin-linked kinase

Abstract
Hepatic fibrosis is a wound-healing process in the liver with acute and chronic injury and is characterized by an excess production and deposition of extracellular matrix components. Hepatic stellate cells as well as portal fibroblasts play a pivotal role in the liver fibrogenesis. Regarding the origin of these mesenchymal cells, two hypotheses emerge. One hypothesis argues in favor of BM-derived progenitor cells and a second hypothesis favors epithelial-mesenchymal transition (EMT) in the local formation of these mesenchymal cells from hepatic epithelium. In this short review, we describe (1) the principle mechanisms of hepatic fibrosis, (2) the cells which play a crucial role in hepatic fibrosis, and (3) the possible involvement of EMT in the process of hepatic fibrosis and carcinogenesis. Copyright © 2007 S. Karger AG, Basel

Abbreviations used in this paper

α-SMA	α-smooth muscle actin
BM	bone marrow
CK	cytokeratin
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
GFAP	glial fibrillary acid protein
HCC	hepatocellular carcinoma
HSC	hepatic stellate cell
ILK	integrin-linked kinase
MF	myofibroblast
N-CAM	neural cell adhesion molecule
V-CAM	vascular cell adhesion molecule

Introduction

Epithelial-mesenchymal transition (EMT) is a central mechanism for diversifying the cells found in complex tissues. This dynamic process helps organize the formation of the body plan, and while EMT is well studied in the context of embryonic development, it also plays a role in the genesis of fibroblasts during organ fibrosis in adult tissues [Iwano et al., 2002]. Emerging evidence from studies of renal fibrosis suggests that more than a third of all disease-related fibroblasts originate from tubular epithelia at the site of injury [reviewed in Kalluri and Neilson, 2003]. In addition, these reports demonstrated that the various events observed in the process of renal fibrosis have also been reported in the process of hepatic fibrosis, suggesting the similarity of these fibrotic diseases. Therefore, the involvement of EMT in hepatic fibrosis is

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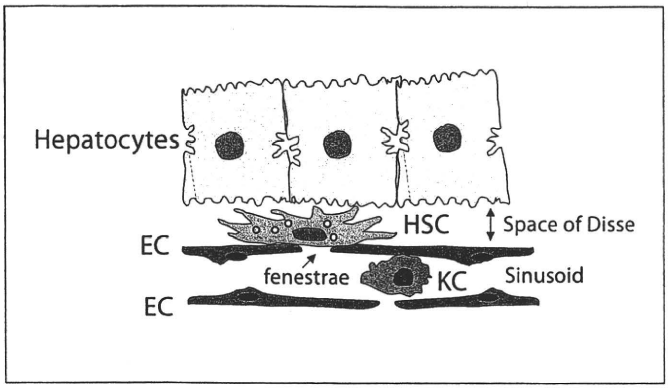

Fig. 1. Hepatic architecture. Following chronic hepatocyte injury, HSCs proliferate and undergo a dramatic phenotypical activation, secreting large amounts of ECM proteins. Sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid. EC = Endothelial cells; KC = Kupffer cells.

likely; however, direct evidence has not yet been provided. This review highlights recent advances in the study of hepatic fibrosis and how it may possibly be mediated by EMT. In this short review, we describe (1) the principle mechanisms of hepatic fibrosis, (2) the cells which play a crucial role in hepatic fibrosis, and (3) the possible involvement of EMT in the process of hepatic fibrosis and carcinogenesis.

Background of Hepatic Fibrosis

Hepatic fibrosis is a wound-healing process in the liver with acute and chronic injury, and is characterized by an excess production and deposition of extracellular matrix (ECM) components [Friedman, 2000]. Its endpoint is cirrhosis, which is responsible for significant morbidity and mortality. Cirrhosis is characterized by the formation of regenerative nodules of liver parenchyma that are separated by and encapsulated in fibrotic septa. The causes of cirrhosis are multiple and include congenital, metabolic, inflammatory, and toxic liver diseases. In addition, the poor prognosis of cirrhosis is in great part consecutive to the frequent occurrence of hepatocellular carcinoma (HCC) in this group.

Hepatic fibrosis was historically thought to be an irreversible process due to the collapse of the hepatic parenchyma and its substitution with a collagen-rich tissue. However, recent evidence indicates that even advanced fibrosis is reversible. In humans, spontaneous resolution of liver fibrosis can occur after successful treatment of the underlying diseases. This observation has been described in patients with alcohol-induced liver injury [Pares et al., 1986], chronic hepatitis C [Arthur, 2002] and B [Malekzadeh et al., 2004], secondary biliary cirrhosis [Hammel et al., 2001], nonalcoholic steatohepatitis (NASH) [Dixon et al., 2004], and autoimmune hepatitis [Dufour et al., 1997; Czaja and Carpenter, 2004]. Although isolated cases of complete fibrosis resolution have been reported, it is conceivable that some degree of fibrosis cannot be removed [Poynard et al., 2002]. Another therapeutic option for end-stage cirrhosis is liver transplantation. However, limited availability of organs, growing lists of patients needing a transplant, issues of compatibility, and comorbid factors mean that not everyone is eligible for transplantation. As a result, effective antifibrotic therapies are urgently needed [reviewed in Iredale, 2003].

Like other parenchyma, the normal liver contains an epithelial component (hepatocytes), an endothelial lining (which in liver is distinguished by fenestrae or pores), tissue macrophages (Kupffer cells), and a perivascular mesenchymal cell called the stellate cell (fig. 1). The cellular elements of liver are organized within the sinusoid, or microvascular unit, with the subendothelial space of Disse separating the epithelium (hepatocytes) from the sinusoidal endothelium. In normal liver, this space contains a basement membrane-like matrix, although it is not electron-dense like a typical basement membrane. The normal subendothelial ECM is essential for maintaining the differentiated function of all resident liver cells. As the liver becomes fibrotic, there are both quantitative and qualitative changes in composition of the hepatic ECM. The total content of collagens and noncollagenous components increases 3- to 5-fold, accompanied by a shift in the type of ECM in subendothelial space from the normal low-density basement membrane-like matrix to an interstitial-type matrix containing fibril-forming collagens. Replacement of the ECM in this space perturbs hepatocyte function and activates fibrogenic cells, which are described below. Accumulation of fibril-forming collagens contributes to the loss of hepatic microvilli and sinusoidal endothelial fenestrae, which result in deterioration of hepatic function [Friedman, 2000].

Major Player in Hepatic Fibrosis

Fibrogenesis is initiated by hepatocyte damage leading to a recruitment of inflammatory blood cells and platelets, as well as activation of Kupffer cells with subsequent

release of different cytokines. During liver fibrogenesis, a subpopulation of the cells from a mesenchymal origin contributes to the wound-healing process. Like the fibrotic process in other organs, myofibroblasts (MFs) play a crucial role in the liver. MFs are practically absent from normal liver; they are derived from the activation of precursor cells. There are several possible sources of hepatic MFs, as described below.

Hepatic Stellate Cells

As a fibrogenic mesenchymal cell in the liver, the best-studied subpopulation is the hepatic stellate cell (HSC) [Reeves and Friedman, 2002]. HSC (also known as lipocytes, fat-storing cells, or Ito cells) reside in the perisinusoidal area in the subendothelial space between hepatocytes and sinusoidal endothelial cells (space of Disse). HSCs have an intriguing embryogenic origin, with evidence suggesting that they are neural crest-derived because they express neural/neuroendocrine features such as glial fibrillary acidic protein (GFAP) [Neubauer et al., 1996], neural cell adhesion molecule (N-CAM) [Knittel et al., 1996] and nestin [Cassiman et al., 1999]. A neural crest origin is further supported by studies in rat neural crest stem cells, which differentiate into MFs that express alpha-smooth muscle actin (α-SMA) [Niki et al., 1999], a classic marker of activated HSCs.

