The present study has some limitations. First, the therapeutic effects of the second and third series of treatments for HCC were not evaluated as prognostic factors. Ten patients were treated by BSC and many patients showed recurrence of HCC during the long-term follow-up. It was thus difficult to evaluate all the therapeutic effects as prognostic factors. Second, out of 184 patients with untreated HCC admitted to our hospital from 1997 to 1998, only 90 serum samples were collected, thereby introducing the possibility of selection bias. Further studies need to be carried out on a larger number of patients. Third, CRP is not useful as a diagnostic tumour marker in the presence of inflammation, such as in HCC patients with chronic hepatitis and liver cirrhosis. The rate of false-positive CRP was higher than that for DCP or AFP measurements (28). H-CRP will also not be useful for evaluation of HCC patients early post-treatment, because of the inflammation induced by the treatment.

We considered that measurement of H-CRP is more suitable than conventional CRP in the patients with HCC. In another study, the cut-off level of conventional CRP was 5–10 mg/L; H-CRP made it possible to measure a lower CRP concentration. We compared serum H-CRP levels in chronic hepatitis type C, liver cirrhosis and HCC patients in this study (data not shown). The median serum H-CRP levels (range) in chronic hepatitis type C, liver cirrhosis and HCC patients were 0.7 mg/L (0.3–3.6), 1.9 mg/L (0.3–8.8) and 2.7 mg/L (0.1–118) respectively. Because no data are available in the literature about H-CRP levels in HCC patients, the cut-off of 3 mg/L for H-CRP was arbitrarily assumed to be suitable based on the following reasons: i, Sattar *et al.* (14) used the same cut-off level in a study of metabolic syndrome and ii, the median H-CRP level in this study was 2.7 mg/L, which agreed with other data from colorectal cancer patients of 2.7–3.4 mg/L (16).

In conclusion, our retrospective study indicates that HCC patients with elevated serum H-CRP had a poor prognosis, and thus strongly implicates serum H-CRP as a valuable prognostic factor in this disease.

References

1. El Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; **340**: 745–50.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: GLOBOCAN 2000. *Int J Cancer* 2001; **94**: 153–6.
3. Mazzaferro V, Regalia E, Doci R, *et al.* Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693–9.
4. Llovet JM, Real MI, Montana X, *et al.* Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002; **359**: 1734–9.
5. Okamoto M, Lee C, Oyasu R. Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. *Cancer Res* 1997; **57**: 141–6.
6. Miki S, Iwano M, Miki Y, *et al.* Interleukin-6 (IL-6) functions as an in vitro autocrine growth factor in renal cell carcinomas. *FEBS Lett* 1989; **250**: 607–10.
7. Miki C, Konishi N, Ojima E, Hatada T, Inoue Y, Kusunoki M. C-reactive protein as a prognostic variable that reflects uncontrolled up-regulation of the IL-1-IL-6 network system in colorectal carcinoma. *Dig Dis Sci* 2004; **49**: 970–6.
8. Nozoe T, Korenaga D, Futatsugi M, Saeki H, Maehara Y, Sugimachi K. Immunohistochemical expression of C-reactive protein in squamous cell carcinoma of the esophagus – significance as a tumor marker. *Cancer Lett* 2003; **192**: 89–95.
9. Nozoe T, Matsumata T, Kitamura M, Sugimachi K. Significance of preoperative elevation of serum C-reactive protein as an indicator for prognosis in colorectal cancer. *Am J Surg* 1998; **176**: 335–8.
10. Ikeda M, Natsugoe S, Ueno S, Baba M, Aikou T. Significant host- and tumor-related factors for predicting prognosis in patients with esophageal carcinoma. *Ann Surg* 2003; **238**: 197–202.
11. Terpos E, Szydlo R, Apperley JF, *et al.* Soluble receptor activator of nuclear factor kappaB ligand–osteoprotegerin ratio predicts survival in multiple myeloma: proposal for a novel prognostic index. *Blood* 2003; **102**: 1064–9.
12. Nakanishi H, Araki N, Kudawara I, *et al.* Clinical implications of serum C-reactive protein levels in malignant fibrous histiocytoma. *Int J Cancer* 2002; **99**: 167–70.
13. Hashimoto K, Ikeda Y, Korenaga D, *et al.* The impact of preoperative serum C-reactive protein on the prognosis of patients with hepatocellular carcinoma. *Cancer* 2005; **103**: 1856–64.
14. Sattar N, Gaw A, Scherbakova O, *et al.* Metabolic syndrome with and without C-reactive protein as a predictor of coronary heart disease and diabetes in the West of Scotland Coronary Prevention Study. *Circulation* 2003; **108**: 414–9.
15. Ivashchenko Y, Kramer F, Schafer S, *et al.* Protein kinase C pathway is involved in transcriptional regulation of C-reactive protein synthesis in human hepatocytes. *Arterioscler Thromb Vasc Biol* 2005; **25**: 186–92.
16. Gunter MJ, Stolzenberg-Solomon R, Cross AJ, *et al.* A prospective study of serum C-reactive protein and colorectal cancer risk in men. *Cancer Res* 2006; **66**: 2483–7.
17. Sobin LH, Wittekind C. *International Union Against Cancer: TNM Classification of Malignant Tumors*, 6th edn. New York: Wiley-Liss, 2002.
18. Edmondson HA, Steiner P. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954; **7**: 462–503.

