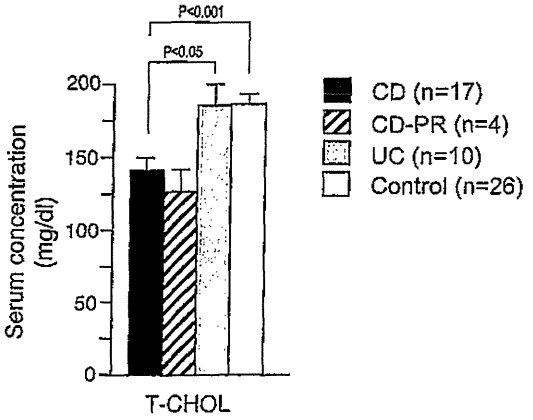

FIGURE 1. Serum total cholesterol concentrations in patients with IBD. T-CHOL, total cholesterol; CD, CD; CD-PR, CD postresection of distal ileum; UC, ulcerative colitis.

(Thermo Fisher Scientific, Waltham, MA) equipped with an HESI-II probe and a Prominence ultra fast liquid chromatography system (Shimazu, Kyoto, Japan).

Serum concentrations of C4 were determined by LC-MS/MS without alkaline hydrolysis.[21] Deuterium-labeled C4 was added to 20 μL of serum, and C4 was extracted with acetonitrile. After derivatization into the picolinyl ester, the sample was analyzed by the LC-ESI-MS/MS system described above.

Calculation of Relative Noncholesterol Sterol Concentrations

In this study, we assayed CYP3A4 activity and steroid metabolism by measuring serum sterol markers. Because most noncholesterol sterols are transported in serum with cholesterol,[22] each sterol level expressed relative to total cholesterol concentration seems to be a more reliable biomarker compared with the absolute concentration especially when dyslipidemia is present.[23] We first compared total serum cholesterol concentrations among CD, UC, and control groups. As shown in Figure 1, total serum cholesterol concentrations in patients with CD were significantly lower than those of UC and control subjects. Therefore, the data of serum noncholesterol sterols were expressed as relative to the total cholesterol concentration.

Quantification of Serum FGF19

Serum FGF19 levels were determined using a commercially available ELISA Kit (Quantikine Human FGF-19 Immunoassay; R&D Systems, Inc., Minneapolis, MN) following the manufacturer's instruction.

Statistics

Data are expressed as the mean ± standard error of the mean. The statistical significance of differences between the results in the different groups was evaluated using nonparametric Mann–Whitney test. Correlation was tested by calculating Pearson's correlation coefficient, r. For all analyses, significance was determined at the level of $P < 0.05$.

RESULTS

Evaluation of CYP3A4 Activity in Patients with CD

Serum oxysterol levels are shown in Figure 2. The concentrations of 4β-hydroxycholesterol (4β-HC) and 25-hydroxycholesterol (25-HC), immediate CYP3A4-induced metabolites of cholesterol, were both reduced in patients with CD compared with UC and control subjects. The reduction was more remarkable in patients with CD with the history of ileal resection and was statistically significant compared with patient with CD without a history of previous surgery. In contrast, the serum concentrations of other major oxysterols, 24S-hydroxycholesterol (24S-HC), and 27-hydroxycholesterol (27-HC) that are products of CYP46A1 and CYP27A1, respectively, were not decreased but rather increased in patients with CD compared with controls.

Bile Acid Metabolism in Patients with CD

Serum concentrations of C4 and FGF19 were compared among CD, UC, and control subjects (Fig. 3). The levels of C4 in patients with CD were significantly higher than that of controls. In particular, the levels in patients with CD with the history of ileal resection were markedly elevated and were significantly higher than that in patients with CD without a history of previous surgery. On the other hand, serum levels of FGF19 in patients with

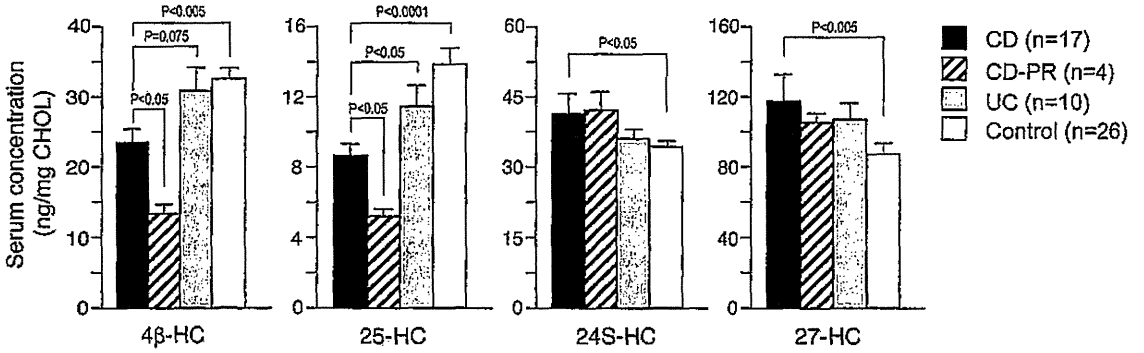

FIGURE 2. Serum oxysterol concentrations in patients with IBD. 4β-HC, 4β-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; CD-PR, CD postresection of distal ileum.

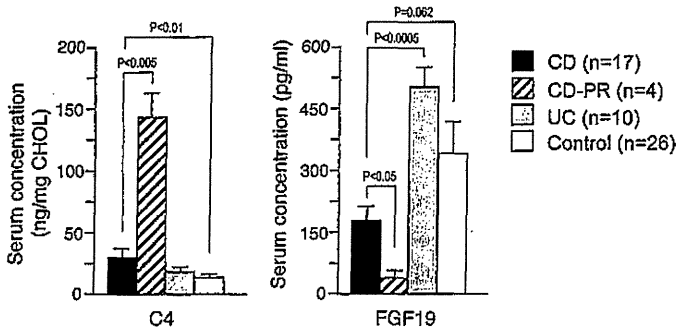

FIGURE 3. Serum C4 and FGF19 concentrations in patients with IBD. C4, 7α-hydroxy-4-cholesten-3-one; FGF19, fibroblast growth factor 19; CD-PR, CD postresection of distal ileum.

CD were significantly lower than those in UC and tended to be low ($0.05 < P < 0.1$) compared with those in control subjects. Patients with CD with the history of ileal resection showed remarkable reduction of serum FGF19 concentrations, and FGF19 levels were significantly lower than those in patients with CD without a history of previous surgery.

The relationships between serum C4 levels and serum 4β-HC and 25-HC concentrations in all 21 patients with CD with and without a history of previous surgery are shown in Figure 4. Both 4β-HC ($r = -0.530$, $P < 0.05$) and 25-HC ($r = -0.553$, $P < 0.01$) concentrations were negatively correlated with C4 concentrations.

Cholesterol Metabolism in Patients with IBD

Serum concentrations of sitosterol, campesterol, and lathosterol in CD, UC, and control subjects are shown in Figure 5.

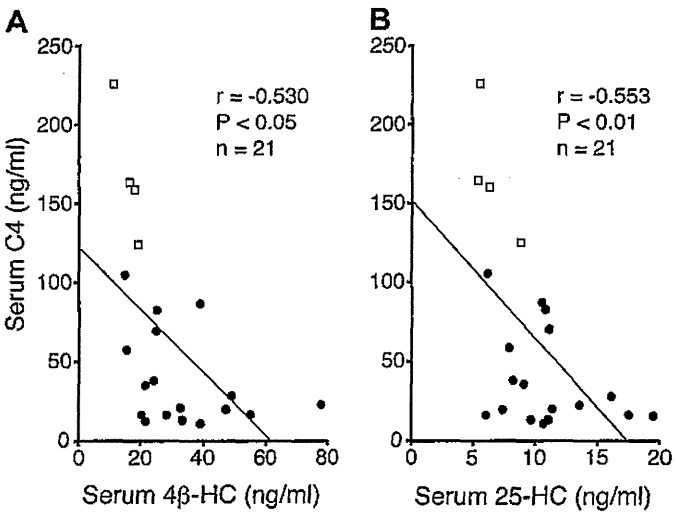

FIGURE 4. Correlations between serum concentrations of C4 and 4β-HC (A) or 25-HC (B) in 21 patients with CD. Closed circles represent patients with CD without a previous history of surgery (n = 17), whereas open squares show patients with CD after resection of distal ileum (n = 4). C4, 7α-hydroxy-4-cholesten-3-one; 4β-HC, 4β-hydroxycholesterol; 25-HC, 25-hydroxycholesterol.

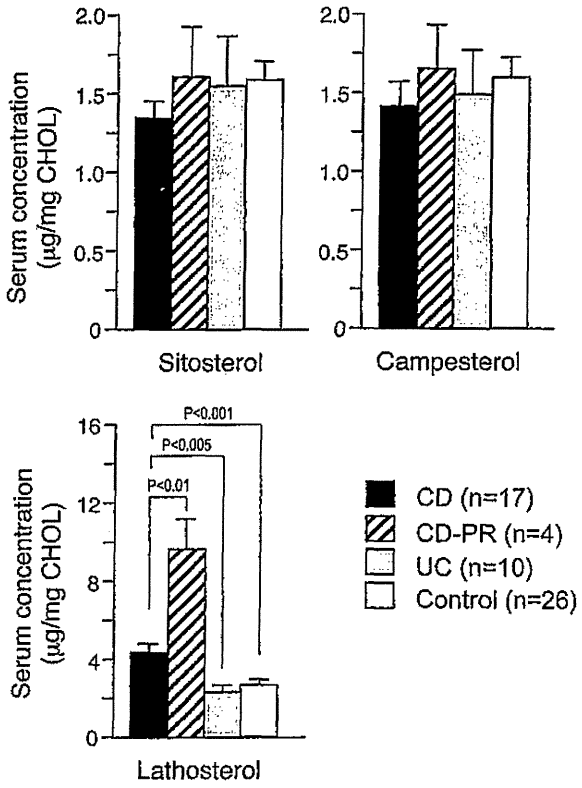

FIGURE 5. Serum plant sterols (sitosterol and campesterol) and lathosterol concentrations in patients with IBD. CD-PR, CD postresection of distal ileum.

The levels of sitosterol and campesterol, markers for intestinal cholesterol absorption,[24] were not significantly different among the groups. In contrast, the concentrations of lathosterol, a marker of whole-body cholesterol biosynthesis,[25] were significantly increased in patients with CD compared with UC and control subjects. In particular, the concentrations in patients with CD with the history of ileal resection were markedly elevated and were significantly higher than those in patients with CD without a history of previous surgery.

Effects of Disease Location and Medications on Serum Markers

Effects of disease location and medications on serum markers in patients with CD are shown in Table 2. Among the 17 patients with CD without a previous history of surgery, 16 were ileum or ileum + colon type and only 1 was colon type. When we compared serum markers in the colon-type patient with those in the other ileum and ileum + colon types, total cholesterol, 4β-HC, and FGF19 concentrations were high, and the lathosterol concentration was low and similar to the UC and control subjects. Effects of medications on serum markers were also studied in the 17 patients with CD without a previous history of surgery. The use of corticosteroids, azathioprine, or infliximab did not significantly affect the serum concentrations of any markers we studied.