In response to a wide variety of liver injuries, HSCs undergo a response known as 'activation' which is the transition of quiescent cells into proliferative, fibrogenic, and contractile MF-like phenotype. This process is associated with the reduction of lipid droplets containing vitamin A from a quiescent form of HSCs, the development of α-SMA, and a marked increase of ECM synthesis. Activated HSCs are known to be the major source of collagens and other matrix proteins that are deposited in liver fibrosis. Resolution of liver fibrosis could be associated with a reversal of activated HSC to a quiescent phenotype, or with a change in the balance of cell death over proliferation resulting in a net loss of activated HSC [Iredale et al., 1998]. Thus, based on the hypothesis that changes in cell fate and behavior of activated HSCs affect the pathogenesis and recovery from hepatic fibrosis/cirrhosis [Bataller and Brenner, 2001], various antibodies and chemicals have been examined for antifibrotic activity in experimental systems.

Portal Fibroblasts

In the last decade, HSCs have been studied most extensively in regard to the pathogenesis of hepatic fibrosis and were thought to be the major precursor population of septal MFs in cirrhosis. In fact, the term 'myofibroblasts' has been taken as synonymous for activated HSCs in many publications. However, previous and recent studies revealed the presence of other MF-like subpopulations of the liver which contribute to ECM synthesis and fibrogenesis. Another mesenchymal fibroblast-like cell type in the liver is the portal (myo)fibroblast, which in normal conditions resides in the portal mesenchyme. Recent clarification of specific markers made the distinction between liver MFs and activated HSCs possible. Depending on the initial cause of liver injury, an increased number of liver MFs at various locations has been observed. Periductal MFs appear to constitute a distinct subpopulation of mesenchymal cells in the portal tract as well as vascular smooth muscle cells residing in the walls of portal vein branches and portal arteries. Periductal MFs have been suggested to proliferate and transdifferentiate in response to bile duct ligation, causing periductal/periductular and periportal 'biliary type' of fibrosis [Kinnman and Housset, 2002; Magness et al., 2004]. Another mesenchymal MF-like cell type of the liver is the MF located around the centrolobular vein. They were suggested to proliferate in the livers of alcohol-fed baboons, causing a typical 'alcoholic type' of pericentral fibrosis [Nakano et al., 1982; Nakano and Lieber, 1982]. Finally, MF residing in Glisson's capsule form a potential source of ECM in the liver and show ultrastructural resemblances to HSC/MF.

Knittel et al. [1999b] reported that HSCs and rat liver MFs were discernible by morphological criteria and growth behavior. Prolonged subcultivation of rat liver MFs was achieved, but HSCs were maintained in culture at maximum until the second passage. HSCs were characterized by the expression of GFAP, desmin, and vascular cell adhesion molecule 1 (V-CAM), which were almost completely absent in rat liver MFs. For synthetic properties, HSCs and rat liver MFs displayed mostly overlapping properties with four striking differences. The complement-activating protease P100 and the protease inhibitor α_2-macroglobulin were preferentially expressed by HSCs, whereas interleukin 6-coding messenger RNAs and the ECM protein fibulin 2 were almost exclusively detectable in rat liver MFs [Knittel et al., 1999b].

Furthermore, Cassiman et al. [2002] demonstrated that three distinct mesenchymal (myo)fibroblastic-like liver cell subpopulations can be discerned: poral/septal MFs, inteface MFs and perisinusoidally located HSCs in advanced fibrosis and cirrhosis, regardless of cause or species. According to the expression pattern of these markers, Cassiman et al. [2002] speculated that septal

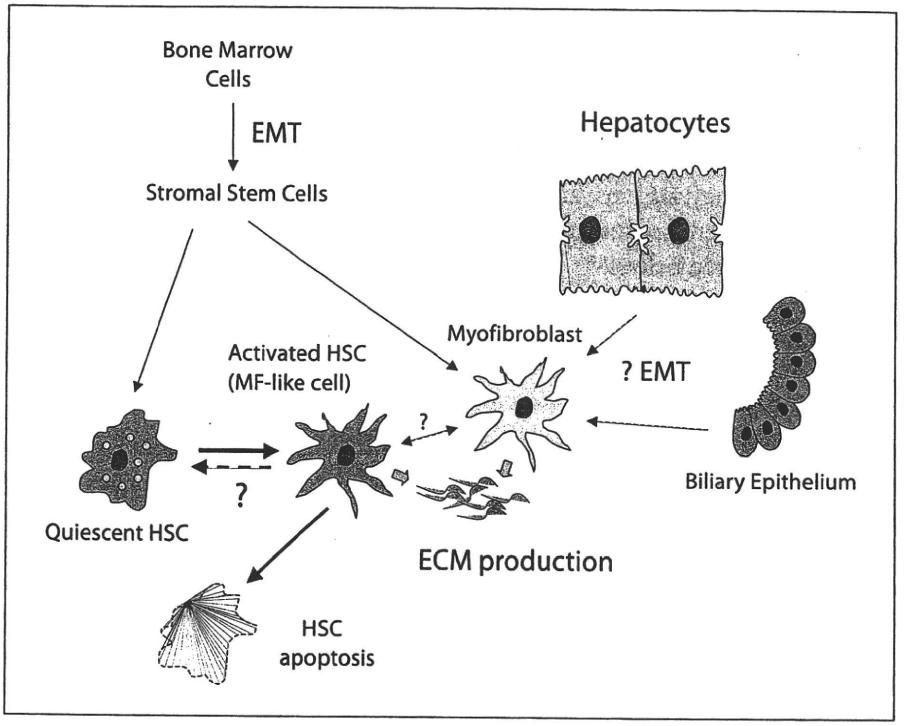

Fig. 2. Origin of hepatic MFs. The supply of hepatic MF occurs on three different levels: activation of HSCs or locally presented MFs, EMT from hepatocytes and/or biliary epithelium (no direct evidence), and BM progenitor cells.

MFs share more characteristics with portal MFs than with HSCs.

The contribution of the portal fibroblasts to hepatic fibrosis was once regarded as minor; however, recent reports demonstrated the opposite. Several reports mentioned that portal fibroblasts are postulated to be the early mediators of hepatic fibrosis after biliary injury, with HSCs comprising a later cellular response. Knittel et al. [1999a] demonstrated that acute liver injury was characterized almost exclusively by an increase in the number of HSCs, while the amount of liver MFs was nearly unchanged. In addition, HSC and MFs were detected within the developing scars in early stages of fibrosis, while HSCs were mainly present at the scar-parenchymal interface in the advanced stage of fibrosis [Knittel et al., 1999a]. Taken together, it is speculated that HSCs and MFs respond differentially to tissue injury. Although the contribution by either HSC or MF to the different type of liver fibrosis seems to be apparent, the previous studies, especially the study using isolated HSCs over several times of passage, should be reconsidered in terms of the possible relevance of liver MFs. Collectively, the origin and role in the hepatic fibrosis of respective cells should be further clarified in future studies.

Origin of Hepatic Mesenchymal Cells

As mentioned above, HSCs and liver MFs represent two similar but not identical cell populations, the latter being comparable to those of other organs. In addition, the question should be answered as to whether these cells (HSCs and MFs) have the same single progenitor cell or transdifferentiated phenotype one to another. MFs may transdifferentiate to HSCs or vice versa, since HSCs are akin to MFs as described in the section above. Understanding the origin and transdifferentiation system of these hepatic mesenchymal cells is important for the establishment of a rational therapeutic strategy (fig. 2).