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19. Nagamatsu H, Itano S, Nagaoka S, *et al.* Prophylactic lamivudine administration prevents exacerbation of liver damage in HBe antigen positive patients with hepatocellular carcinoma undergoing transhepatic arterial infusion chemotherapy. *Am J Gastroenterol* 2004; **99**: 2369–75.
20. Albert MA, Glynn RJ, Ridker PM. Plasma concentration of C-reactive protein and the calculated Framingham Coronary Heart Disease Risk Score. *Circulation* 2003; **108**: 161–5.
21. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **10**: 749–59.
22. Warzocha K, Ribeiro P, Bienvenu J, *et al.* Genetic polymorphisms in the tumor necrosis factor locus influence non-Hodgikin's lymphoma outcome. *Blood* 1998; **91**: 3574–81.
23. El-Omar EM, Carrington M, Chow WH, *et al.* Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 2000; **404**: 398–402.
24. Chao Y, Li CP, Chau GY, *et al.* Prognostic significance of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin in patients with resectable hepatocellular carcinoma after surgery. *Ann Surg Oncol* 2003; **4**: 355–62.
25. Ren Y, Poon RT, Tsui HT, *et al.* Interleukin-8 serum levels in patients with hepatocellular carcinoma: correlations with clinicopathological features and prognosis. *Clin Cancer Res* 2003; **9**: 5996–6001.
26. Parasole R, Izzo F, Perrone F, *et al.* Prognostic value of serum biological markers in patients with hepatocellular carcinoma. *Clin Cancer Res* 2001; **11**: 3504–9.
27. Yoshida T, Yamagishi S, Nakamura K, *et al.* Pigment epithelium-derived factor (PEDF) inhibits advanced glycation end product (AGE)-induced C-reactive protein expression in hepatoma cells by suppressing Rac-1 activation. *FEBS Lett* 2006; **580**: 2788–96.
28. Lin ZY, Wang LY, Yu ML, *et al.* Role of serum C-reactive protein as a marker of hepatocellular carcinoma in patients with cirrhosis. *J Gastroenterol Hepatol* 2000; **15**: 417–21.
29. Shimada H, Nabeya Y, Okazumi S, *et al.* Elevation of preoperative serum C-reactive protein level is related to poor prognosis in esophageal squamous cell carcinoma. *J Surg Oncol* 2003; **83**: 248–52.

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肝細胞癌における細胞外基質

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アークメディア

肝細胞癌における細胞外基質

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索引用語：転移・浸潤，肝癌細胞，ラミニン-5，基底膜，上皮・間葉移行