TABLE 2. Effects of Disease Location and Medications on Serum Markers for Cholesterol Metabolism

	Location of CD		Medications					
			Corticosteroid		Azathioprine		Infliximab	
Serum markers	Ileum or ileum + colon n = 16	Colon n = 1	With n = 4	Without n = 13	With n = 8	Without n = 9	With n = 9	Without n = 8
T-CHOL (mg/dL)	140 ± 9 (120–160)	166[a]	132 ± 25	144 ± 9	131 ± 12	151 ± 13	138 ± 13	145 ± 13
Lathosterol (μg/mg CHOL)	4.4 ± 0.5 (3.3–5.5)	2.3[a]	3.4 ± 0.7	4.6 ± 0.6	5.1 ± 0.9	3.6 ± 0.3	4.8 ± 0.8	3.7 ± 0.4
Sitosterol (μg/mg CHOL)	1.3 ± 0.1 (1.1–1.6)	1.6	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.4 ± 0.2
Campesterol (μg/mg CHOL)	1.4 ± 0.1 (1.1–1.7)	1.5	1.4 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.5 ± 0.2	1.4 ± 0.1	1.4 ± 0.2
4β-HC (ng/mg CHOL)	23 ± 2 (18–27)	29[a]	25 ± 4	23 ± 2	21 ± 3	25 ± 3	23 ± 3	23 ± 3
25-HC (ng/mg CHOL)	8.2 ± 1.0 (6.1–10.4)	9.8	12 ± 3	7.3 ± 0.7	9.6 ± 1.7	7.2 ± 1.0	9.3 ± 1.5	7.3 ± 1.1
24S-HC (ng/mg CHOL)	44 ± 3 (38–50)	28[a]	53 ± 9	40 ± 2	47 ± 5	39 ± 3	47 ± 5	38 ± 3
27-HC (ng/mg CHOL)	120 ± 14 (91–149)	92	124 ± 37	116 ± 14	137 ± 25	102 ± 9	130 ± 23	105 ± 10
C4 (ng/mg CHOL)	28 ± 6 (15–40)	16	21 ± 6	29 ± 7	29 ± 6	25 ± 9	27 ± 6	27 ± 10
FGF19 (ng/mL)	162 ± 36 (85–239)	356[a]	160 ± 76	178 ± 42	120 ± 41	221 ± 54	110 ± 37	245 ± 55

[a]Outside the 95% confidence intervals for means of ileum or ileum + colon types of CD.
CHOL, cholesterol; 4β-HC, 4β-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; C4, 7α-hydroxy-4-cholesten-3-one. Mean ± standard error of the mean values are given. Ninety-five percent confidence intervals for means are in parenthesis.

DISCUSSION

Our data clearly demonstrated that serum 4β-HC and 25-HC concentrations were significantly decreased in patients with CD especially in those with a history of ileal resection. Since both 4β-HC and 25-HC are synthesized from cholesterol by CYP3A4[26] and 4β-HC is considered as a biomarker of whole-body CYP3A4 activity,[20] the reduction of these oxysterols strongly suggests that CYP3A4 activity is downregulated in patients with CD. In contrast to CD, patients with UC did not show significant reduction of these oxysterol concentrations, which suggests that the inhibition of CYP3A4 activity is not a common feature of all IBD.

There were some possibilities that the drugs used in patients with CD affected the CYP3A4 activity. However, 5-aminosalicylates had been used in all patients with IBD (Table 1). When we compared serum markers in patients with CD with and without taking corticosteroids, azathioprine, or infliximab, they were not significantly affected by these drugs (Table 2). Therefore, the inhibition of CYP3A4 activity in patients with CD was not explained by these medications.

CYP3A4 is one of the most important enzymes involved in the metabolism of xenobiotics and is found in many organs in the human body. The expression of CYP3A4 in both the intestine and liver is a clinically important determinant of serum concentrations of certain medicines. In patients with CD, however, the destruction of intestinal mucosa does not seem to be a direct cause of the reduced CYP3A4 activity measured by the serum markers because this enzyme activity in the ileum is much lower than that in duodenum and in jejunum,[27] and the main locus of inflammation in our patients was the ileum. In addition, Paine et al[28] have demonstrated that median intestinal microsomal CYP3A protein content is several times lower than that in the liver, and the total microsomal mass for the whole intestine is about 26 times lower than that for the liver. Therefore, total expression amount of CYP3A4 in the intestine is calculated to be about 100 times lower than that in the liver.[29] Thus, decreased serum 4β-HC and 25-HC concentrations observed in patients with CD seems to reflect mainly the decreased CYP3A4 and PXR activities in the liver.

Why is hepatic PXR deactivated in this intestinal disease? It has been reported that proinflammatory cytokines, such as TNF-α, can downregulate the gene expression of CYP3A.[30] However, our results showed that treatment of CD with infliximab, a monoclonal antibody against TNF-α, did not affect serum concentrations of either 4β-HC or 25-HC (Table 2). Since bile acids are ligands of PXR and induce CYP3A4 expression through the activation of PXR,[18,19] reduced enterohepatic circulation of bile acids due to BAM was strongly suggested as the mechanism of PXR deactivation in the liver. Although we did not measure hepatic bile acid concentrations directly in our patients with CD, these levels seemed to be reduced irrespective of the history of ileal resection. Serum concentrations of C4, a marker for hepatic bile acid biosynthesis,[16] were significantly elevated to compensate for the reduced bile acid pool, and the levels of FGF19, a marker for intestinal bile acid flux,[17] were reduced in both ileal-resected and nonresected patients with CD.

A previous report by Lenicek et al[15] also showed that serum concentrations of C4 were high not only in ileal-resected patients with CD but also in nonresected patients with CD with ileitis. However, it was still controversial if the elevation of serum C4 concentrations commonly occurs in the nonresected patients with CD or not.[31] While Lenicek et al measured serum C4 concentrations

by a less sensitive HPLC-UV method, we quantified the levels with 1000 times more sensitive HPLC-MS/MS method using [$^2H_7$]C4 as an internal standard.[32] Our data support their result that the elevation of serum C4 concentrations commonly occurs in nonresected patients with CD with ileitis. In addition, serum 4β-HC and 25-HC levels were significantly reduced in our nonresected patients with CD. When we plotted serum C4 concentrations against serum 4β-HC or 25-HC concentrations in all patients with CD, a significant inverse correlation was observed (Fig. 4A, B). The results suggest that BAM develops not only in ileal-resected but also in patient with nonresected CD with ileitis, and the inhibition of CYP3A4 or PXR activity in patients with CD is associated with the severity of BAM.

Although we do not have any data on intestinal CYP3A4 or PXR activities in patients with CD, these molecules are likely deactivated by the reduced enterohepatic bile acid pool. Since the activation of PXR inhibits the production of TNF-α,[8,9] deactivated PXR in patients with CD may relate to the continuous overproduction of this cytokine. In addition, the reduced bile acid pool deactivates not only PXR but also another nuclear receptor, FXR. Similar to PXR, FXR activation also inhibits proinflammatory cytokine production and may ameliorate intestinal inflammation.[33] Deactivation of FXR in CD but not in patients with UC was reported by demonstrating reduced intestinal mRNA expression levels of the FXR target gene small heterodimer partner (SHP).[34] Our data also suggest that FXR is deactivated in CD but not in patients with UC because the expression of FGF19, another target gene of FXR,[35] was decreased only in CD.

CYP3A4 is the most abundantly expressed form of P450 in human liver (as much as 60% of all hepatic P450)[36] and small intestine,[27] and it has been estimated that more than 50% of prescription drugs are metabolized by this P450 enzyme. Our results showed that bile acids were the critical molecules for maintaining baseline activity of hepatointestinal CYP3A4 and that BAM significantly downregulated CYP3A4 activity in patients with CD. These results suggest that bioavailability of CYP3A4-metabolizing drugs in patients with CD can be increased by BAM and by direct injury of intestinal mucosa.

Finally, hypocholesterolemia is one of the biochemical features of patients with CD. Our data also showed that total serum cholesterol concentrations were significantly lower in patients with CD compared with control and patients with UC. However, intestinal cholesterol absorption measured by serum sitosterol and campesterol concentrations and cholesterol biosynthesis measured by serum lathosterol concentration were not significantly reduced in our patients with CD (Fig. 5). Therefore, it is important to note that hypocholesterolemia in patients with CD is caused not only by cholesterol malabsorption but also by increased bile acid synthesis due to BAM.

In summary, serum biomarker analyses strongly suggested that hepatointestinal CYP3A4 activity was downregulated in patients with CD with ileal inflammation or resection. Enterohepatic circulation of bile acids is a key factor for the preservation of baseline activity of hepatointestinal PXR. The deactivation of PXR can affect the pharmacokinetics of CYP3A4-metabolizing drugs and may relate to the continuous overproduction of proinflammatory cytokines in patients with CD.

REFERENCES

1. Podolsky DK. Inflammatory bowel disease. *N Engl J Med.* 2002;347:417–429.
2. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet.* 2007;369:1641–1657.
3. Zhou C, Verma S, Blumberg B. The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism. *Nucl Recept Signal.* 2009;7:e001.
4. Faucette SR, Sueyoshi T, Smith CM, et al. Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor. *J Pharmacol Exp Ther.* 2006;317:1200–1209.
5. Lehmann JM, McKee DD, Watson MA, et al. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest.* 1998;102:1016–1023.
6. Zhou C, Tabb MM, Nelson EL, et al. Mutual repression between steroid and xenobiotic receptor and NF-κB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest.* 2006;116:2280–2289.
7. Shah YM, Ma X, Morimura K, et al. Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF-κB target gene expression. *Am J Physiol Gastrointest Liver Physiol.* 2007;292:G1114–G1122.
8. Mencarelli A, Migliorati M, Barbanti M, et al. Pregnane-X-receptor mediates the anti-inflammatory activities of rifaximin on detoxification pathways in intestinal epithelial cells. *Biochem Pharmacol.* 2010;80:1700–1707.
9. Mencarelli A, Renga B, Palladino G, et al. Inhibition of NF-κB by a PXR-dependent pathway mediates counter-regulatory activities of rifaximin on innate immunity in intestinal epithelial cells. *Eur J Pharmacol.* 2011;668:317–324.
10. Dring MM, Goulding CA, Trimble VI, et al. The pregnane X receptor locus is associated with susceptibility to inflammatory bowel disease. *Gastroenterology.* 2006;130:341–348; quiz 592.
11. Glas J, Seiderer J, Fischer D, et al. Pregnane X receptor (PXR/NR1I2) gene haplotypes modulate susceptibility to inflammatory bowel disease. *Inflamm Bowel Dis.* 2011;17:1917–1924.
12. Shafran I, Burgunder P. Adjunctive antibiotic therapy with rifaximin may help reduce Crohn's disease activity. *Dig Dis Sci.* 2010;55:1079–1084.
13. Cheng J, Shah YM, Ma X, et al. Therapeutic role of rifaximin in inflammatory bowel disease: clinical implication of human pregnane X receptor activation. *J Pharmacol Exp Ther.* 2010;335:32–41.
14. Akerlund JE, Reihner E, Angelin B, et al. Hepatic metabolism of cholesterol in Crohn's disease. Effect of partial resection of ileum. *Gastroenterology.* 1991;100:1046–1053.
15. Lenicek M, Duricova D, Komarek V, et al. Bile acid malabsorption in inflammatory bowel disease: assessment by serum markers. *Inflamm Bowel Dis.* 2011;17:1322–1327.
16. Sauter G, Berr F, Beuers U, et al. Serum concentrations of 7α-hydroxy-4-cholesten-3-one reflect bile acid synthesis in humans. *Hepatology.* 1996;24:123–126.
17. Lundasen T, Galman C, Angelin B, et al. Circulating intestinal fibroblast growth factor 19 has a pronounced diurnal variation and modulates hepatic bile acid synthesis in man. *J Intern Med.* 2006;260:530–536.
18. Xie W, Radominska-Pandya A, Shi Y, et al. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A.* 2001;98:3375–3380.
19. Staudinger JL, Goodwin B, Jones SA, et al. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A.* 2001;98:3369–3374.
20. Diczfalusy U, Nylen H, Elander P, et al. 4β-Hydroxycholesterol, an endogenous marker of CYP3A4/5 activity in humans. *Br J Clin Pharmacol.* 2011;71:183–189.
21. Honda A, Miyazaki T, Ikegami T, et al. Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC-ESI-MS/MS. *J Steroid Biochem Mol Biol.* 2010;121:556–564.
22. Babiker A, Diczfalusy U. Transport of side-chain oxidized oxysterols in the human circulation. *Biochim Biophys Acta.* 1998;1392:333–339.
23. Honda A, Yoshida T, Xu G, et al. 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.* 2004; 53:42–48.
24. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol.* 1990;131: 20–31.
25. Kempen HJ, Glatz JF, Gevers Leuven JA, et al. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. *J Lipid Res.* 1988;29:1149–1155.
26. Honda A, Miyazaki T, Ikegami T, et al. Cholesterol 25-hydroxylation activity of CYP3A. *J Lipid Res.* 2011;52:1509–1516.
27. Zhang QY, Dunbar D, Ostrowska A, et al. Characterization of human small intestinal cytochromes P-450. *Drug Metab Dispos.* 1999;27:804–809.
28. Paine MF, Khalighi M, Fisher JM, et al. Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism. *J Pharmacol Exp Ther.* 1997;283:1552–1562.
29. Galetin A, Gertz M, Houston JB. Contribution of intestinal cytochrome p450-mediated metabolism to drug-drug inhibition and induction interactions. *Drug Metab Pharmacokinet.* 2010;25:28–47.
30. Abdel-Razzak Z, Loyer P, Fautrel A, et al. Cytokines down-regulate expression of major cytochrome P-450 enzymes in adult human hepatocytes in primary culture. *Mol Pharmacol.* 1993;44:707–715.
31. Kunstar E, Hegyi P, Rakonczay Z Jr, et al. Is bile acid malabsorption really a common feature of Crohn's disease or is it simply a consequence of ileal resection? *Front Physiol.* 2011;2:28.
32. Honda A, Yamashita K, Numazawa M, et al. 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.
33. Gadaleta RM, van Erpecum KJ, Oldenburg B, et al. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. *Gut.* 2011;60:463–472.
34. Nijmeijer RM, Gadaleta RM, van Mil SW, et al. Farnesoid X receptor (FXR) activation and FXR genetic variation in inflammatory bowel disease. *PLoS One.* 2011;6:e23745.
35. Holt JA, Luo G, Billin AN, et al. Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev.* 2003;17:1581–1591.
36. Guengerich FP. Mechanism-based inactivation of human liver microsomal cytochrome P-450 IIIA4 by gestodene. *Chem Res Toxicol.* 1990;3:363–371.