Two hypotheses emerge regarding the origin of adult fibroblasts. One hypothesis argues that bone marrow (BM) cells are progenitors for tissue fibroblasts that then shuttle through the circulation to populate peripheral organs. One study revealed that the collagen-producing lung fibroblasts in pulmonary fibrosis can be derived from BM progenitor cells [Hashimoto et al., 2004]. In the liver, Forbes et al. [2004] recently demonstrated the possible contribution of extrahepatically derived MF to human liver cirrhosis by examining human liver after sex-mismatched orthotropic liver or BM transplantation. They tracked male cells of extrahepatic origin through the use of in situ hybridization for the Y chromosome in

a female patient who received a BM transplant from a male donor and subsequently developed hepatitis C-induced cirrhosis. In this patient, 12.4% of the MFs in the tested liver section were Y chromosome positive, indicating a BM origin [Forbes et al., 2004]. Furthermore, the additional investigation by Russo et al. [2006] demonstrated a contribution of BM cells to liver fibrosis. In their report, the contribution of BM cells to parenchymal regeneration is minor, whereas the BM contributed significantly to HSC and MF populations. These hematopoietic stem cells have also been known as progenitors for hepatic hepatocytes and biliary epithelial cells. However, it is unknown at which level the fate of these cells as progeny (e.g. hepatocytes, biliary epithelial cells, mesenchymal cells) is decided. For instance, is it possible that intrahepatic stem cells, which have a multilineage plasticity, can transdifferentiate to mesenchymal cells at the injured site? Or can BM cells differentiate to more professional stromal stem cells (marrow stromal cells) and be delivered to the liver? According to the study mentioned above, these BM-derived MFs did not occur through cell fusion between BM-derived cells and indigenous hepatic cells but, instead, originated largely from the mesenchymal stem cells of the BM [Russo et al., 2006].

A second hypothesis favors EMT in the local formation of interstitial fibroblasts from organ epithelium. The findings supporting the involvement of EMT in hepatic fibrosis are summarized below.

EMT from Local Epithelial Cell Lineage

It is well recognized that in some pathologic conditions, including reactive and neoplastic conditions, epithelial cells may transform into cells with mesenchymal characteristics that can migrate and form connective tissue. Renal fibrosis has been best studied in terms of EMT occurring from tubular epithelium to mesenchymal phenotype [Iwano et al., 2002; Liu, 2004]. As epithelial cells transform into fibroblasts, epithelial proteins [e.g., E-cadherin] are downregulated and mesenchymal markers [e.g., matrix metalloproteinase-2 and α-SMA] are induced. Although not all of the mechanisms for EMT have been elucidated, several key molecules, including integrin-linked kinase (ILK) [Li et al., 2003], Smad3 [Saika et al., 2004] and Snail [Cano et al., 2000], have been identified. Recent investigation revealed that upregulation of these factors is also closely associated with the EMT in the hepatic fibrosis.

Smad3, a factor mediating the majority of the profibrotic activities of TGF-β, also contributes to inducing EMT [Flanders, 2004]. In chronic liver injury, the consti-

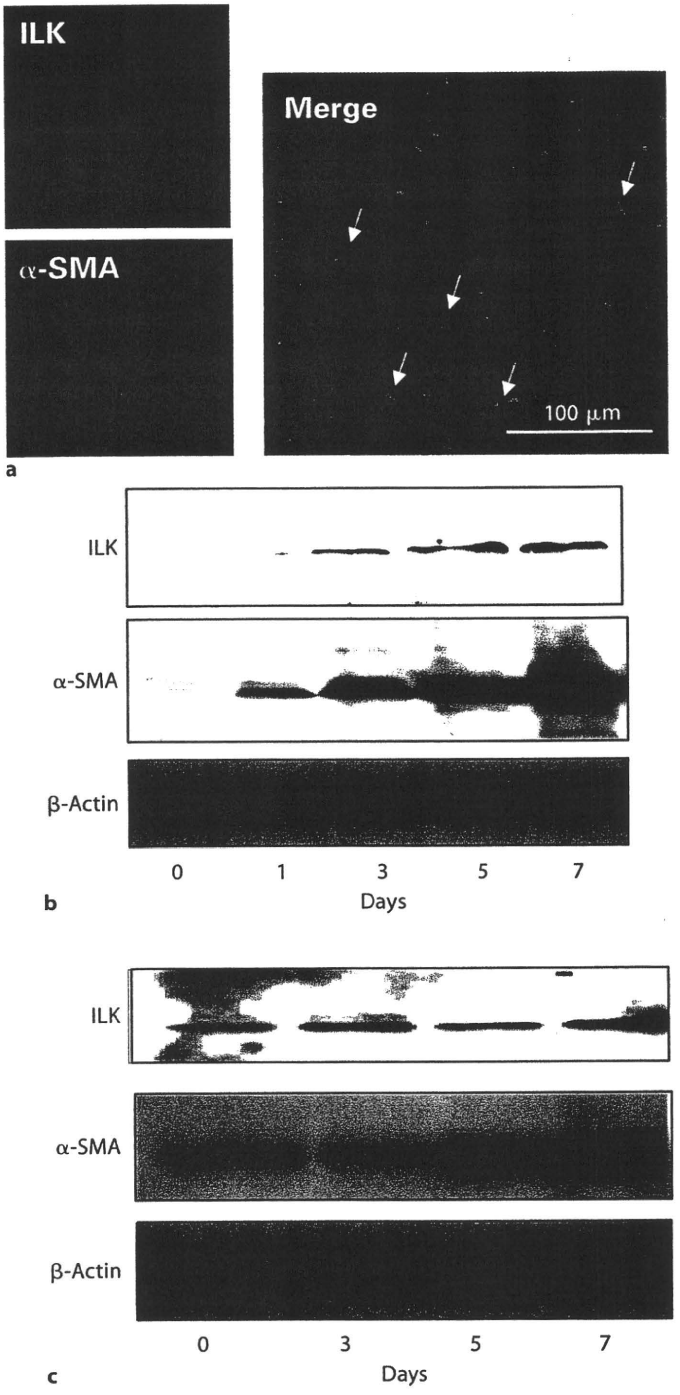

Fig. 3. Expression of ILK in activated HSC. **a** Liver sections from rats treated for 3 weeks with a weekly injection of CCl_4 were immunohistochemically stained red for α-SMA and green for ILK individually or combined (merged). Arrows point to ILK-expressing sites. **b** HSC isolated from control rats seeded in plastic dishes and cultured for up to 7 days. **c** HSC isolated from rats that received CCl_4 treatment for 2 weeks seeded in plastic dishes and cultured for up to 7 days. Expression of ILK, α-SMA, and β-actin were determined by Western blotting [Zhang et al., 2006].

tutive phosphorylation and nuclear localization of Smad3 were observed [Inagaki et al., 2001]. When acute liver injury was induced by administration of carbon tetrachloride (CCl_4), Smad3 KO mice showed approximately one half of the induction of hepatic collagen type I mRNA as Smad3 WT mice [Schnabl et al., 2001].

We and another group reported the expression of ILK in the HSCs in vivo and in vitro [Shafiei and Rockey, 2006; Zhang et al., 2006]. ILK has been considered as a critical mediator for tubular EMT [Dedhar et al., 1999], and plays a crucial role in the pathogenesis of renal fibrosis [Liu, 2004]. In our study, ILK is strongly expressed in the perisinusoidal area of CCl_4-induced liver fibrosis in rats (fig. 3a). Furthermore, HSC isolated from fibrotic livers expressed high levels of ILK and α-SMA, and their expression was sustained in culture. In contrast with these findings, HSC isolated from normal rat liver did not express ILK but its expression was increased when the cells were activated in culture (fig. 3b, c). These results are consistent with the findings reported by Shafiei and Rockey [2006]. They also employed another hepatic fibrosis model using bile duct ligation and confirmed the upregulation of ILK, suggesting that the upregulation of ILK is a universal event occurring in the various types of hepatic fibrosis. In addition, the role of ILK in hepatic fibrosis has been determined by using small interfering RNA in both studies. The inhibition of ILK resulted in the significant decrease in the phosphorylation of the MAPK family and PKB. These changes were accompanied by significant inhibition of proliferation, cell spreading and migration of HSCs [Zhang et al., 2006] which are characteristic features of mesenchymal cells. Regression of hepatic fibrosis by the inhibition of ILK expression was also confirmed using an in vivo study [Shafiei and Rockey, 2006], showing the crucial role of ILK in hepatic fibrosis, and indirectly suggesting the possible involvement of EMT process.