1 はじめに

肝組織における細胞外基質は主にI,III,IV,V,XVIII型コラーゲン，フィブロネクチン，ラミニン，テネイシン，ヒアルロン酸，ヘパラン硫酸，コンドロイチン硫酸などから構成されている．また，物理的な肝組織の保持の他に，インテグリンファミリーを介し肝臓を構成する細胞と接着し，これら細胞の増殖，分化，各種遺伝子の発現などに関与しているといわれている[1]．一方，肝細胞癌における細胞外基質は機能的役割における特徴として，上記機能の他に肝癌細胞の運動能，特に転移・浸潤に関与しているといわれている[2]．肝癌細胞が転移・浸潤をするためには，血管周囲に存在する基底膜への移動，接着し，これを破壊して血管内へ浸潤し転移部位へ移動したのち増殖することが要求され，これら一連の過程の内いずれの能力が肝癌細胞に欠けていても転移・浸潤は成立しない．今回は，肝細胞癌組織における基底膜成分の分布，肝癌細胞の基底膜成分に対する接着，基底膜の分解に重要な役割を果たすMatrix metalloproteinase産生能，細胞増殖や運動能と基底膜成分との関係に関するわれわれの研究結果と，肝癌細胞の上皮・間葉移行と細胞外基質との関係などの最近の知見を紹介したい．

2 肝細胞癌組織における基底膜成分の分布

正常肝組織において基底膜は肝動脈，門脈，胆管，および中心静脈周囲に存在するが類洞にそっては存在しない．基底膜の構成成分であるIV型コラーゲン，ラミニンのうちIV型コラーゲンは肝動脈，門脈，胆管，および中心静脈周囲の他，類洞に沿っても観察されるが，ラミニンは類洞にはほとんど認められない．肝細胞癌組織においても高分化肝細胞癌で腫瘍径が10 mm以下の発生初期の腫瘍

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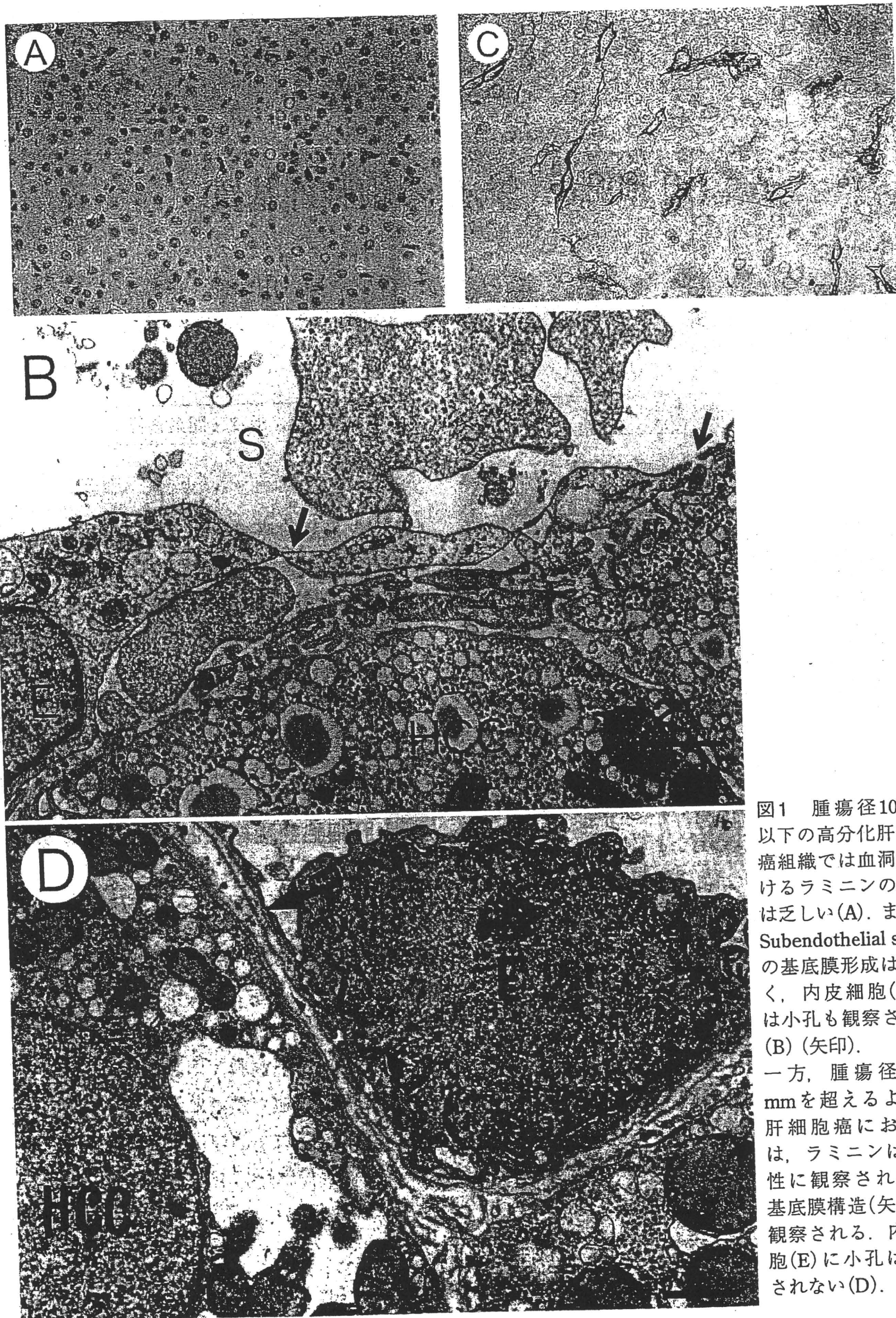
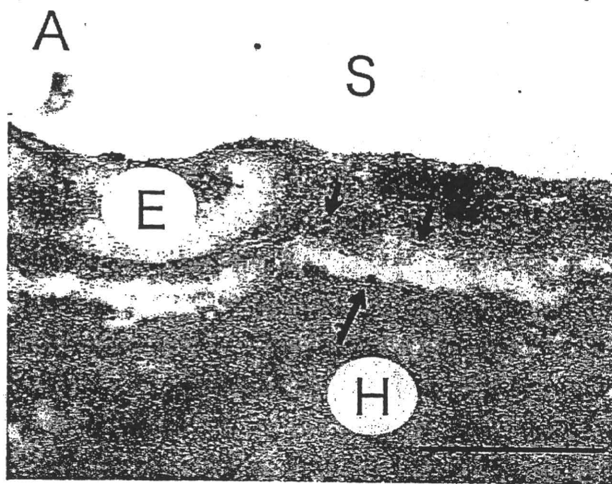