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Scientific Research

Bicarbonate Attenuates Irinotecan-Induced Cytotoxicity through Regulation of Both Extracellular and Intracellular *pHs* in Intestine Cell Line

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ABSTRACT

The anti-cancer therapy of irinotecan (CPT-11) is often limited due to severe late-onset diarrhea. Because the higher toxic form of CPT-11/its active metabolite (SN-38) is produced at acidification, the usefulness of oral sodium bicarbonate treatment against the CPT-11/SN-38-induced intestinal injuries and diarrhea has been confirmed. However, the roles of bicarbonate have been suggested to affect not only intestinal *pH* environment but also intracellular *pH* and CPT-11/SN-38 dynamics. The present study proposed to clarify the hypothesis in CPT-11/SN-38-exposed colon cell line in various *pH* conditions adjusted by bicarbonate. HT29 cell pre-exposed to ~1.0 μM SN-38 lactone or carboxylate forms was incubated at different *pH* adjusted by either bicarbonate or HCl/NaOH. The degrees of SN-38-induced cell injury depended on the higher proportion of the toxic form (lactone) of SN-38 rather than mere *pH* condition of medium. Apoptosis and cell injury induced by SN-38 were significantly inhibited by bicarbonate in a dose-dependent manner. Intercellular *pH* acidification induced by SN-38 was significantly prevented by 30 mM bicarbonate. Cell cytotoxicity of SN-38 depended on not only extracellular but also intracellular *pH* that converts the SN-38 form, while the intracellular acidification was prevented by bicarbonate. The multiple regulations of bicarbonate on both exracellular and intracellular *pH* would be essential mechanism against intestinal cell injury by CPT-11/SN-38.

Keywords: Bicarbonate; SN-38; CPT-11; *pHi*; Anti-Cancer Drug; Intestine

1. Introduction

Irinotecan hydrochloride (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin) is a water-soluble derivative of camptothecin, an alkaloid isolated from Camptotheca acuminata, and presents a wide spectrum of antitumor activity through the inhibition of DNA topoisomerase I [1]. This chemotherapeutic agent has been approved by the FDA for treatment of colorectal cancer in the United States, but has shown clinical responses for many other malignancies such as lung, gastric, pancreatic, cervical, and ovarian cancers as well as leukemia and lymphoma [2-6]. However, this agent has major limitation in the therapeutic use for the patients with cancer due to severe side effect as the lethal delayed diarrhea [7,8] including the marked intestinal injury and the presence of pseudomembranous jejunoileitis [9].

CPT-11 is hydrolyzed to active 7-ethyl-10-hydrocy-camptothecin (SN-38) by hepatic carboxylesterase [10], and SN-38 has at least a 100-fold more potent antitumor effect than CPT-11 [1]. The ability of both CPT-11 and SN-38 relies on *pH*. Both CPT-11 and SN-38 have a labile α-hydroxy-3-lactone ring, which undergoes reversible hydrolysis at a rate that is mainly *pH*-dependent [11]. At acidic condition, the lactone ring of CPT-11/SN-38 closes, and this form is called as a lactone form. On the other hand, the lactone ring opens at physiological *pH* and higher, and yields a carboxylate form. The carboxy-

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late form is a less potent inhibition of the topoisomerase I and has much weaker antitumor activity than its lactone counterpart. Therefore, one of the possible strategies for prevention from the CPT-11-induced side effects might be manipulating the *pH*-dependent interconversion. Indeed, we and others have previously confirmed the oral bicarbonate (HCO_3^-) administration prior to and/or during CPT-11 treatment avoided acidifications in intestinal lumen and stool, and consequently, could decrease the incidence of side effects including delayed diarrhea, nausea, vomiting, myelotoxicity, and lymphocytopenia in the patients with lung cancer [12], advanced gastrointestinal cancer [13] or colorectal cancer [14], and the experimental animal model [12,15]. Therefore, it has been believed that oral HCO_3^- treatment is effectiveness on the attenuation of the side effects in CPT-11 anticancer therapy through the keeping of intestinal alkalization.

Additionally, our previous studies also confirmed that there was significant difference in the initial uptake rate of CPT-11/SN-38 by isolated hamster intestinal cells between the respective lactone and carboxyrate forms [9,15]. The uptake rate of the lactone form that is passively transported was higher than the carboxyrate form that is actively absorbed. Therefore, the regulation of ambient *pH* environment is also important factor for the intracellular uptake of CPT-11/SN-38.

Furthermore, intracellular *pH* (*pHi*) is also associated with anti-cancer action of the agent, because it generally influences the cellular proliferation and death [16-19]. Because intracellular acidification is an early event in apoptosis and also essential in genomic DNA destruction [17], it has been also considered as one of the multiplier factors to emphasize the inhibitive action of SN-38 on the topoisomerase I [20]. In addition to the regulation of ambient *pH*, HCO_3^- has been reported to act as a regulator of *pHi* in some cells through Na^+-dependent Cl^-/HCO_3^- exchange, Na^+-independent Cl^-/HCO_3^- exchange, and Na^+-HCO_3^- cotransport [21-23]. So, it is suggested that HCO_3^- might have another potential protective action against SN-38 through the regulation of the *pHi*.

While the oral HCO_3^- treatment has demonstrated scientific and clinical benefits on CPT-11 treated anticancer therapy, there is few understanding of its action. In the present study, we purposed to clarify the implication of HCO_3^- on *pH*-dependent CPT-11/SN-38 toxicity in the human colon adenocarcinoma HT29 cell line under various culture *pH* environments adjusted with/without HCO_3^-.

2. Materials and Methods

2.1. Materials

SN-38 was supplied by Yakult Honsha Co., Ltd. (Tokyo, Japan) and was 98% - 99% pure as judged by gas-liquid chromatography. Nigericine was purchased from Sigma (St. Louis, MO). Snarf1-AM was from Calbiochem (San Diego, CA). Other chemicals were of the highest purity available. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco®, Invitrogen Corporation (Carlsbad, CA). For the culture conditions, the HT29 cell line (ATCC®, Manas- sas, VA) was maintained in DMEM supplemented with 10% heat-inactivated FBS, as previously described [9,24, 25]. The HT29 model was selected since we have previ- ously reported a similar respective mechanism of uptake of SN-38 lactone and carboxylate to that reported in isolated intestinal cells [26]. Moreover, HT29 cells allow for long-term studies that are not possible using the isolated enterocyte model.

2.2. Chemical (SN-38) Preparation

SN-38 stock solution was prepared by dissolution with DMSO due to highly hydrophobicity. The SN-38 stock solution was incubated with 50 mM PBS, *pH* 3.0 or 9.0, overnight at RT, as a 100-fold dilution, to obtain the lactone and carboxylate forms, respectively [27]. At this final concentration, DMSO was confirmed to have no effect on the initial uptake of the agent [26]. SN-38 at concentrations up to 5 μM did not significantly change the *pH* of the culture medium. The resultant solution was 98% lactone form at *pH* 3.0 and 97% carboxylate form at *pH* 9.0 (HPLC determination by Dr. K. Arimori and T. Hidaka, Dept. Pharmacy, Miyazaki Medical School, Miyazaki, Japan). For cell culture, SN-38 was diluted to final concentrations of 0.5 or 1.0 μM in DMEM.

2.3. Effect of Extracellular *pH* on SN-38-Induced Cytotoxicity

HT29 cells seeded on a 96-well plate as density at 1×10^4 cells/well were cultured for 24 h, and then exposed to either 0 or 0.5 μM SN-38 lactone/carboxylate in DMEM for 2 h that the exposed period does not affect the interconversion of SN-38 [10,28]. The cells were then washed twice with PBS, and further incubated in DMEM without the SN-38 at *pH* 6.2 or 7.2 set by HCl/NaOH for 96 h. Thereafter, cell viability was measured by the MTT assay, and expressed as a relative ratio to control (0 μM SN-38) in each culture condition.

2.4. The Effect of Bicarbonate-Adjusted *pH* before and after SN-38 Exposure

HT29 cells seeded on a 24-well plate at a density of 5×10^4 cells/well were cultured in standard condition for 24 h, and then, were incubated with DMEM containing 25 mM HEPES and increasing concentrations (5, 20, 44, 100 mM) of HCO_3^- for 2 h prior to SN-38 exposure.

The HCO_3^- concentrations resulted in the *pH* adjustment at 7.0, 7.2, 7.4, and 7.8, respectively. The osmolality was maintained constant among the different HCO_3^- buffers. After the incubation with HCO_3^-, cell was exposed to either 0.5 or 1.0 μM SN-38 lactone for an additional 2 h. Thereafter, cell was washed twice with PBS, and further incubated for an additional 96 h with the respective HCO_3^- containing culture medium without SN-38. In parallel experiments, HT29 cell was cultured without HCO_3^- prior to the SN-38 exposure, and were incubated with the different concentrations of HCO_3^- during the additional 96 h exposure.

2.5. Cell Viability in Various *pH* Conditions with/without Bicarbonate

HT29 cell cultured under standard condition containing DMEM with 44 mM HCO_3^-, at *pH* 7.4, was exposed to either 0 or 1.0 μM SN-38 lactone for 2 h. Thereafter, the washed cell was further incubated for an additional 48 h with 5-set points of *pH* at 6.8, 7.0, 7.4, 7.8, or 8.2 in culture medium adjusted by either increasing concentrations of HCO_3^- [5, 10, 20, 44, 100 mM, respectively; HCO_3^- (+)] or NaOH with 5 mM HCO_3^- [HCO_3^- (−)]. During cell culture, acidification of medium is usually induced due to respiration of cells. Therefore, in the present experiment, the cell was cultured with different media in each condition to maintain *pH* during culture. DMEM contained 2.5 mM·L-glutamate, 1 mM sodium pyruvate, and 10% FBS, and each culture medium was constituted as shown in **Table 1**. The constitution of the medium was based on a previous study by Eagle *et al.* [29] and further modified in the present study. Cell proliferation after culture in these conditions was determined by the MTT assay.