In the liver, major subpopulations belonging to the epithelium are hepatocytes and bile duct epithelium (cholangiocytes). It has been thought that these two cell lineages originate from a single source, such as hematopoietic progenitor cells [Masson et al., 2004] or oval cells [Paku et al., 2001]. Several works have already demonstrated the potential of neonatal rat hepatocytes [Pagan et al., 1995, 1997] as well as Met murine hepatocytes [Spagnoli et al., 2000] (derived from transgenic mice expressing a truncated constitutively active form of c-Met) to change into mesenchymal cells. Furthermore, Ju et al. [2006] also demonstrated the morphological change as well as E-cadherin expression in isolated mouse hepatocytes. Lim et al. [2002] previously demonstrated the expression of both cytokeratin 18 (CK18) and CK19 in cultured human HSCs, and progressive reduction of these CKs during prolonged cultivations. CK18 and CK19 have been thought to be cell markers of epithelial origin. Therefore, they concluded that HSCs might be of epithelial origin, and undergo transdifferentiation to mesenchymal phenotype during activation. In addition, Lim et al. [2006] showed that resting rat HSCs express E-cadherin and β-catenin both in vivo and in vitro, and E-cadherin switches to N-cadherin during HSC activation. Furthermore, a recent report by Sicklick et al. [2006] demonstrated that the HSC and the epithelial progenitors accumulated in the injured liver coexpress epithelial and mesenchymal markers, providing evidence that EMT occurs in adult liver cells. In this report, they demonstrated that E-cadherin, a specific epithelial cell marker, was expressed in an established rat HSC cell line. In addition, they isolated the HSC from a mouse and determined the expression of several cell markers following culture activation. Neural cell markers (GFAP and nestin) decreased during culture activation in their mouse HSC cultures. In contrast, freshly isolated HSC expressed high levels of Mpk, a classic oval cell marker, over time. Expression of CK19, a biliary marker, also remained relatively constant during culture, suggesting either the survival of biliary epithelial cells during culture or the capability of some HSC to convert into cholangiocytes via EMT. The overlapped expression of both epithelial and mesenchymal cell markers in a single cell strongly suggests that the cell is in the EMT process.

Although it should be discussed further whether the activation of HSC can be regarded as EMT process or not, it is likely that HSC possess the characteristics of the cells in EMT process. Taken together, it is likely that EMT is an important source of hepatic mesenchymal cells, and contributes to the progression of hepatic fibrosis. As already shown in a renal fibrosis model [Iwano et al., 2002], the direct evidence of EMT in hepatic fibrosis should be demonstrated by using transgenic animals in future studies.

EMT and Hepatic Carcinogenesis

The progress of hepatic fibrosis is closely associated with the hepatic carcinogenesis. HCC is clinically the most important cancer of the liver because of its association with chronic hepatitis B and C, which has a high prevalence in countries of the Middle East, Asia and Af-

rica [Fattovich et al., 2004]. Common to all forms of HCC is advanced fibrosis or cirrhosis, although the strength of the association varies by etiology. In chronic hepatitis C, as much as 95% of HCC is in the cirrhotic liver, whereas in chronic hepatitis B, the figure is less than 60%. In general, changes in hepatocyte growth factor expression, somatic mutations, protease and matrix metalloproteinase overexpression, and oncogene expression are seen in hepatic inflammation and chronic hepatitis, and become more extensive as liver injury progresses through fibrosis, cirrhosis and dysplastic foci and nodules to overt HCC. Several reports demonstrate that the suppression of chronic inflammation or fibrosis by the administration of interferon is effective in the prevention of hepatic carcinogenesis [Heathcote, 2004; Omata et al., 2005]. MFs play a major role in the formation of septa observed in liver fibrosis and cirrhosis as mentioned above, and are also a major component of the stroma reaction which develops around HCC [Desmouliere et al., 2004]. After implanting rat HCC cells into normal and cirrhotic rat liver, it has been shown that they grow more quickly in cirrhotic liver than in normal liver [Tsujimoto et al., 2001]. Neaud et al. [1997] reported that human liver MFs act on HCC cells to increase their invasiveness and these effects are blocked by addition of an antibody to HGF, suggesting that MF-derived HGF can be involved in the pathogenesis of HCCs. MF-like cells derived from HSC can play a role in the capillarization of the sinusoids and in the neovascularization of the tumor which facilitates the formation of metastasis [Olaso et al., 2003]. At the same time, it has been hypothesized that tumor cells themselves can control the behavior of hepatic MFs. An in vitro experiment demonstrated that tumor-activated MFs prevent penetration of T lymphocytes and macrophages within tumor nodules [Lieubeau et al., 1999]. By developing the large sheath surrounding the tumor, MFs may reduce physical contact between cancer cells and immune cells, an essential phenomenon for effective destruction of cancer cells. Taken together, controlling the fibrogenic cells in tumor-bearing liver could be a potential strategy to enhance the immunotherapy against the cancer.

Recent reports revealed that the EMT is responsible for the invasive properties of HCC [Giannelli et al., 2005]. In this report, the authors suggest that blocking a specific integrin by antibody may suppress the ILK activation, and finally inhibit the EMT of the cancer cells. In addition, Plante et al. [2005] showed the activation of the ILK signaling pathway and suppression of E-cadherin expression in the chemical-induced rat hepatocarcinogenic model. Whether ILK is an important gene in cancer, the coinvolvement of ILK in cancer and fibrosis not only suggests a mechanistic connection between the two processes, but also that the two may be amenable to common treatment by ILK inactivation.

On the other hand, one report studied the role of macrophages in the EMT of hepatocarcinoma cells. Lin et al. [2006] investigated the effect of activated macrophages on the metastatic behavior of HepG2 cells. The conditioned media from macrophages activated in different ways significantly increased the migration and invasiveness of HepG2 cells. In accordance with this finding, levels of E-cadherin and β-catenin in the membrane fraction of HepG2 were decreased. Therefore, hepatoma itself can acquire the invasive property via EMT through the interaction with surrounding mesenchymal cells and inflammatory cells.

Conclusion and Future Perspective

More evidence is presently available supporting the idea that many important biological phenomena depend on the concerted action of epithelial and mesenchymal phenotypes. The discovery of the EMT process in renal fibrosis has further provided a new paradigm to understand the mechanism of fibrosis. However, the relevance of EMT in hepatic fibrosis has been less investigated despite the presence of an abundant number of similarities between renal and hepatic fibrosis. A series of recent studies strongly suggested the actual role of EMT in the hepatic fibrosis as mentioned above. Unlike the direct presentation of the dynamic process in vivo which was demonstrated for renal fibrosis [Li et al., 2003], these supportive reports for hepatic fibrosis provide indirect evidence. In addition, it seems to be very challenging to claim an EMT process in the liver since the origin of fibrogenic cells in the liver (e.g. HSCs, MFs) is still debated since the precise role as well as the process of development of each fibrogenic cell (HSCs, MFs) have not been elucidated completely. However, further investigation will clarify the dynamic process of EMT during hepatic fibrosis in the near future since the number of new findings further supporting this hypothesis is increasing.

The study of liver fibrosis has progressed mainly through the investigation of HSC cell biology in the last two decades, furthering advances in a new stage by the addition of a new fibrogenic cell lineage in the liver. Although it is supposed to be a long way off, the utilization

of the common concept in the pathophysiology of fibrosis and of the therapeutic strategies by highlighting the specificity and uniformity of hepatic fibrosis in comparison to other fibrotic diseases is possible. In this context, EMT, an emerging concept for further understanding of the mechanism of fibrosis and wound healing, may give us the breakthrough.