図1　腫瘍径10 mm以下の高分化肝細胞癌組織では血洞におけるラミニンの局在は乏しい(A)．また，Subendothelial spaceの基底膜形成は乏しく，内皮細胞(E)には小孔も観察される(B)(矢印)．
一方，腫瘍径が20 mmを超えるような肝細胞癌においては，ラミニンは連続性に観察され(C)，基底膜構造(矢印)も観察される．内皮細胞(E)に小孔は監察されない(D)．



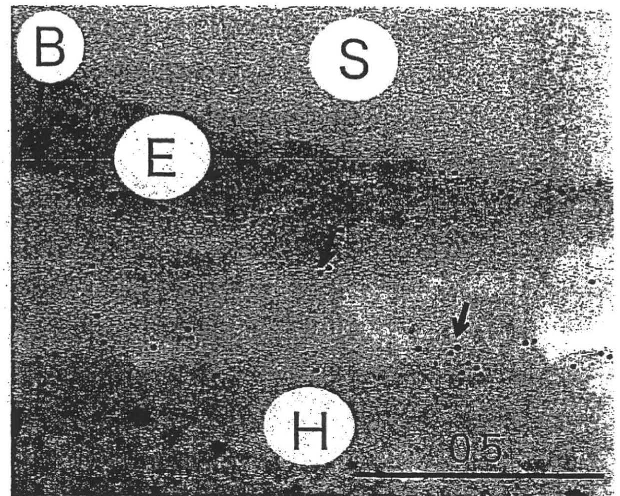
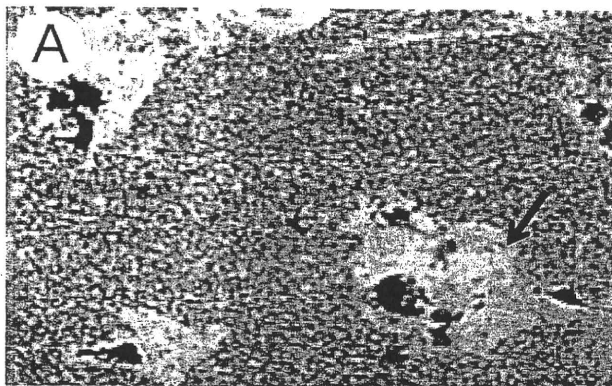
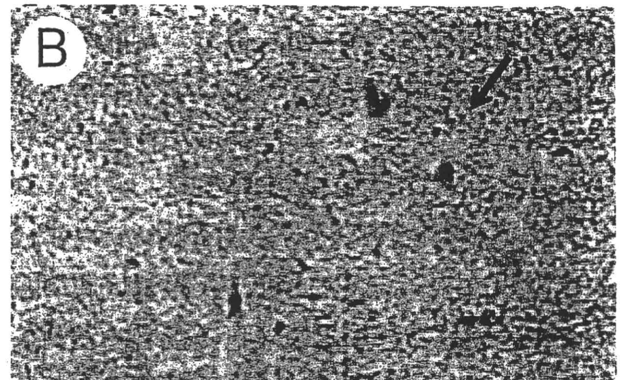