For detection of apoptotic cells in the various pH conditions either with or without HCO_3^-, following exposure to SN-38 lactone, the cell was incubated with 3-set points of *pH* at 6.8, 7.0, or 7.8 with increasing HCO_3^- concentrations [HCO_3^- (+)] or without HCO_3^- [HCO_3^- (−)] for an additional 48 h.

Table 1. Conditions of culture medium at different *pHs*.

HCO_3^- (+)	*pH* 6.8	*pH* 7.0	*pH* 7.4	*pH* 7.8	*pH* 8.2
$NaHCO_3$ (mM)	5	10	20	44	100
Hepes (mM)	25	25	25	25	25
HCO_3^- (−)	*pH* 6.8	*pH* 7.0	*pH* 7.4	*pH* 7.8	*pH* 8.2
$NaHCO_3$ (mM)	5	5	5	5	5
Trice (mM)	10	0	0	0	0
Hepes (mM)	25	25	20	20	20
Tris-base (mM)	0	0	25	25	25
NaH_2PO_4 (mM)	10	10	0	0	0

The cell was incubated in 5% and 0% CO_2, in HCO_3^- (+) and HCO_3^- (−), respectively.

2.6. Cell Viability Assay

Cell viability was analyzed as cell proliferation and apoptosis by the MTT assay [30] and flow cytometry, respectively. In the MTT assay, following incubation of the cell with the indicated concentration of the agents, the medium was removed and the cell was further incubated with 0.5 mg/mL MTT in medium for 4 h at 37°C. The blue formazan compound was solubilized with methanol containing 0.01 N HCl. The absorbance was determined at 560 nm, with 670 nm used as a reference in a spectrophotometer. In the flow cytometry, apoptotic HT29 cell was determined by annexin V and propidium iodide (PI) double staining using a commercially available kit (Annexin V-EGFP Apoptosis Detection Kit, BioVision, Inc., Mountain View, CA). Following the respective culture condition, the cell was harvested with trypsin and suspended with annexin V-enhanced green fluorescent protein (EGFP) and PI, and then analyzed by flow cytometry (FacSort, Becton Dickinson, San Jose, CA).

2.7. Intracellular *pH* Determination

HT29 cell plated at 1×10^6 cells per well was incubated with and without 0.5 μM SN-38 lactone for 2 h in culture medium containing increasing concentrations of HCO_3^- (10 - 58 mM). After removal of SN-38, the cell was maintained under the same culture conditions for an additional 4 h period before being loaded with 5 μM Snarf-1 acetoxymethyl acetate for 20 - 40 min at 37°C. The cell was then washed, and the respective cellular fluorescence level was determined with the ACAS 570 laser scanner cytometer (Meridian Instruments Inc., Okemos, MI). A *pH* standard curve (6.8, 7.2, 7.4, 7.6, and 8.0) using high potassium concentration (100 mM) was determined for each series of experiments using either PIPES for buffer $pH < 7$ or HEPES for buffer $pH > 7$ and 10 μM nigericin. The cell membrane potassium ionophore nigericin causes the interior of the cells to equilibrate with the external *pH* of the buffer. The cells were excited with a 514 nm argon laser beam (5 W, Innova 90/5, Coherent) and the 587 nm and 640 nm emissions were simultaneously collected. The fluorescence intensity at each wavelength was detected using a photomultiplier tube. Images were acquired by scanning several fields of cells. Each image consisted of 3000 - 5000 discrete sample points. The ratio of the 640/587 nm emission data was used to determine the *pHi*. On

average, the data were collected from 12 - 20 successive scans.

2.8. Statistical Analysis

Statistically significant differences were assessed by applying the Student's unpaired *t*-test, paired *t*-test, or analysis of variance (ANOVA) where applicable with an α level of 0.05. Control experiments, using only the vehicle for solubilizing the respective agent, were performed in parallel. The results were expressed as the mean ± SD of the mean based on the number of studies. Each experiment was performed in duplicate or triplicate.

3. Results

3.1. Effect of Extracellular *pH* on SN-38-Induced Cellular Toxicity

Table 2 shows the cell viability of HT29 cell after exposure to either the SN-38 lactone or carboxylate form in different two *pH* conditions at 7.4 or 6.2 adjusted by NaOH/HCl. At *pH* 7.4, cell viability was significantly decreased in the exposure to carboxylate form compared to that in the control, and was further and significantly decreased in the exposure to lactone form compared to that in other conditions. The significantly decreased cell viability in the lactone form rather than the carboxylate form was also observed at *pH* 6.2. In addition, the viability in lactone form at *pH* 6.2 was significantly lower than carboxylate form at *pH* 7.4. Compared between *pH* 7.4 and 6.2, the toxic level of SN-38 lactone was almost similar. These results are in agreement with the previous study reporting that cell cytotoxicity of SN-38 depended on the form, *i.e.*, lactate or carboxylate forms rather than extracellular *pH* [15].

3.2. The Difference of Bicarbonate Treatment between before and after SN-38 Exposure at Various *pH* Conditions in the Cellular Toxicity

Figure 1 presents the cell viability of HT29 cells cultured with increasing concentrations of HCO_3^- before to or after SN-38 exposures (0.5 or 1.0 μM) for 2 h. With HCO_3^- treatment before 0.5 μM SN-38 exposure, cell viability was significantly higher at *pH* 7.2, 7.4, and 7.8 than at *pH* 7.0, and at *pH* 7.8 was the highest. Similarly, with HCO_3^- treatment after 0.5 μM SN-38, cell viability at *pH* 7.4 and 7.8 was significantly higher compared to that at *pH* 7.0. Moreover, these characteristic patterns of viability seen in the 0.5 μM SN-38 were also observed in the exposure to 1.0 μM SN-38. There was no difference in SN-38-induced toxicity in the respective *pH* condition between HCO_3^- treatment before and after SN-38 exposures. The results indicate that HCO_3^- does not affect the conversion of SN-38 in the relatively short 2 h exposure period that is similar to small intestine transit time.

In addition, HT29 cell was incubated with the different HCO_3^- buffers for 4 h before and during 1.0 μM SN-38 lactone exposure. The cell was then washed and incubated in fresh DMEM containing 44 mM HCO_3^- (*pH*

Table 2. Effect of medium *pH* on SN-38 toxicity.

Agents	*pH* Culture medium	Cell viability (%)
Control	7.4	100
SN-38 Lactone	7.4	45.3 ± 1.1*†
SN-38 Carbonate	7.4	66.6 ± 3.8*
SN-38 Lactone	6.2	44.8 ± 1.6*†
Control	6.2	100

The values of cell viability are shown as the mean ± SD (%) relative to the respective control. *$p < 0.05$; Significantly difference from the respective control, †$p < 0.05$; Significantly difference from SN-38 Carbonate.

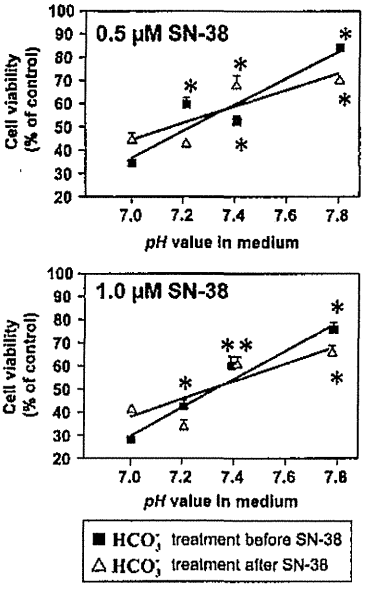

Figure 1. Effect of bicarbonate on SN-38-induced toxicity in HT29 cell. The cell was incubated with SN-38 (0.5 or 1.0 μM) for 2 h and increasing concentrations of bicarbonate (HCO_3^-; 5, 20, 44, and 100 mM) corresponding to *pH* 7.0, 7.2, 7.4, and 7.8, respectively, either for 2 h before (Closed squares) or after (Opened triangles) the SN-38 exposure. The cell in both conditions was further cultured in the medium with these increasing concentrations of HCO_3^- for additional 96 h. Cell viability was determined by the MTT assay. Data are shown as the mean ± SD of the percent of the respective control (0 μM SN-38 treatment). *Significantly different from the respective control determined in the presence of 5 mM bicarbonate, $p < 0.05$.

7.4) for 96 h. Because the result showed that SN-38-induced toxicity was around 50% under all the different conditions (data not shown), it implied that HCO_3^- could not modify the influence of SN-38 on cell viability.

3.3. Effect of Bicarbonate-Adjusted *pH* on Cell Viability

Figure 2(a) shows the viability of HT29 cell treated with various *pH* conditions (6.8 - 8.2) adjusted by increasing concentrations of HCO_3^- or NaOH for 48 h following a 2 h SN-38 exposure. In the HCO_3^- treatment, the cell viability was significantly enhanced at *pH* 7.4, 7.8, and 8.2 compared to that at *pH* 6.8. On the other hand, there was no difference in the viability of HT29 cell treated without HCO_3^- among these *pH* conditions. In these experiments, the *pH* in the medium was almost perfectly maintained even after cell culture, and there was no difference in *pH* after culture between control and SN-38 exposure in both experiments (**Figure 2(b)**).

Figure 3 shows the flow cytometry image (**Figure 3(a)**) and the percentage of apoptotic cells analyzed by flow cytometry (**Figure 3(b)**) in the different *pH* conditions (48 h) with or without HCO_3^- following a 2 h exposure to 1 μM SN-38 lactone. In the control, the per centage of necrotic cells (double positive for annexin V-EGFP and PI) was higher at *pH* 6.8 with 5 mM HCO_3^- than at *pH* 7.8 with 44 mM HCO_3^- (**Figure 3(a)**). The necrosis at *pH* 6.8 was further aggravated by exposure to SN-38. Furthermore, the percentage of apoptotic cells (annexin V-EGFP positive) was increased. In the SN-38-exposed cells, the numbers of apoptotic and necrotic cells were lower at *pH* 7.8 than at *pH* 6.8. In the control cells, apoptotic cells were around 10% in all 3-set *pH* conditions despite of HCO_3^- treatment (**Figure 3(b)**). In case of SN-38-exposed cells, the apoptosis was enhanced by around 40% in all three *pH* conditions of HCO_3^- (−). On the other hand, the SN-38-induced apoptosis was significantly inhibited at *pH* 7.0 and 7.8 with increasing HCO_3^- concentrations compared to that at *pH* 6.8. These results show that higher *pH* conditions adjusted by HCO_3^- could inhibit the cellular toxicity induced by SN-38.