In summary, the identification of EMT in the liver with chronic inflammation that mediates liver repair justifies research to clarify how pathways that regulate EMT influence the evolution of HCC and cirrhosis. Such work offers the promise of identifying novel pathways that might be targeted to improve the outcome of many types of liver damage.

References

Arthur, M.J. (2002) Reversibility of liver fibrosis and cirrhosis following treatment for hepatitis C. Gastroenterology *122:* 1525–1528.

Bataller, R., D.A. Brenner (2001) Hepatic stellate cells as a target for the treatment of liver fibrosis. Semin Liver Dis *21:* 437–451.

Cano, A., M.A. Perez-Moreno, I. Rodrigo, A. Locascio, M.J. Blanco, M.G. del Barrio, F. Portillo, M.A. Nieto (2000) The transcription factor Snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol *2:* 76–83.

Cassiman, D., L. Libbrecht, V. Desmet, C. Denef, T. Roskams (2002) Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. J Hepatol *36:* 200–209.

Cassiman, D., J. van Pelt, R. De Vos, F. Van Lommel, V. Desmet, S.H. Yap, T. Roskams (1999) Synaptophysin: a novel marker for human and rat hepatic stellate cells. Am J Pathol *155:* 1831–1839.

Czaja, A.J., H.A. Carpenter (2004) Decreased fibrosis during corticosteroid therapy of autoimmune hepatitis. J Hepatol *40:* 646–652.

Dedhar, S., B. Williams, G. Hannigan (1999) Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. Trends Cell Biol *9:* 319–323.

Desmouliere, A., C. Guyot, G. Gabbiani (2004) The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol *48(5–6):* 509–517.

Dixon, J.B., P.S. Bhathal, N.R. Hughes, P.E. O'Brien (2004) Nonalcoholic fatty liver disease: improvement in liver histological analysis with weight loss. Hepatology *39:* 1647–1654.

Dufour, J.F., R. DeLellis, M.M. Kaplan (1997) Reversibility of hepatic fibrosis in autoimmune hepatitis. Ann Intern Med *127:* 981–985.

Fattovich, G., T. Stroffolini, I. Zagni, F. Donato (2004) Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology *127(5 suppl 1):* S35–50.

Flanders, K.C. (2004) Smad3 as a mediator of the fibrotic response. Int J Exp Pathol *85:* 47–64.

Forbes, S.J., F.P. Russo, V. Rey, P. Burra, M. Rugge, N.A. Wright, M.R. Alison (2004) A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology *126:* 955–963.

Friedman, S.L. (2000) Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. J Biol Chem *275:* 2247–2250.

Giannelli, G., C. Bergamini, E. Fransvea, C. Sgarra, S. Antonaci (2005) Laminin-5 with transforming growth factor-beta1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. Gastroenterology *129:* 1375–1383.

Hammel, P., A. Couvelard, D. O'Toole, A. Ratouis, A. Sauvanet, J.F. Flejou, C. Degott, J. Belghiti, P. Bernades, D. Valla, P. Ruszniewski, P. Levy (2001) Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. N Engl J Med *344:* 418–423.

Hashimoto, N., H. Jin, T. Liu, S.W. Chensue, S.H. Phan (2004) Bone marrow-derived progenitor cells in pulmonary fibrosis. J Clin Invest *113:* 243–252.

Heathcote, E.J. (2004) Prevention of hepatitis C virus-related hepatocellular carcinoma. Gastroenterology *127(5 suppl 1):* S294–302.

Inagaki, Y., M. Mamura, Y. Kanamaru, P. Greenwel, T. Nemoto, K. Takehara, P. ten Dijke, A. Nakao (2001) Constitutive phosphorylation and nuclear localization of Smad3 are correlated with increased collagen gene transcription in activated hepatic stellate cells. J Cell Physiol *187:* 117–123.

Iredale, J.P. (2003) Cirrhosis: new research provides a basis for rational and targeted treatments. BMJ *327(7407):* 143–147.

Iredale, J.P., R.C. Benyon, J. Pickering, M. McCullen, M. Northrop, S. Pawley, C. Hovell, M.J. Arthur (1998) Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest *102:* 538–549.

Iwano, M., D. Plieth, T.M. Danoff, C. Xue, H. Okada, E.G. Neilson (2002) Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest *110:* 341–350.

Ju, W., A. Ogawa, J. Heyer, D. Nierhof, L. Yu, R. Kucherlapati, D.A. Shafritz, E.P. Bottinger (2006) Deletion of Smad2 in mouse liver reveals novel functions in hepatocyte growth and differentiation. Mol Cell Biol *26:* 654–667.

Kalluri, R., E.G. Neilson (2003) Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest *112:* 1776–1784.

Kinnman, N., C. Housset (2002) Peribiliary myofibroblasts in biliary type liver fibrosis. Front Biosci *7:* d496–503.

Knittel, T., S. Aurisch, K. Neubauer, S. Eichhorst, G. Ramadori (1996) Cell-type-specific expression of neural cell adhesion molecule (N-CAM) in Ito cells of rat liver. Up-regulation during in vitro activation and in hepatic tissue repair. Am J Pathol *149:* 449–462.

Knittel, T., D. Kobold, F. Piscaglia, B. Saile, K. Neubauer, M. Mehde, R. Timpl, G. Ramadori (1999a) Localization of liver myofibroblasts and hepatic stellate cells in normal and diseased rat livers: distinct roles of (myo-)fibroblast subpopulations in hepatic tissue repair. Histochem Cell Biol *112:* 387–401.

Knittel, T., D. Kobold, B. Saile, A. Grundmann, K. Neubauer, F. Piscaglia, G. Ramadori (1999b) Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. Gastroenterology *117:* 1205–1221.

Li, Y., J. Yang, C. Dai, C. Wu, Y. Liu (2003) Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. J Clin Invest *112:* 503–516.

Lieubeau, B., M.F. Heymann, F. Henry, I. Barbieux, K. Meflah, M. Gregoire (1999) Immunomodulatory effects of tumor-associated fibroblasts in colorectal-tumor development. Int J Cancer *81:* 629–636.

Lim, Y.S., K.A. Kim, J.O. Jung, J.H. Yoon, K.S. Suh, C.Y. Kim, H.S. Lee (2002) Modulation of cytokeratin expression during in vitro cultivation of human hepatic stellate cells: evidence of transdifferentiation from epithelial to mesenchymal phenotype. Histochem Cell Biol *118:* 127–136.