図2　肝細胞癌組織の内皮細胞と肝癌細胞の細胞表面に
インテグリン$\alpha 6$ (A) (矢印)，$\beta 1$ (B)の表出を認める(矢印)．



Autocrine motility factor



Autocrine motility factor + 抗インテグリンβI 抗体

図3　合成基底膜であるマトリゲル上での肝癌細胞の運動能は，autocrine motility factor 添加により亢進するが(A) (矢印)，抗インテグリン$\beta 1$抗体を加えると抑制される(B) (矢印)．

ではIV型コラーゲンは血洞に沿って観察されるが，ラミニンはほとんど認められない．また，基底膜の形成も乏しく，血洞を構成する血管内皮細胞には正常肝組織の類洞内皮細胞と同様にfenestrationも観察される．この時点での肝細胞癌は動脈性腫瘍血管の発達も不完全であり血洞の毛細血管化も認めず，周囲に線維製被膜の形成も無く肉眼的には境界不明瞭型を呈することが多い．しかし，腫瘍が増大し腫瘍径が20 mmを超える頃になると，血洞にそってIV型コラーゲンとともにラミニンも明瞭に観察されるようになり，基底膜も形成され血洞内皮細胞のfenestrationもなくなり血洞が毛細血管化をきたしてくる(図1)[3]．この頃の肝細胞癌は動脈性の腫瘍血管も腫瘍組織内に観察されるようになり，線維性被膜も形成され肉眼分類では単純結節型が多い．これらの結果から，発生初期の高分化肝細胞癌では増殖能が低く，周囲の肝組織に対して置換性に発育するため線維性被膜の形成はみられない．この時点での肝細胞癌組織内の血洞の構造は，正常肝組織の類洞構造に類似しラミニンの蓄積と基底膜の形成はほとんど認められない．しかし，腫瘍が増大するにしたがい周囲の肝組織に対して膨張性に発育するようになると，癌組織と非癌部との境