3.4. Effect of Bicarbonate on *pHi* Alternation Induced by SN-38

The level of *pHi* was determined in the presence of either 10 or 30 mM HCO_3^- following SN-38 (**Figure 4**). In the 10 mM HCO_3^-, *pHi* level in SN-38-exposed cells was significantly decreased compared to that in the control. On the other hand, there was no significant difference in the *pHi* between the control and SN-38-exposed cells in the presence of 30 mM HCO_3^-. Similar results

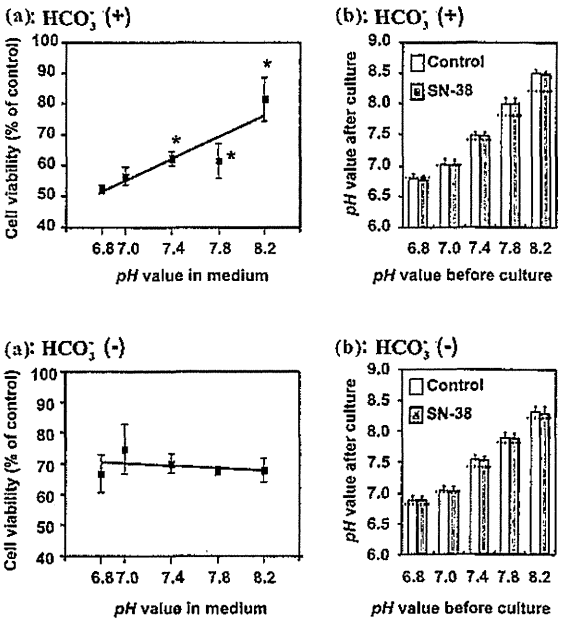

Figure 2. Effect of bicarbonate-adjusted *pH* on cell viability (a) and *pH* values in medium after cell culture (b). The cells incubated with/without 1.0 μM SN-38 for 2 h were cultured with *pH* set media at 5 points (6.8, 7.0, 7.4, 7.8, 8.2) adjusted by either different concentrations (5, 10, 20, 44, 100 mM, respectively) of bicarbonate [HCO_3^- (+)] or NaOH [HCO_3^- (-)]. (a) Cell viability evaluated by MTT assay was shown as the percent of the respective control (0 μM SN-38). $^*p < 0.05$; Significantly different from *pH* 6.8; (b) The 5-set points of culture media adjusted by either increasing concentrations of bicarbonate [HCO_3^- (+)] or NaOH [HCO_3^- (−)] were measured after the cell culture. The dotted lines on each column show the set-*pH* values before the cell culture. The *pH* in each culture medium was measured using a standard *pH* meter in triplicate from three samples immediately after culture.

were obtained with a higher concentration of HCO_3^- (58 mM) as those obtained with 30 mM HCO_3^- (no data shown). The results indicate that higher concentrations of HCO_3^- could prevent intracellular acidification caused by SN-38.

4. Discussion

Diarrhea is one of the lethal major side effects in the cancer patients receiving CPT-11 [7,31]. We and others have previously demonstrated that intestinal alkalization by HCO_3^- could reduce CPT-11/SN-38-induced cell injuries and diarrhea in both *in vitro* and *in vivo* studies [12,13,15]. These studies were based on the role of HCO_3^- on intestinal *pH* regulation because of the *pH*-dependent cytotoxicity of CPT-11/SN-38 [1,9,10,12,14, 15,28,32,33]. In addition to the regulation of *pH* in the

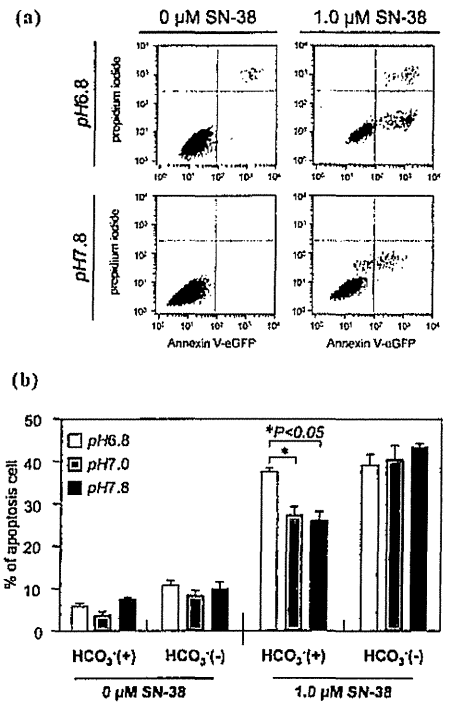

Figure 3. Effect of bicarbonate-adjusted *pH* on apoptosis. The cell following exposure to SN-38 was incubated for an additional 48 h in 3-set *pH* conditions (6.8, 7.0, or 7.8) either in HCO_3^- (+) or HCO_3^- (−) medium. See the legend of Figure 2 for the bicarbonate concentrations that were used to set the *pH* in the medium. Figure 3(a) shows the images of flow cytometry in the cells exposed to either 0 or 1.0 μM SN-38 lactone in HCO_3^- (+) conditions set at *pH* 6.8 or 7.8. The apoptotic cells were expressed as the percentage of cells stained with annexin V-EGFP positive and PI negative to the total numbers of cells, and shown as the mean ± SD in Figure 3(b).

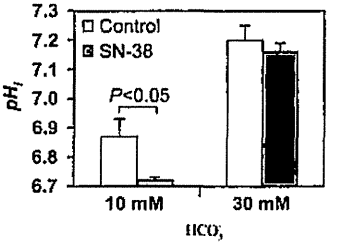

Figure 4. Effect of bicarbonate on SN-38-induced change in intracellular *pH*. Changes in *pHi* was assessed following incubation of the HT29 cell in the presence and absence of 0.5 μM SN-38 for 2 h and in a culture medium containing either 10 or 30 mM bicarbonate. The *pHi* was determined 4 h after removal of SN-38.

intestinal lumen, we suggested that HCO_3^- might play other roles on cytoprotection against CPT-11/SN-38 through influence on *pHi* environment. Palissot *et al.* have reported that the CPT-11/SN-38-induced apoptosis was less in the multidrug-resistant HL60-Vinc leukemic cells that could keep *pHi* higher, while apoptosis was much in drug-sensitive HL60 cell which intercellular acidification was induced following CPT-11/SN-38 treatment [34]. Therefore, the regulation of *pHi* level would be associated with the sensitive to CPT-11/SN-38 [18].

Accordingly, we designed a series of experiments in the large intestine cell line to test the hypothesis that the HCO_3^- might influence on not only extracellular *pH* but also *pHi* environments, and consequently, disturb the intracellular conversion of SN-38 from the less toxic carboxylate form to the more toxic lactone form [9,35]. In the result, the cytotoxicity induced by SN-38 lactone form was attenuated in a dose-dependent matter of HCO_3^-, but not by the respective pH condition merely adjusted by NaOH/HCl with low concentration of HCO_3^-. Although the HCO_3^- itself changes medium *pH*, the result implies that the cell damages induced by SN-38 would depend on not only ambient *pH* environment because the exposed time to SN-38 was only 2 hours that is too short to cause interconversion of SN-38 forms, and therefore, HCO_3^- might play other roles on the protection against SN-38. One of the possibilities is that HCO_3^- would be uptaked into cells, and then, would affect the *pHi*. Indeed, the present study confirmed that a higher concentration of HCO_3^- (30 mM) could inhibit SN-38-induced intracellular acidification (**Figure 4**).

Furthermore, cellular acidification becomes a trigger in the early phase of apoptosis because of activation of endonucleases, and thus, regulation of *pHi* is critically important for tumor development [21]. The present study showed that the SN-38 itself has the action to cause intercellular acidification in the intestinal cell. Besides the inhibition of DNA topoisomerase I, it means that intercellular SN-38 also behaves as a trigger of apoptosis. Therefore, the regulation of *pHi* should be further important for attenuation of the side effects on CPT-11/SN-38 therapy. In addition to the effect of HCO_3^- on SN-38-induced intercellular acidification, Flow Cytometry assay showed that apoptosis following SN-38 exposure was significantly decreased by increasing concentrations of HCO_3^-, but not in the respective pH level adjusted by NaOH/HCl. These findings implied that the regulation of *pHi* by HCO_3^- is effectiveness on the prevention of SN-38 induced apoptosis in intestinal cells.

In addition to the interconversion of CPT-11/SN-38, *pH* environment is also associated with other characteristics of the agents. Gabr *et al.* have reported in L1210 leukemia cells that the uptake of radiolabeled CPT-11 was more rapidly at *pH* 6.2 than at physiological *pH* 7.4, and the intercellular retention was predominant at *pH* 6.2

[36]. Similarly, we have previously reported that the uptake of SN-38 into HT29 cells was significantly increased at ambient lower *pH* [9]. In this point, HCO_3^- is indirectly contributed on the cellular uptake of SN-38 by regulation of ambient *pH* level.

In conclusion, the present study confirmed that HCO_3^- could keep alkalization/neutralization in not only extracellular but also intracellular *pH* environments, and consequently, cytotoxicity in large intestine cells would be attenuated through the reductions of interconversion to more toxic form and cellular uptake of SN-38. These results would certify the multiple roles of HCO_3^- on the attenuation of side effects; cell injuries and diarrhea, in CPT-11/SN-38 antitumor therapy.

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REFERENCES

[1] Y. Kawato, M. Aonuma, Y. Hirota, H. Kuga and K. Sato, "Intracellular Roles of SN-38, a Metabolite of the Camptothecin Derivative CPT-11, in the Antitumor Effect of CPT-11," *Cancer Research*, Vol. 51, No. 16, 1991, pp. 4187-4191.

[2] S. Kudoh, Y. Fujiwara, Y. Takada, H. Yamamoto, A. Kinoshita, Y. Ariyoshi, K. Furuse, M. Fukuoka and West Japan Lung Cancer Group, "Phase II Study of Irinotecan Combined with Cisplatin in Patients with Previously Untreated Small-Cell Lung Cancer," *Journal of Clinical Oncology*, Vol. 16, No. 3, 1998, pp. 1068-1074.

[3] W. P. Irvin, F. V. Price, H. Bailey, M. Gelder, R. Rosenbluth, H. J. Durivage and R. K. Potkul, "A Phase II Study of Irinotecan (CPT-11) in Patients with Advanced Squamous Cell Carcinoma of the Cervix," *Cancer*, Vol. 82, No. 2, 1998, pp. 328-333. doi:10.1002/(SICI)1097-0142(19980115)82:2<334::AID-CNCR13>3.0.CO;2-#

[4] C. F. Verschraegen, T. Levy, A. P. Kudelka, E. Llerena, K. Ende, R. S. Freedman, C. L. Edwards, M. Hord, M. Steger, A. L. Kaplan, D. Kieback, A. Fishman and J. J. Kavanagh, "Phase II Study of Irinotecan in Prior Chemotherapy-Treated Squamous Cell Carcinoma of the Cervix," *Journal of Clinical Oncology*, Vol. 15, No. 2, 1997, pp. 625-631.

[5] Y. Shimizu, S. Umezawa and K. Hasumi, "Successful Treatment of Clear Cell Adenocarcinoma of the Ovary (OCCA) with a Combination of CPT-11 and Mitomycin C," *Gan to Kagaku Ryoho*, Vol. 23, No. 5, 1996, pp. 587-593.

[6] H. C. Pitot, D. B. Wender, M. J. O'Connell, G. Schroeder, R. M. Goldberg, J. Rubin, J. A. Mailliard, J. A. Knost, C. Ghosh, R. J. Kirschling, R. Levitt and H. E. Windschitl, "Phase II Trial of Irinotecan in Patients with Metastatic Colorectal Carcinoma," *Journal of Clinical Oncology*, Vol. 15, No. 8, 1997, pp. 2910-2919.

[7] D. J. Sargent, D. Niedzwiecki, M. J. O'Connell and R. L. Schilsky, "Recommendation for Caution with Irinotecan, Fluorouracil, and Leucovorin for Colorectal Cancer," *The New England Journal of Medicine*, Vol. 345, No. 2, 2001, pp. 144-145. doi:10.1056/NEJM200107123450213

[8] L. Saltz, "Irinotecan-Based Combinations for the Adjuvant Treatment of Stage III Colon Cancer," *Oncology (Williston Park)*, Vol. 14, No. 12, 2000, pp. 47-50.