Lim, Y.S., H.C. Lee, H.S. Lee (2006) Switch of cadherin expression from E- to N-type during the activation of rat hepatic stellate cells. Histochem Cell Biol, in press.
Lin, C.Y., C.J. Lin, K.H. Chen, J.C. Wu, S.H. Huang, S.M. Wang (2006) Macrophage activation increases the invasive properties of hepatoma cells by destabilization of the adherens junction. FEBS Lett *580:* 3042–3050.
Liu, Y. (2004) Epithelial to mesenchymal transition in renal fibrogenesis: pathologic significance, molecular mechanism, and therapeutic intervention. J Am Soc Nephrol *15:* 1–12.
Magness, S.T., R. Bataller, L. Yang, D.A. Brenner (2004) A dual reporter gene transgenic mouse demonstrates heterogeneity in hepatic fibrogenic cell populations. Hepatology *40:* 1151–1159.
Malekzadeh, R., M. Mohamadnejad, N. Rakhshani, S. Nasseri-Moghaddam, S. Merat, S.M. Tavangar, A.A. Sohrabpour (2004) Reversibility of cirrhosis in chronic hepatitis B. Clin Gastroenterol Hepatol *2:* 344–347.
Masson, S., D.J. Harrison, J.N. Plevris, P.N. Newsome (2004) Potential of hematopoietic stem cell therapy in hepatology: a critical review. Stem Cells *22:* 897–907.
Nakano, M., C.S. Lieber (1982) Ultrastructure of initial stages of perivenular fibrosis in alcohol-fed baboons. Am J Pathol *106:* 145–155.
Nakano, M., T.M. Worner, C.S. Lieber (1982) Perivenular fibrosis in alcoholic liver injury: ultrastructure and histologic progression. Gastroenterology *83:* 777–785.
Neaud, V., S. Faouzi, J. Guirouilh, B. Le Bail, C. Balabaud, P. Bioulac-Sage, J. Rosenbaum (1997) Human hepatic myofibroblasts increase invasiveness of hepatocellular carcinoma cells: evidence for a role of hepatocyte growth factor. Hepatology *26:* 1458–1466.
Neubauer, K., T. Knittel, S. Aurisch, P. Fellmer, G. Ramadori (1996) Glial fibrillary acidic protein – a cell type specific marker for Ito cells in vivo and in vitro. J Hepatol *24:* 719–730.
Niki, T., M. Pekny, K. Hellemans, P.D. Bleser, K.V. Berg, F. Vaeyens, E. Quartier, F. Schuit, A. Geerts (1999) Class VI intermediate filament protein nestin is induced during activation of rat hepatic stellate cells. Hepatology *29:* 520–527.
Olaso, E., C. Salado, E. Egilegor, V. Gutierrez, A. Santisteban, P. Sancho-Bru, S.L. Friedman, F. Vidal-Vanaclocha (2003) Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis. Hepatology *37:* 674–685.
Omata, M., H. Yoshida, Y. Shiratori (2005) Prevention of hepatocellular carcinoma and its recurrence in chronic hepatitis C patients by interferon therapy. Clin Gastroenterol Hepatol *3(10 suppl 2):* S141–143.
Pagan, R., M. Llobera, S. Vilaro (1995) Epithelial-mesenchymal transition in cultured neonatal hepatocytes. Hepatology *21:* 820–831.
Pagan, R., I. Martin, M. Llobera, S. Vilaro (1997) Epithelial-mesenchymal transition of cultured rat neonatal hepatocytes is differentially regulated in response to epidermal growth factor and dimethyl sulfoxide. Hepatology *25:* 598–606.
Paku, S., J. Schnur, P. Nagy, S.S. Thorgeirsson (2001) Origin and structural evolution of the early proliferating oval cells in rat liver. Am J Pathol *158:* 1313–1323.
Pares, A., J. Caballeria, M. Bruguera, M. Torres, J. Rodes (1986) Histological course of alcoholic hepatitis. Influence of abstinence, sex and extent of hepatic damage. J Hepatol *2:* 33–42.
Plante, I., D.G. Cyr, M. Charbonneau (2005) Involvement of the integrin-linked kinase pathway in hexachlorobenzene-induced gender-specific rat hepatocarcinogenesis. Toxicol Sci *88:* 346–357.
Poynard, T., J. McHutchison, M. Manns, C. Trepo, K. Lindsay, Z. Goodman, M.H. Ling, J. Albrecht (2002) Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. Gastroenterology *122:* 1303–1313.
Reeves, H.L., S.L. Friedman (2002) Activation of hepatic stellate cells – a key issue in liver fibrosis. Front Biosci *7:* d808–826.
Russo, F.P., M.R. Alison, B.W. Bigger, E. Amofah, A. Florou, F. Amin, G. Bou-Gharios, R. Jeffery, J.P. Iredale, S.J. Forbes (2006) The bone marrow functionally contributes to liver fibrosis. Gastroenterology *130:* 1807–1821.
Saika, S., S. Kono-Saika, Y. Ohnishi, M. Sato, Y. Muragaki, A. Ooshima, K.C. Flanders, J. Yoo, M. Anzano, C.Y. Liu, W.W. Kao, A.B. Roberts (2004) Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury. Am J Pathol *164:* 651–663.
Schnabl, B., Y.O. Kweon, J.P. Frederick, X.F. Wang, R.A. Rippe, D.A. Brenner (2001) The role of Smad3 in mediating mouse hepatic stellate cell activation. Hepatology *34:* 89–100.
Shafiei, M.S., D.C. Rockey (2006) The role of integrin-linked kinase in liver wound healing. J Biol Chem *281:* 24863–24872.
Sicklick, J.K., S.S. Choi, M. Bustamante, S.J. McCall, E.H. Perez, J. Huang, Y.X. Li, M. Rojkind, A.M. Diehl (2006) Evidence for epithelial-mesenchymal transitions in adult liver cells. Am J Physiol Gastrointest Liver Physiol *291:* G575–G583.
Spagnoli, F.M., C. Cicchini, M. Tripodi, M.C. Weiss (2000) Inhibition of MMH (Met murine hepatocyte) cell differentiation by TGF(beta) is abrogated by pre-treatment with the heritable differentiation effector FGF1. J Cell Sci *113:* 3639–3647.
Tsujimoto, T., S. Kuriyama, M. Yamazaki, Y. Nakatani, H. Okuda, H. Yoshiji, H. Fukui (2001) Augmented hepatocellular carcinoma progression and depressed Kupffer cell activity in rat cirrhotic livers. Int J Oncol *18:* 41–47.
Zhang, Y., T. Ikegami, A. Honda, T. Miyazaki, B. Bouscarel, M. Rojkind, I. Hyodo, Y. Matsuzaki (2006) Involvement of integrin-linked kinase in carbon tetrachloride-induced hepatic fibrosis in rats. Hepatology *44:* 612–622.

特集

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肝胆膵の癌に対する放射線療法

本邦における肝細胞癌に対する放射線治療の現況と今後の展望*

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要約：本邦における放射線療法は，phase Ⅰ/Ⅱ試験での有用性により施行されているのが現状である。本邦におけるHCCに対する放射線照射療法は従来の照射方法に加え，conformal radiotherapyと体幹部定位放射線治療，さらに陽子線，重粒子線などの新しい粒子線治療は，効果的かつ安全で耐用性に富み，さらには繰り返し可能であることが第二相試験で示されている。これらの利用により，今後根治目的の治療選択肢の一つとして用いられる可能性があり，また腫瘍径や局在，門脈塞栓，合併症など，幅広い適応を有する可能性が示唆されると考えられる。しかし，RCTによる科学的根拠に基づく有効性の確立や，コストや保険診療など，いくつかの課題も抱えており，今後本邦における放射線照射療法の位置付けを明確にしていく必要があろう。さらに放射線照射療法の発表症例を増やし，全国原発性肝癌追跡調査用紙の項目に放射線治療の照射条件や線質などの項目を加えよりupdateのものに改訂していく必要があると考えられる。

Key words：conformal radiotherapy，定位放射線治療，粒子線治療，門脈塞栓

はじめに

本邦における第17回全国原発性肝癌追跡調査報告によると肝癌に対する治療施行率において，原発性肝細胞癌（HCC）の根治的治療法とされる手術施行率は33.6%である。外科手術以外の治療法の状況は，局所療法31.2%（RFA 65.8%，PEI 21.4%，MCT 11.6%），塞栓療法29.6%であり，局所療法と塞栓術が主流を占める。一方これらに比べ放射線照射療法はわずか1.3%である[1)]。

現在，HCCに対する多くの治療法は，それぞれ適応と限界があり厚生労働省からも治療指針が出されている[2)]。肝臓は放射線への耐容性が低く，従来は肝細胞癌に対する放射線療法が試みられてきたが，照射による肝機能低下のため積極的な治療法とはならなかった。