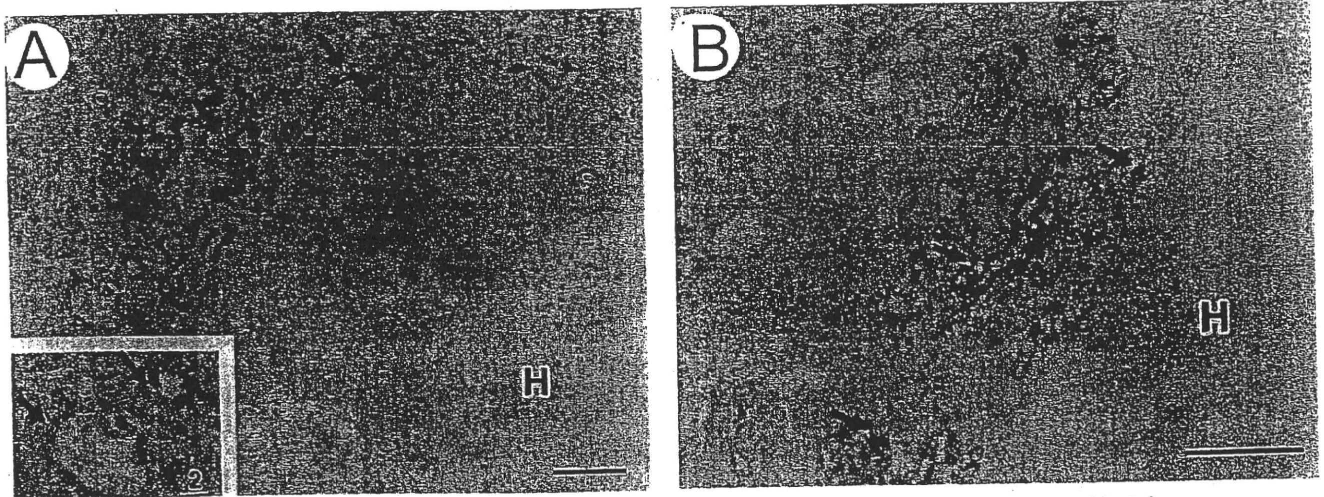


図4　肝細胞癌組織における肝癌細胞によるMT1-MMP (A), MMP-2 (B) の産生 (矢印).

界に線維性被膜が形成される. 肝細胞癌組織内の血洞にはラミニンが蓄積し, 基底膜構造が形成され毛細血管化を呈するようになると考えられる[4,5]. ラミニンはα, β, γ鎖から構成されているが現在15種類のアイソフォームの存在が知られている. この内, $\alpha3\beta3\gamma2$で構成されるラミニン-5は成人の基底膜の主たる構成成分であり腫瘍細胞の接着, 浸潤, 生存, 増殖などに関与しているといわれている[6]. Giannelliらは正常肝や肝硬変組織には存在しないラミニン-5が肝細胞癌組織には観察され, 特に転移巣の辺縁に強く観察されラミニン-5の3本の鎖のうち$\gamma2$の発現程度と転移・予後に相関が認められたと報告している[7].

3 肝癌細胞の基底膜への接着・運動能亢進

細胞の細胞外基質への接着にはインテグリンが重要な役割を果たしている. インテグリンはα鎖とβ鎖からなり, 現在までに24種類の存在が明らかにされている. 細胞はインテグリンを介して細胞外基質に接着すると, さまざまな細胞内シグナル伝達が活性化され, さらに増殖因子レセプターからのシグナル伝達とも協調して, 細胞の生存, 増殖, 運動能が亢進する[8]. われわれは, 肝細胞癌組織においてインテグリン$\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$が発現しており, 特にインテグリン$\alpha6\beta1$とラミニンの発現が協調して観察されることを明らかにした(図2)[9]. さらに, Ⅳ型コラーゲン, ラミニンおよびⅣ型コラーゲンとラミニンを混合した基質はいずれも肝癌細胞の接着, および運動能を亢進させるが, この内Ⅳ型コラーゲンとラミニンを混合した基質が最も強力に肝癌細胞の接着, および運動能を亢進させた[10]. また, 肝癌細胞の基底膜への浸潤能は癌細胞自身が産生するautocrine motility factorがsmall GTPaseであるRhoの活性化とMEK1/2のリン酸化を促進することにより亢進するが, この時$\beta1$インテグリンの活性化が重要であることを明らかにした(図3)[11]. Yangらは肝癌細胞株を用いた検討でTGF-β, EGF, bFGFに対する細胞運動においてインテグリン$\alpha1\beta1$, $\alpha2\beta1$は細胞外基質への浸潤に重要であると報告している[12]. また, Carloniらは肝癌細胞をコラーゲンやラミニンをコートしたディッシュで培養すると, 細胞の運動に関係するFAK/MAP kinaseが活性化されるがこの時インテグリン$\alpha6\beta1$

表1　浸潤型肝癌細胞におけるSnail mRNAとSlug mRNAの発現

	無刺激	ラミニン-5	ラミニン-5 + 抗インテグリン α3抗体	ラミニン-5 + 抗インテグリン α6抗体	抗インテグリン α3抗体	抗インテグリン α6抗体
Snail	0.91 ± 0.33	5.61 ± 2.02	3.72 ± 1.34	8.11 ± 2.92	1.12 ± 0.40	1.08 ± 0.39
Slug	6,285 ± 2,262	14,489 ± 4,057	12,332 ± 2,836	15,153 ± 4,697	6,511 ± 1,953	8,071 ± 2,905