[9] K. Kobayashi, B. Bouscarel, Y. Matsuzaki, S. Ceryak, S. Kudoh and H. Fromm, "pH-Dependent Uptake of Irinotecan and Its Active Metabolite, SN-38, by Intestinal Cells," *International Journal of Cancer*, Vol. 83, No. 4, 1999, pp. 491-496. doi:10.1002/(SICI)1097-0215(19991112)83:4<491::AID-IJC10>3.3.CO;2-D

[10] L. P. Rivory, M. R. Bowles, J. Robert and S. M. Pond, "Conversion of Irinotecan (CPT-11) to Its Active Metabolite, 7-Ethyl-10-hydroxycamptothecin (SN-38), by Human Liver Carboxylesterase," *Biochemical Pharmacology*, Vol. 52, No. 7, 1996, pp. 1103-1111. doi:10.1016/0006-2952(96)00457-1

[11] J. Fassberg and V. J. Stella, "A Kinetic and Mechanistic Study of the Hydrolysis of Camptothecin and Some Analogues," *Journal of Pharmaceutical Sciences*, Vol. 81, No. 7, 1992, pp. 676-684. doi:10.1002/jps.2600810718

[12] Y. Takeda, K. Kobayashi, Y. Akiyama, T. Soma, S. Handa, S. Kudoh and K. Kudo, "Prevention of Irinotecan (CPT-11)-Induced Diarrhea by Oral Alkalization Combined with Control of Defecation in Cancer Patients," *International Journal of Cancer*, Vol. 92, No. 2, 2001, pp. 269-275. doi:10.1002/1097-0215(200102)9999:9999<::AID-IJC1179>3.0.CO;2-3

[13] V. Valenti Moreno, J. Brunet Vidal, H. Manzano Alemany, A. Salud Salvia, M. Llobera Serentill, I. Cabezas Montero, S. Servitja Tormo, E. Sopena Bert and J. Guma Padro, "Prevention of Irinotecan Associated Diarrhea by Intestinal Alkalization. A Pilot Study in Gastrointestinal Cancer Patients," *Clinical and Translational Oncology*, Vol. 8, No. 3, 2006, pp. 208-212. doi:10.1007/s12094-006-0012-1

[14] T. Tamura, K. Yasutake, H. Nishisaki, T. Nakashima, K. Horita, S. Hirohata, A. Ishii, K. Hamano, N. Aoyama, D. Shirasaka, T. Kamigaki and M. Kasuga, "Prevention of

Irinotecan-Induced Diarrhea by Oral Sodium Bicarbonate and Influence on Pharmacokinetics," *Oncology*, Vol. 67, No. 5-6, 2004, pp. 327-337. doi:10.1159/000082915

[15] T. Ikegami, L. Ha, K. Arimori, P. Latham, K. Kobayashi, S. Ceryak, Y. Matsuzaki and B. Bouscarel, "Intestinal Alkalization as a Possible Preventive Mechanism in Irinotecan (CPT-11)-Induced Diarrhea," *Cancer Research*, Vol. 62, No. 1, 2002, pp. 179-187.

[16] R. Belhoussine, H. Morjani, R. Gillet, V. Palissot and M. Manfait, "Two Distinct Modes of Oncoprotein Expression during Apoptosis Resistance in Vincristine and Daunorubicin Multidrug-Resistant HL60 Cells," *Advances in Experimental Medicine and Biology*, Vol. 457, 1999, pp. 365-381. doi:10.1007/978-1-4615-4811-9_39

[17] R. A. Gottlieb, J. Nordberg, E. Skowronski and B. M. Babior, "Apoptosis Induced in Jurkat Cells by Several Agents Is Preceded by Intracellular Acidification," *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 93, No. 2, 1996, pp. 654-658. doi:10.1073/pnas.93.2.654

[18] A. K. Larsen, A. E. Escargueil and A. Skladanowski, "Resistance Mechanisms Associated with Altered Intracellular Distribution of Anticancer Agents," *Pharmacology & Therapeutics*, Vol. 85, No. 3, 2000, pp. 217-229. doi:10.1016/S0163-7258(99)00073-X

[19] K. H. Sit, B. H. Bay and K. P. Wong, "Effect of Genistein, a Tyrosine-Specific Protein Kinase Inhibitor, on Cell Rounding by Ph Upshifting," *In Vitro Cellular & Developmental Biology—Animal*, Vol. 29A, No. 5, 1993, pp. 395-402. doi:10.1007/BF02633988

[20] J. F. Goossens, J. P. Henichart, L. Dassonneville, M. Facompre and C. Bailly, "Relation between Intracellular Acidification and Camptothecin-Induced Apoptosis in Leukemia Cells," *European Journal of Pharmaceutical Sciences*, Vol. 10, No. 2, 2000, pp. 125-131. doi:10.1016/S0928-0987(99)00091-3

[21] H. Izumi, T. Torigoe, H. Ishiguchi, H. Uramoto, Y. Yoshida, M. Tanabe, T. Ise, T. Murakami, T. Yoshida, M. Nomoto and K. Kohno, "Cellular pH Regulators: Potentially Promising Molecular Targets for Cancer Chemotherapy," *Cancer Treatment Reviews*, Vol. 29, No. 6, 2003, pp. 541-549. doi:10.1016/S0305-7372(03)00106-3

[22] R. D. Vaughan-Jones and K. W. Spitzer, "Role of Bicarbonate in the Regulation of Intracellular pH in the Mammalian Ventricular Myocyte," *Biochemistry and Cell Biology*, Vol. 80, No. 5, 2002, pp. 579-596. doi:10.1139/o02-157

[23] D. Gleeson, N. D. Smith and J. L. Boyer, "Bicarbonate-Dependent and -Independent Intracellular pH Regulatory Mechanisms in Rat Hepatocytes. Evidence for Na^+-HCO_3^- Cotransport," *Journal of Clinical Investigation*, Vol. 84, No. 1, 1989, pp. 312-321. doi:10.1172/JCI114156

[24] B. Bouscarel, C. Cortinovis, C. Carpene, J. C. Murat and H. Paris, "Alpha 2-Adrenoceptors in the HT 29 Human Colon Adenocarcinoma Cell Line: Characterization with [3H]clonidine; Effects on Cyclic AMP Accumulation," *European Journal of Pharmacology*, Vol. 107, No. 2, 1985, pp. 223-231. doi:10.1016/0014-2999(85)90062-7

[25] H. Paris, B. Bouscarel, C. Cortinovis and J. C. Murat, "Growth-Related Variation of Alpha 2-Adrenergic Receptivity in the HT 29 Adenocarcinoma Cell-Line from Human Colon," *FEBS Letters*, Vol. 184, No. 1, 1985, pp. 82-86. doi:10.1016/0014-5793(85)80658-X

[26] K. Kobayashi, B. Bouscarel, Y. Matsuzaki, S. Ceryak and H. Fromm, "Uptake Mechanism of Irinotecan (CPT-11) and Its Metabolite (SN-38) by Hamster Intestinal Cells," *Gastroenterology*, Vol. 114, Supplement 1, 1998, p. A626. doi:10.1016/S0016-5085(98)82560-2

[27] X. Y. Chu, Y. Kato, K. Niinuma, K. I. Sudo, H. Hakusui and Y. Sugiyama, "Multispecific Organic Anion Transporter Is Responsible for the Biliary Excretion of the Camptothecin Derivative Irinotecan and Its Metabolites in Rats," *Journal of Pharmacology and Experimental Therapeutic*, Vol. 281, No. 1, 1997, pp. 304-314.

[28] K. Akimoto, A. Kawai and K. Ohya, "Kinetic Studies of the Hydrolysis and Lactonization of Camptothecin and Its Derivatives, CPT-11 and SN-38, in Aqueous Solution," *Chemical and Pharmaceutical Bulletin (Tokyo)*, Vol. 42, No. 10, 1994, pp. 2135-2138. doi:10.1248/cpb.42.2135

[29] H. Eagle, "Buffer Combinations for Mammalian Cell Culture," *Science*, Vol. 174, No. 4008, 1971, pp. 500-503. doi:10.1126/science.174.4008.500

[30] F. Denizot and R. Lang, "Rapid Colorimetric Assay for Cell Growth and Survival. Modifications to the Tetra- zolium Dye Procedure Giving Improved Sensitivity and Reliability," *Journal of Immunological Methods*, Vol. 89, No. 2, 1986, pp. 271-277. doi:10.1016/0022-1759(86)90368-6

[31] P. Rougier, R. Bugat, J. Y. Douillard, S. Culine, E. Suc, P. Brunet, Y. Becouarn, M. Ychou, M. Marty, J. M. Extra, J. Bonneterre, A. Adenis, J. F. Seitz, G. Ganem, M. Namer, T. Conroy, S. Negrier, Y. Merrouche, F. Burki, M. Mousseau, P. Herait and M. Mahjoubi, "Phase II Study of Irinotecan in the Treatment of Advanced Colorectal Cancer in Chemotherapy-Naive Patients and Patients Pretreated with Fluorouracil-Based Chemotherapy," *Journal of Clinical Oncology*, Vol. 15, No. 1, 1997, pp. 251-260.

[32] J. L. Flowers, R. M. Hoffman, T. A. Driscoll, M. E. Wall, M. C. Wani, G. Manikumar, H. S. Friedman, M. Dewhirst, O. M. Colvin and D. J. Adams, "The Activity of Camptothecin Analogues Is Enhanced in Histocultures of Human Tumors and Human Tumor Xenografts by Modulation of Extracellular pH," *Cancer Chemotherapy and Pharmacology*, Vol. 52, No. 3, 2003, pp. 253-261. doi:10.1007/s00280-003-0635-7

[33] L. P. Rivory, E. Chatelut, P. Canal, A. Mathieu-Boue and J. Robert, "Kinetics of the *in Vivo* Interconversion of the Carboxylate and Lactone Forms of Irinotecan (CPT-11) and of Its Metabolite SN-38 in Patients," *Cancer Research*, Vol. 54, No. 24, 1994, pp. 6330-6333.

[34] V. Palissot, R. Belhoussine, Y. Carpentier, S. Sebille, H. Morjani, M. Manfait and J. Dufer, "Resistance to Apoptosis Induced by Topoisomerase I Inhibitors in Multidrug-Resistant HL60 Leukemic Cells," *Biochemical and Biophysical Research Communications*, Vol. 245, No. 3,

1998, pp. 918-922. doi:10.1006/bbrc.1998.8550

[35] Y. Kawato, M. Sekiguchi, K. Akahane, Y. Tsutomi, Y. Hirota, H. Kuga, W. Suzuki, H. Hakusui and K. Sato, "Inhibitory Activity of Camptothecin Derivatives against Acetylcholinesterase in Dogs and Their Binding Activity to Acetylcholine Receptors in Rats," *Journal of Pharmacy and Pharmacology*, Vol. 45, No. 5, 1993, pp. 444-448. doi:10.1111/j.2042-7158.1993.tb05573.x

[36] A. Gabr, A. Kuin, M. Aalders, H. El-Gawly and L. A. Smets, "Cellular Pharmacokinetics and Cytotoxicity of Camptothecin and Topotecan at Normal and Acidic pH," *Cancer Research*, Vol. 57, No. 21, 1997, pp. 4811-4816.

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Original Article

Post-therapeutic needle biopsy in patients with hepatocellular carcinoma is a useful tool to evaluate response to proton irradiation

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Aim: Proton beam therapy is safe and more effective than conventional radiation therapy for the local control of nodular hepatocellular carcinoma (HCC). However, evaluating therapeutic response by imaging is not accurate during the early post-irradiation period. Therefore, we examined whether the histopathological study of biopsy specimens obtained at 3 weeks after irradiation can be used to more accurately assess therapeutic response.

Methods: Fifteen HCC lesions from 13 patients were treated with proton beam irradiation. Tissue biopsy samples were obtained using abdominal ultrasound-guided percutaneous fine-needle aspiration from the center of the tumor before, 3 weeks after and 1 year post-proton therapy. The specimens were examined after staining with hematoxylin–eosin (HE) and a MIB-1 antibody.

Results: MIB-1 labeling indices (LI) before treatment were 13.0 ± 8.5% (mean ± SD; range, 0.6–27.0), whereas those 3 weeks after proton therapy were significantly reduced to 3.2 ± 2.4% (range, 0.6–8.9) ($P < 0.05$). Although the tumor size was reduced, we did not observe a reduction in tumor blood flow by dynamic computed tomography or degenerative changes by HE. All lesions that displayed reduced MIB-1 LI at 3 weeks post-proton treatment were ultimately diagnosed as complete response at 1 year after treatment. In contrast, one case with increased MIB-1 LI at 3 weeks had significant tumor size progression at 1 year post-treatment.