近年，放射線治療は限局部位への線量集中技術の進歩とともに適応も拡大され，HCCに対する放射線治療の報告がされるようになった[3～6)]。さらに選択的腫瘍照射法として従来の放射線を利用したconformal radiotherapyや，定位放射線治療による放射線照射療法の有効性も報告されるようになった[7～12)]。さらに近年，新しい陽子線，炭素線などの重荷電粒子線照射療法など本邦におけるHCCに対する放射線照射療法の進歩は著しく，有効性を示す成績も散見される[13～17)]。

このように今日放射線療法に関し，残念ながら科学的根拠に基づく多施設無作為試験（RCT）報告はないが，HCCの集学的治療の一環としての放射線照射療法の有効性は，現段階ではphase Ⅱ試験として有効性を示唆する報告により支持されている。

本稿においては，放射線照射療法の最先端の重荷電粒子線照射療法の現状も含め，日本肝癌研究会の追跡調査を参考にし，現在の本邦におけるHCCに対する放射線治療の現状を明らかにしたい。

* Radiotherapy for Hepatocellular Carcinoma in the Present and Future Status in Japan

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Ⅰ．肝細胞癌に対する従来の放射線外部照射法

HCCに対する局所治療法として放射線照射療法は従来より試みられてきた。しかし，外部照射である全肝照射が中心であったために，肝不全出現のため有効性が示唆されながらも，なかなか積極的な治療法としては確立されてこなかった。

HCCに対する光子線治療の外部照射は年代を追ってみると，高良ら[3]によれば30–50 Gyの照射にて，病理組織学的に一部に腫瘍細胞の残存を認めたことからこの線量では根治性の面からは不十分とした。さらにOhtoら[4]は，39例のHCC患者にlinacを30–50 Gy照射し，直径5 cm以下のHCCの場合，縮小率は90%に認められ，5 cm以上では，50%以上の腫瘍縮小率が約60%に認められるという良好な成績を報告している。吉川ら[5]は，肝動脈塞栓療法とリニアックX線約50 Gyを局所照射併用し，良好な成績を報告している。Matsuuraら[6]は，肝動脈塞栓療法後の再発腫瘍に58–64 Gyの照射し6ヵ月後で75%，2年後で45%，3年後で36%という良好な局所制御率を示している。同様の報告は近年においてもなされている。

このような従来の外部肝照射法の最大の難点は，正常組織への照射による副作用である。非LC例での全照射の耐容線量は30–40 Gyとされ，一方HCCに対する根治線量は50 Gy以上必要とされる。したがって，従来の外部照射法ではHCCに対し抗腫瘍効果を得るのに十分な線量を局所に照射しえず，安全かつ確実な治療効果が得られなかった。

Ⅱ．肝細胞癌に対するconformal radiotherapyと定位放射線治療

CT検査の進歩により，3次元的位置情報をもとにコンピューター上においてシミュレーションをし，最適な照射方向，範囲，線量を考慮し行われる。原体照射，多門照射法，回転照射法などの照射法の工夫や，呼吸位相同調照射装置の開発[18]など，より限局した部位への高線量照射が試みられている。conformal radiotherapyは，回転する線源に連動し照射野を変化させ，色々な方向から腫瘍の3次元的形態に合わせて照射する方法である。最近はCTを利用し，切除不能HCCに照射効果があったと報告も散見され照射治療計画により，安全にかつ正確に治療できるとしている[7~9]。一方，定位放射線治療（stereotactic multiple arcs radiotherapy）とは多数の角度から，細い高エネルギーX線の線束を腫瘍に集中させ照射する照射術である[10~12]。最近，本邦ではTakedaら[10]は，短期間の定位放射線治療が，HCCの良好な局所制御を得られることを報告している。これらの照射法の利用により良好な成績が得られるようになってきているのが現状である。

Ⅲ．門脈腫瘍塞栓に対する放射線療法

HCCの門脈腫瘍塞栓PVTTに対する放射線照射療法の報告も散見される[19~25]。本邦においては，Ohtoら[4]，高良ら[3]はPVTTに対する放射線照射療法の有効性を1980年代にすでに報告している。その後，TAEとの併用での有効性を吉川ら[5]が報告している。本邦においてYamadaら[21]は，3D–CT下conformal radiotherapyにての高線量照射の有効性を報告している。Tazawaら[22]も，経カテーテル的肝動脈化学塞栓術とliniac放射線治療の併用でChild A症例でPVTTに有効であると報告している。Hataら[20]も陽子線照射療法にて，門脈腫瘍塞栓を伴う高度進行肝硬変合併HCCを照射し，塞栓も縮小し急速に悪化する肝機能障害も止めることが可能と報告している。このように，患者さんのQOLを損なうことなく，高度進行肝硬変合併HCCの治療が可能となる。

Ⅳ．新しい放射線療法—重荷電粒子線治療—

1．粒子線治療の理論的根拠

重粒子とは電子より重い粒子のことをいい，これを加速器で高速にしたものを広義の重粒子と呼ぶ。重粒子線は，さらに非荷電粒子と重荷電粒子の2つに分類される。前者は中性子であり，後はさらに，陽子，重イオン，p中間子の3つに分類される。新しい放射線療法として現在臨床応用されているものは，重荷電粒子線として陽子線，重イオン（炭素，アルゴン，ネオンなど）が挙げられる。陽子線やさらにエネルギーの強い炭素線が体内の一定深度で高線量域（Bragg–peak）を形成し，線量が表面で少なく体内深部で大きくなる理由である。

2．重荷電粒子線治療の現状

2006年10月現在で，陽子線治療が施行されているところは，世界で18施設，本邦で6施設である。炭素線は，世界で3施設，本邦で2施設稼働しているのが現状である。2005年6月現在全世界で陽子線治療では，42,766名，重イオンで4,520名の患者さんが治療を受けている[26]。この中でもHCCに対する粒子線治療は圧倒的に本邦で施行されている。

表 1　症例（n＝86）

症例の適格項目		症例の適格条件		
		放治単独（n＝30）	放治＋TACE（n＝29）	放治＋TAI（n＝27）
1	臨床診断	肝細胞癌	肝細胞癌	肝細胞癌
2	肝外転移	無	無	無
3	主な治療	その他の治療法	その他の治療法	その他の治療法
4	その他の治療法/放射線療法	有	有	有
5	塞栓療法（TACE）	無	有	無
6	化学療法（TAI）	無	無	有
7	手術	無	無	無
8	局所療法	無	無	無

放治：放射線治療

HCCに対する重荷電粒子線治療として現在，HCCに対する陽子線治療症例数と，観察期間の長さにおける臨床治験の蓄積は筑波大学の施設において施行されたものが最大規模である[15]。これについで，国立がんセンター東病院[16]，米国ロマリンダ大学も報告をしている[27]。陽子線は，最も実用に近い新しい放射線治療というのが大方の一致した見解である。福井県の敦賀にも陽子線専用施設，兵庫県の播磨公園科学都市の兵庫粒子線センター，静岡県立がんセンターなどで陽子線治療専用施設が完成しすでに稼働している。また重イオン治療に関しての臨床試験は1995年より放射線医学総合研究所において炭素イオンによって開始され，HCCに対してもphase Ⅱ臨床成績がすでに放射線医学総合研究所より報告されている[17]。兵庫粒子線センターにおいても炭素イオンを用い試行している。

Ⅴ．本邦における肝細胞癌に対する放射線治療の現状

前述のごとく第17回全国原発性肝癌追跡調査報告によると，肝細胞癌（HCC）の手術以外の非観血的治療法として，RFA，PEI，TACEが主流を占める。一方，放射線照射療法はわずか1.5%である[1]。しかも詳細な内容は明らかにされていない。上述のごとく近年，肝細胞癌に対する放射線治療の進歩は著しく，有効性を示す成績も散見される。そこで，本邦におけるHCCに対する放射線照射療法の現状を明らかにするため全国追跡調査の資料を検討した[28]。