Conclusion: The percutaneous fine-needle aspiration biopsy of HCC is a safe and useful tool that can be used to evaluate the response to proton irradiation. In particular, MIB-1 LI may provide additional information to assess the therapeutic response of HCC during the early post-irradiated period.

Key words: dynamic computed tomography, hepatocellular carcinoma, MIB-1, needle biopsy, proton radiotherapy

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the fourth most common cause of cancer in Japan, and its incidence is increasing worldwide because of the increase in chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV).[1,2] Curative surgical resection of HCC offers the best treatment outcome, but fewer than 30% of all patients are able to undergo surgical resection because of the presence of underlying cirrhosis. For unresectable HCC, radiofrequency ablation (RFA) or transcatheter arterial chemoembolization (TACE) is performed;[3–8] however, the former is only suitable for small tumors (<3 cm), and the latter is not suitable for patients with poor liver function or those with associated portal vein thrombus.

Whereas conventional radiation is almost useless for treating HCC, proton irradiation has recently been successful in treating unresectable HCC.[9–12] The proton beam has a Bragg peak, which limits beam distribution

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and reduces the amount of radiation that reaches surrounding non-cancer tissues. Therefore, this therapeutic option is available to treat patients with poor liver function or portal vein thrombus. In addition, even large tumor volumes (>10 cm) were effectively irradiated at high doses without significant dose exposure to non-targeted areas.[12]

In the future, proton irradiation therapy will become more common for the treatment of HCC, and it is necessary to develop a powerful diagnostic option to evaluate HCC after therapy. Computed tomography (CT), magnetic resonance imaging and contrast-enhanced color Doppler ultrasonography (CECDU) are all useful methods to evaluate the therapeutic response of HCC after proton irradiation. However, these imaging methods are not accurate during the early post-irradiated period because the reduction in tumor size or reduced blood flow in the tumor are not clear during this period using these techniques.[13]

MIB-1 is an anti-Ki-67 monoclonal antibody developed by Cattoretti *et al.*, and it is used for the easy and accurate evaluation of cell proliferation potential in formalin-fixed paraffin-embedded tissues using an antigen retrieval technique.[14,15] We previously reported that the MIB-1 labeling indices of HCC samples obtained using ultrasound-guided, fine-needle liver biopsies were significantly correlated with tumor volume doubling times and were better indicators of tumor aggressiveness than the conventional indices of histological atypia.[16]

The present study was undertaken to compare the histological changes of HCC before and after proton irradiation therapy. In addition, MIB-1 labeling indices in these tissues were used to examine if the indices during the early post-irradiated period can assess HCC therapeutic response for longer periods after proton irradiation.

METHODS

Patients and treatment

THIRTEEN PATIENTS DIAGNOSED with a single HCC or multinodular HCC (up to three) from 1988–1993 at the Department of Gastroenterology, Tsukuba University Hospital were selected for proton therapy because they either refused hepatectomy or had unresectable HCC with complications of advanced liver cirrhosis, renal failure or heart failure. Insufficient accumulation of lipiodol (Laboratorie Guerbert, Paris, France) and the presence of viable cells (evaluated by histological examination of needle biopsy specimen) in the lesion by TACE was also included in the selection criteria. Informed consent was obtained from all subjects, and the study protocol was approved by the ethics committee of Tsukuba University.

The patient characteristics are displayed in Table 1. Cases 2, 8 and 15 had ectopic or asynchronous HCC that appeared in the same patient. All patients had HCV antibody positive liver cirrhosis, and Child–Pugh classification of the disease severity[18] was A in seven patients, B in three patients and C in three patients. The size of the treated HCC ranged 15–60 mm in maximum diameter with an average size of 34.7 ± 14.5 mm (mean ± standard deviation [SD]). Twelve of the 15 cases (80.0%) were treated using proton irradiation monotherapy, while the other three cases (20.0%) were treated using proton radiotherapy after unsatisfactory TACE results. The total proton beam irradiation dose ranged 68.8–84.0 Gy (physical dose) with an average dose of 79.2 ± 4.0 Gy. HCC in patients treated using proton monotherapy were followed using CT after the insertion of an iridium marker needle under the ultrasound guide. For patients who received lipiodol-targeted chemotherapy, the locations of the HCC were determined using CT on the lipiodol-contrasted lesions.

CT follow up

In 14 cases, the size and contrast enhancement of the HCC before, 3 weeks after and 1 year post-proton therapy were compared using dynamic CT. Tumor treatment response was judged according to the Response Evaluation Criteria in Cancer of the Liver (RECICL).[19] In the remaining case, because we could only analyze the size of the HCC using plain CT because of renal failure, the response was judged only by tumor size reduction. The size reduction rate was calculated using the equation below after calculating the product of the major axis of the maximum cross-section by the maximum diameter crossing the major axis at a right angle: size reduction rate = ([product before treatment] – [product after treatment]) / (product before treatment) × 100.

Standard histological examination

Hepatocellular carcinoma tissue samples were obtained using abdominal ultrasound-guided, percutaneous fine-needle aspiration biopsies (Majima needle, 21G; Top, Tokyo, Japan) taken from the center of the tumor before, 3 weeks after and 1 year post-proton therapy. The biopsy specimens were fixed in 10% neutral buffered formalin and were routinely processed by dehydration in graded ethanol, clearing in xylenes and embedding in paraffin blocks. Each paraffin block was

Table 1 Patient characteristics

Case no.	Age (years)	Sex	Tumor size (mm)	Child–Pugh class	Treatment	Total dose (Gy)	Histological differentiation
1	59	M	60 × 57	B	TACE + proton	84.0	Well
2†	67	M	50 × 50	A	Proton	83.0	Poorly
3	66	M	46 × 20	A	TACE + proton	77.0	Moderately
4	70	F	25 × 25	C	Proton	78.0	Well
5	73	M	37 × 37	A	Proton	78.0	Moderately
6	59	M	30 × 29	A	Proton	78.0	Moderately
7	73	F	44 × 39	A	Proton	78.0	Moderately
8†	67	M	20 × 20	A	Proton	83.0	Well
9	62	M	30 × 30	B	Proton	68.8	Moderately
10	66	M	38 × 30	C	Proton	80.0	Moderately
11	73	M	15 × 15	B	Proton	80.5	Well
12	58	M	15 × 15	A	TACE + proton	84.0	Well
13	59	M	50 × 50	A	Proton	82.0	Moderately
14	57	M	45 × 40	C	Proton	78.0	Well
15†	67	M	15 × 15	A	Proton	75.5	Well

†Ectopic or asynchronous HCC appeared in the same patient.
Histological differentiation was determined according to a modified version of Edmondson–Steiner classification: well, grade I (well differentiated); moderately, grade II (moderately differentiated); poorly, grade III and IV (poorly differentiated).[17]
F, female; HCC, hepatocellular carcinoma; M, male; proton, proton irradiation; TACE, transcatheter arterial chemoembolization.

sectioned at 3 μm and stained using hematoxylin–eosin (HE). We examined the degrees of histological differentiation (before treatment) and pathological changes (after treatment).[17]

Immunohistochemistry for MIB-1

The lesions that displayed the presence of viable cancer cells by HE staining 3 weeks after proton therapy (11 cases) were further examined by immunohistochemical staining using a MIB-1 antibody, as previously described.[16] Briefly, paraffin sections were dewaxed in xylenes, rehydrated in graded ethanol and subjected to heat-induced antigen retrieval in citrate buffer (pH 6.0) at 120°C for 10 min. After the inactivation of endogenous peroxidase activity by incubating with 0.3% H_2O_2 at room temperature for 15 min, the sections were stained using the labeled streptavidin-biotin peroxidase technique using the Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan) and the MIB-1 monoclonal antibody (mouse immunoglobulin G, no. 0607; Immunotech, Marseille, France) at a dilution of 1:50. Finally, the sections were stained with diaminobenzidine-hydrogen peroxide (Sigma-Aldrich, St Louis, MO, USA) and counterstained with hematoxylin. A normal lymph node was simultaneously stained for use as a positive control.

For all specimens, three or more fields were randomly selected, and 500 or more tumor cells were examined using a CAS-200 Image Analyzer (Cell Analysis Systems, Lombard, IL, USA) at a magnification of ×400. The MIB-1 labeling index (LI) was calculated by the number of immunoreactive nuclei divided by the total number of neoplastic nuclei.

Statistical analysis

MIB-1 LI obtained before and 3 weeks post-proton treatment were compared using the Wilcoxon rank sum test, and $P < 0.05$ was considered to be significant.

RESULTS

Treatment evaluation using CT

TUMOR SIZE AND histological findings before and after proton radiotherapy are compared in Figure 1. One year post-proton irradiation, a reduction in tumor size with pathological coagulative necrosis was observed in 14 out of 15 lesions (93.3%). While five out of the 14 lesions disappeared (100% reduction), the reduction rate of the other nine lesions did not reach 100% by 1 year post-proton irradiation. However, hypervascularity disappeared in these nine lesions; therefore, all 14 lesions were diagnosed as having complete response (CR). In contrast, one case revealed tumor expansion with hypervascularity at 1 year post-proton therapy. This

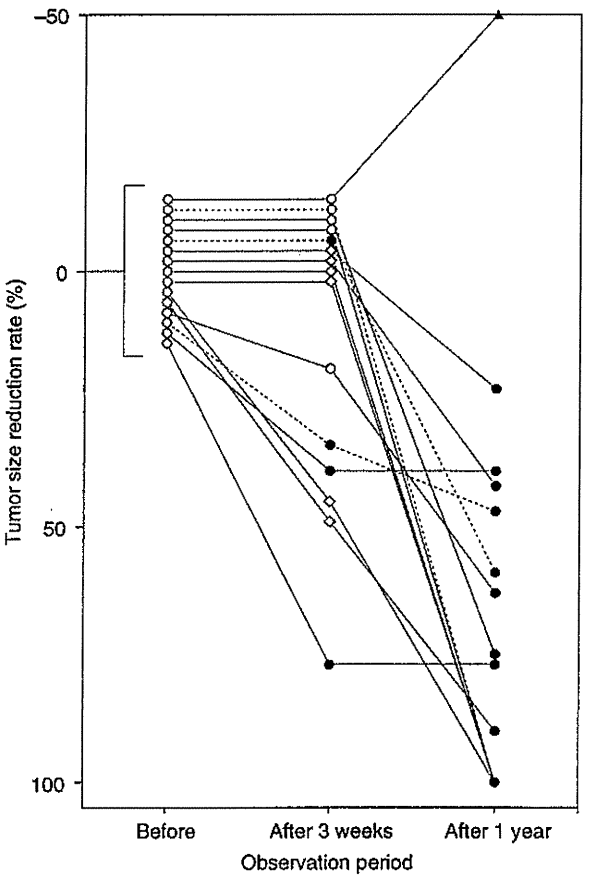

Figure 1 Change in tumor size and histological findings before and after proton beam irradiation. TACE, transcatheter arterial chemoembolization. O, viable; ◊, degenerative change; ●, necrosis; ▲, not done; —, proton; - - -, TACE + proton.

case was diagnosed as having progressive disease (PD), and additional treatment was started without a follow-up biopsy.

At 3 weeks post-proton therapy, a reduction in tumor size had already been observed in six out of the 14 CR lesions (42.9%); however, tumor size was not reduced in the other eight CR lesions (57.1%). The vascularity of 13 CR lesions was also studied using dynamic CT at 3 weeks post-proton therapy. All lesions preserved hypervascularity and no sign of tumor necrosis was detected at this time.

Treatment evaluation by standard histological examination

Histological findings of the 14 CR lesions at 3 weeks post-proton therapy are displayed in Figure 1. Coagulative necrosis in the whole specimen was observed in four lesions (28.6%), and sporadic cellular swelling due to degeneration in viable cancer cells (Fig. 2d) was observed in six lesions (42.8%). The other four lesions (28.6%) did not show significant pathological changes, and these specimens were diagnosed as being viable HCC.

Treatment evaluation by immunohistochemical staining for MIB-1

As shown in Figure 2(a,b), although both specimens were diagnosed as viable HCC by standard HE staining, the MIB-1 positive cell numbers in post-treatment specimens were clearly reduced compared to the pretreatment specimens. MIB-1 LI obtained before and 3 weeks after proton treatment were compared in 11 lesions that did not have coagulative necrosis at 3 weeks (Fig. 3). MIB-1 LI before treatment were 13.0 ± 8.5% (mean ± SD; range, 0.6–27.0) whereas those 3 weeks after proton treatment were 3.2 ± 2.4% (mean ± SD; range, 0.6–8.9). Thus, at 3 weeks post-proton treatment, a reduction of MIB-1 LI had been observed in all lesions that had achieved CR at 1 year after treatment ($P < 0.05$). In contrast, one case that was diagnosed as having PD at 1 year after treatment did not show a change in tumor size or changes in histology at 3 weeks post-treatment. The MIB-1 LI from this case at 3 weeks after treatment was relatively high compared to before treatment. There was no significant relation between the histological or immunohistological changes observed at 3 weeks post-treatment and a history of TACE.

DISCUSSION

OUR DATA DEMONSTRATED that at 3 weeks post-proton therapy, local control of HCC could be expected when: (i) tumor size reduction was observed by CT; (ii) a degenerative change in the biopsy specimen was observed by HE staining; or (iii) MIB-1 LI in the biopsy specimen was reduced. However, there were many cases that did not demonstrate: (i) a reduction tumor size; or (ii) degenerative changes at 3 weeks, and then diagnosed as having a CR at 1 year post-proton therapy.

During the early post-irradiated period it may be difficult to assess the therapeutic response of HCC using an imaging diagnosis such as CT. Upon assessment of the direct effect of proton irradiation on the target HCC tumor, both size reduction and the disappearance of hypervascularity of the nodule are important findings by dynamic CT.[19] However, the tumor size determined by

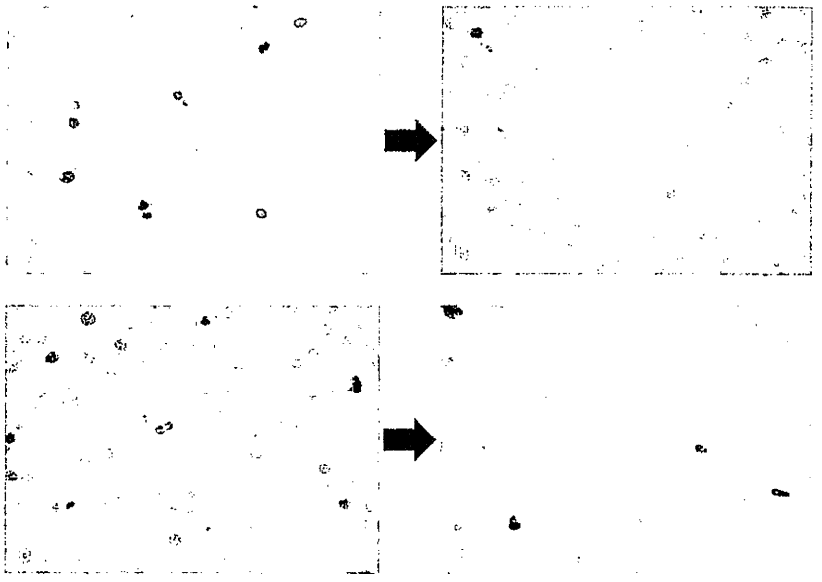

Figure 2 Comparison of immunohistochemical findings using the MIB-1 monoclonal antibody before and after proton beam irradiation. Cells with clear brown staining of the entire nucleus or nucleolus were defined as being MIB-1 positive cells. (a) Case 7. Before proton irradiation; moderately differentiated hepatocellular carcinoma (HCC); MIB-1 labeling index (LI) = 12.0%. (b) Case 7. Three weeks after proton irradiation, cancer cells appear viable but MIB-1 LI is markedly reduced to 2.2%. (c) Case 13. Before proton irradiation; moderately differentiated HCC; MIB-1 LI = 15.2%. (d) Case 13. Three weeks after proton irradiation, cancer cells are swelling due to degenerative change; MIB-1 LI = 1.7%. (Original magnification, ×400.)

CT often remains unchanged even though the tumor is completely necrotic.[10] Furthermore, hypervascularity is not always a characteristic feature of undamaged HCC because an increase in blood flow in the nodule (due to radiation-induced liver injury) is often observed during the early post-irradiated period.[10,20–22] We previously studied tumor blood flow using CECDU and reported that a transient increase in tumor blood flow was also recognized by this method in more than half of the patients immediately after proton therapy.[13]

Because of the insufficient diagnosability using CT and CECDU for the degeneration of HCC during the early post-irradiated period, percutaneous fine-needle aspiration biopsy was performed. Taking HCC biopsies is not generally recommended because there is the possibility of the tumor seeding outside of the liver.[23] However, percutaneous biopsy using the coaxial cutting needle technique was demonstrated to prevent seeding risk.[24] We still need to pay attention to other complication risks such as bleeding, but recent reports demonstrated that a needle biopsy of HCC is a safe and useful method for tumor grading[25] and gathering prognostic information.[26] In our cases, no patient had post-biopsy bleeding or detectable seeding within 1 year after treatment.

The effects of conventional radiation therapy on tumor histopathology are well-documented by Shimosato *et al.*[27] After irradiation, cancer tissue scars via a degenerative change that includes both viable and non-viable cancer cells. In contrast, proton beam irradiation causes ballooning degeneration in uveal melanoma;[28] however, histopathological reports about the effects of proton therapy on cancer tissues are limited. In the present study, cellular swelling due to degenerative changes was observed by HE staining in 42.8% of the 14 CR lesions at 3 weeks post-proton beam irradiation. However, no degenerative change was observed in the other 28.6% of the lesions, which means the effect of proton therapy cannot be completely evaluated by the HE staining of early post-irradiated biopsy samples.

In contrast, significant reduction of MIB-1 LI at 3 weeks post-proton therapy was observed in all lesions that had CR at 1 year after treatment. In spite of the lesions that were diagnosed as viable HCC by standard HE staining, the MIB-1 positive cell number was clearly reduced, which suggested that most cancer cells were no longer in growth phase at 3 weeks after proton therapy. Indeed, one case diagnosed as having PD at 1 year after treatment demonstrated elevated MIB-1 LI at 3 weeks after therapy relative to the before treatment measurement. Thus, MIB-1 LI was a simple and useful index to evaluate response to proton irradiation during the early post-irradiation period when tumor size reduction or the disappearance of hypervascularity of the nodule cannot be observed using imaging analysis. We could not set up the cut-off value of MIB-1 LI using the early post-therapeutic needle biopsy specimen. Comparison between pre- and post-therapeutic needle biopsies is

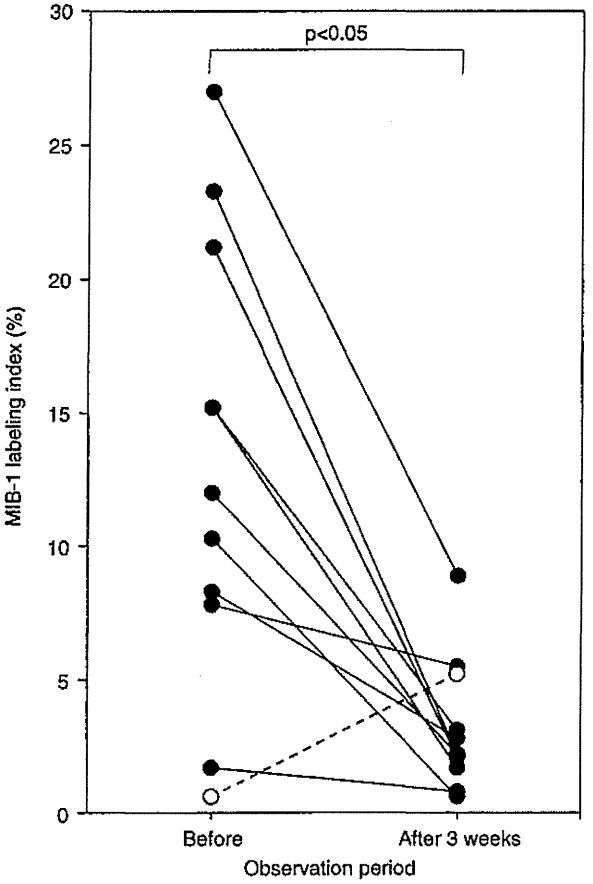

Figure 3 Comparison of MIB-1 labeling indices before and after proton beam irradiation. CR, complete response; PD, progressive disease. -●-, CR after 1 year; -○-, PD after 1 year.

needed to assess the therapeutic response of HCC by MIB-1 LI. However, the evaluation by MIB-1 LI may be difficult in cases with low MIB-1 LI of the pre-therapeutic needle biopsy.

The proton beam irradiation technique has one advantage in that a large amount of radiation can be focused directly on the lesion. Therefore, it is necessary to set the irradiation field accurately and to synchronize with the organ motion that accompanies breathing. Our previous study has suggested that a few lesions did not achieve local control after proton therapy because of a geographic miss due to imperfect treatment planning.[11] The case diagnosed as having PD in the present study is also thought to have uncontrolled disease for this same reason.

In summary, the percutaneous fine-needle aspiration biopsy of HCC is a safe and useful tool to evaluate response to proton irradiation during the early post-irradiated period. In particular, MIB-1 immunostaining of biopsy specimens was significantly associated with radiation effects after proton beam irradiation. When tumor size reduction or the disappearance of hypervascularity of the nodule was not detected during the early post-irradiated stage by imaging, comparing the MIB-1 LI before and after proton irradiation therapy may provide additional information to assess the therapeutic response of HCC.

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REFERENCES

1 Research Group for Population-based Cancer Registration in Japan. Cancer incidence and incidence rates in Japan in 1995: estimates based on date from nine population-based cancer resistries. *Jpn J Clin Oncol* 2000; 32: 1224–9.
2 Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; 362: 1907–17.
3 Bruix J, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; 42: 1208–36.
4 Izumi N. Recent advances of radiofrequency ablation for early hepatocellular carcinoma. *J Gastroenterol Hepatol* 2011; 26 (Suppl 1): 115–22.
5 Waki K, Aikata H, Katamura Y *et al.* Percutaneous radiofrequency ablation as first-line treatment for small hepatocellular carcinoma: results and prognostic factors on long-term follow up. *J Gastroenterol Hepatol* 2010; 25: 597–604.
6 Huang J, Yan L, Cheng Z *et al.* A randomized trial comparing radiofrequency ablation and surgical resection for HCC conforming to the Milan criteria. *Ann Surg* 2010; 252: 903–12.
7 Hsu CY, Huang YH, Choiou YY *et al.* Comparison of radiofrequency ablation and transarterial chemoembolization for hepatocellular carcinoma within the Milan criteria: a propensity score analysis. *Liver Transpl* 2011; 17: 556–66.
8 Olivo M, Valenza F, Buccelato A *et al.* Transcatheter arterial chemoembolization for hepatocellular carcinoma in cirrhosis: survival rate and prognostic factors. *Dig Liver Dis* 2010; 42: 515–19.
9 Tanaka N, Matsuzaki Y, Chuganji Y *et al.* Proton irradiation for hepatocellular carcinoma. *Lancet* 1992; 340: 1358.
10 Matsuzaki Y, Osuga T, Saito Y *et al.* A new effective, and safe therapeutic option using proton irradiation for hepatocellular carcinoma. *Gastroenterology* 1994; 106: 1032–41.