2000年1月から2003年12月（第16～17回追跡調査）の4年間に，手術，TACE，局所療法を同時期に併用しなかった37,645例の肝細胞癌中，肝外転移を合併しない症例は34,067例であった。放射線照射療法が313例に施行され，予後因子の解析項目を満足する症例は86例であった。放射線照射療単独例は30例，TACE併用は29例，TAI併用27例であった。各群間における生存率，さらに生存率に寄与する因子について多変量解析を行った。対象を表1に示す。

検討結果は以下のようであった。①86例全体の生存率は，1生率：58%，2生率：58%，3生率：52%（2年9ヵ月）であった。②86例全体の生存率に関与する背景因子の単変量解析においては，年齢，Child-Pugh分類，腫瘍数，門脈侵襲，肝静脈浸潤，AFP値，TNM分類で有意差を認めた。③予後に寄与する因子をCox比例ハザードにて多変量解析を行った。その結果，肝障害度（AがBに比し），HCV抗体陽性率（HBsAg陽性に比し），Stage分類が有意な予後良好な因子であった。

肝細胞癌に対する放射線照射療法の効果は，3年生存率50%以上が得られ，併存肝障害の程度，脈管浸潤が生存率を規定する因子として重要であった。以上より，肝細胞癌に対し放射線照射療法を用いることは今後の新たな治療選択肢として，適応を考慮することで有用な位置を占める可能性が示唆された。今回の結果からでは数値からみると生存率がよい。その理由としては，1つには，陽子線，炭素線治療の成績が相当数となっていること，しかし一方放射線照射療例の頻度が313/37,645例（0.8%）と低く，解析例が86/313例（27.5%）と少ないという問題を抱えている。その根本的問題は，調査用紙において検討項目の無記入，無効が多く，脱落する例が多いことが挙げられる。

今後，放射線照射療法の発表症例を増やし，追跡調査用紙の項目に放射線治療の照射条件や線質などの項目を加えてよりupdateのものに改訂していく必要があると考えられた[27]。

ま と め

残念ながら，科学的根拠に基づく肝癌診療ガイドライン（2005年2月）には放射線療法に関する科学的根拠に関する項目はない。phase Ⅰ/Ⅱ試験での有用性で

もって施行されているのが現状である。本邦におけるHCCに対する放射線照射療法は従来の照射方法に加え，conformal radiotherapyと体幹部定位放射線治療，さらに陽子線，重粒子線などの新しい粒子線治療は，効果的かつ安全で耐用性に富み，さらには繰り返し可能であることが第二相試験で示されている。これらの利用により，外科手術同様，根治目的の治療選択肢の一つとして用いられる可能性があり，また腫瘍径や局在，血流，門脈塞栓，合併症などの条件に制限が少なく，HCCに対して幅広い適応を有する可能性が示唆されると考えられる。しかし，コストや保険診療，RCTによる科学的根拠に基づく有効性の確立など，いくつかの課題も抱えており，今後本邦における放射線照射療法の位置付けを明確にしていく必要があろう。

参考文献

1) 日本肝癌研究会：第17回原発性肝癌追跡調査報告(2002～2003)．2006.
2) 科学的根拠に基づく肝癌診療ガイドライン作成に関する研究班/編：科学的根拠に基づく肝癌診療ガイドライン2005年版．100-102，金原出版，2005.
3) 高良健司，大藤正雄，吉川正治：肝細胞癌に対する放射線療法の治療効果に関する検討．日消病会誌 **83**：1473-1482, 1986.
4) Ohto M, Ebara M, Yoshikawa M, et al.：Radiation therapy and percutaneous ethanol injection for the treatment of hepatocellular carcinoma. [In Okuda K, Ishak KG editors], Neoplasms of the liver. 335-341, Springer-Verlag, Tokyo, 1987.
5) 吉川正治，江原正明，大藤正雄，ほか：放射線療法と肝動脈塞栓療法の併用による肝細胞癌の治療—とくに単独療法との比較による治療効果の検討—．日消病会誌 **87**：255-234, 1990.
6) Matsuura M, Ishikawa A, Nakajima N, et al.：Radical radiation therapy for hepatocellular carcinoma. Nippon Acta Radiologica **54**：628-635, 1994.
7) Kim TH, Kim DY, Park JW, et al.：Three-dimensional conformal radiotherapy of unresectable hepatocellular carcinoma patients for whom transcatheter arterial chemoembolization was ineffective or unsuitable. Am J Clin Oncol **29**：568-75, 2006.
8) Mornex F, Girard N, Beziat C, et al.：Feasibility and efficacy of high-dose three-dimensional-conformal radiotherapy in cirrhotic patients with small-size hepatocellular carcinoma non-eligible for curative therapies-mature results of the French Phase II RTF-1 trial. Int J Radiat Oncol Biol Phys **66**：1152-1158. 2006.
9) Seong J, Park HC, Han KH, et al.：Clinical results of 3-dimensional conformal radiotherapy combined with transarterial chemoembolization for hepatocellular carcinoma in the cirrhotic patients. Hepatol Res **27**：30-35, 2003.
10) Takeda A, Takahashi M, Kunieda E, et al.：Hypofractionated stereotactic radiotherapy with and without transarterial chemoembolization for small hepatocellular carcinoma not eligible for other ablation therapies：Preliminary results for efficacy and toxicity. Hepatol Res. [Epub ahead of print]. 2007.
11) Choi BO, Jang HS, Kang KM, et al：Fractionated stereotactic radiotherapy in patients with primary hepatocellular carcinoma. Jpn J Clin Oncol **36**：154-158, 2006.
12) Cheng SH, Lin Y-M, Chuang VP et al.：A pilot study of three-dimensional conformal radiotherapy in unresectable hepatocellular carcinoma. J Gastroenterol Hehatol **14**：1025-1033, 1999.
13) Tanaka N, Matsuzaki Y, Chuugannji Y, et al.：Proton irradiation for hepatocellular carcinoma. The Lancet **28**：1358, 1992.
14) Matsuzaki Y, Osuga T, Saito Y, et al.：A new effective and safe therapeutic option using proton irradiation for hepatocellular carcinoma. Gastroenterology **106**：1032-1041, 1994.
15) Chiba T, Tokuuye K, Matsuzaki Y, et al.：Proton beam therapy for hepatocellular carcinoma：a retrospective review of 162 patients. Clin Cancer Res **11**：3799-3805, 2005.
16) Kawashima M, Furuse J, Nishio T, et al.：Phase II study of radiotherapy employing proton beam for hepatocellular carcinoma. J Clin Oncol **23**：1839-1846, 2005.
17) Kato H, Tsujii H, Miyamoto T, et al.：Results of the first prospective study of carbon ion radiotherapy for hepatocellular carcinoma with liver cirrhosis. Liver Cancer Working Group. Int J Radiat Oncol Biol Phys **59**：1468-1476, 2004.
18) 大原　潔，菅原信二，吉田次男，ほか：肝癌集学的治療における肝部分照射の放射線耐容．日本医放会誌 **50**：146-154, 1990
19) Lin CS, Jen YM, Chiu SY, et al.：Treatment of portal vein tumor thrombosis of hepatoma patients with either stereotactic radiotherapy or three-dimensional conformal radiotherapy. Jpn J Clin Oncol **36**：212-217, 2006.
20) Hata M, Tokuuye K, Sugahara S, et al.：Proton beam therapy for hepatocellular carcinoma with portal vein tumor thrombus. Cancer **104**：794-801, 2005.
21) Yamada K, Izaki K, Sugimoto K, et al.：Prospective trial of combined transcatheter arterial chemoembolization and three-dimensional conformal radiotherapy for portal vein tumor thrombus in patients with unresectable hepatocellular carcinoma. Int J Radiat Oncol Biol Phys **57**：113-119, 2003.
22) Tazawa J, Maeda M, Sakai Y, et al.：Radiation ther-