（文献25より改変）

の発現が必要であると述べている[13]．さらにGiannelliらは，TGF-β1はインテグリンα3β1の発現を刺激し非浸潤型の肝癌細胞を浸潤型に変換する．浸潤型の肝癌細胞はラミニン-5上での運動能が亢進していると報告していると述べている[14]．このように肝癌細胞は細胞外基質への接着因子である幾種類ものインテグリンを発現している．これらインテグリンは細胞外基質への接着のみならず，細胞内のシグナル伝達を活性化し細胞の運動などにも深く関係している．

4 細胞外基質の分解

癌細胞が浸潤・転移する際に周囲の細胞外基質，特に基底膜を分解する必要がある．基底膜の主な構成成分であるIV型コラーゲンとラミニンを分解するマトリクスメタロプロテナーゼ（MMP）-2は，通常非活性型で分泌され，低濃度のティッシュインヒビターオブメタロプロテナーゼ（MT）-2によって膜貫通ドメインを有する膜型MMPと接着し細胞表面で活性化される[15,16]．肝細胞癌においてMMP-2とMT1-MMPは非癌部に比べて発現が亢進していた．MMP-2とMT1-MMPは肝癌細胞や肝星細胞で産生されており，脱分化するにつれ発現の亢進が認められた（図4）[17]．MMP-2とMT1-MMPは腫瘍の浸潤先端部に強く発現しており，MT1-MMP, MT3-MMPは肝細胞癌の周囲に存在する線維性被膜内へ浸潤を認める肝細胞癌でより強く発現していた[18,19]．また，MMP-2の発現の強い肝細胞癌の方が外科的切除後の再発率が高かった[20]．AriiらはMMP-9に関しても腫瘍の浸潤先端部に強く発現しており，MMP-9の発現程度とMMP-9のTIMP-1に対する比は線維性被膜内へ浸潤を認める肝細胞癌でより高かったと報告している[21]．*in vitro*の検討において，肝癌細胞をTGF-β, EGF, bFGFやautocrine motility factorで刺激し運動能が亢進すると癌細胞のMMP-2分泌が亢進する．さらにMMP-2の活性を抑制したり，インテグリンβ1抗体で肝癌細胞と細胞外基質の接触を抑制すると肝癌細胞の運動能の低下が観察される[11,12]．肝癌細胞の細胞運動能，各種インテグリンを介した細胞外基質との接着および細胞外基質の分解能には，互いに密接な関連があると考えられる．

5 細胞外基質による肝癌細胞の増殖

われわれは以前に*in vitro*の検討において，基底膜成分であるIV型コラーゲン，ラミニンおよびIV型コラーゲンとラミニンを混合して，コートした培養ディッシュで肝癌細胞を培養すると，いずれの場合もこれら細胞外基質をコートしていないディッシュで培養した肝癌細部に比べて有意に細胞の増殖能が亢進し，特にIV型コラーゲンとラミニンを混合したディッシュで培養した場合が最も増殖力が

強かったことを明らかにした[10]．近年，Bergaminiらは肝細胞癌組織においてラミニン-5が分布している近傍の肝癌細胞の増殖が盛んであること．*in vitro*の検討において，肝癌細胞が表出しているインテグリン$\alpha3\beta1$を介して，ラミニン-5がAktやErk1/2をリン酸化することで，ラミニン-5はEGFとほぼ同程度に肝癌細胞を増殖させることを明らかにした[22]．

6 肝癌細胞の上皮・間葉移行（EMT）と細胞外基質

近年，種々の癌細胞の転移・浸潤過程においてEMTが重要な役割を果たしていることが明らかになってきた．EMTでは細胞間接着因子であるE-カドヘリンの発現低下に伴い，E-カドヘリンの裏打ち蛋白であるβ-カテニンが核内に移行し，WNT-1やc-mycなどが活性化されるといわれている[23]．E-カドヘリンの発現を低下させる転写因子がSnailやSlugといわれている．EMTの結果，癌細胞は浸潤能の亢進が認められるようになる．肝細胞癌組織におけるEMTに関する検討として，SugimachiらはSnailが発現している肝細胞癌ではE-カドヘリンの発現が低下しており，癌部と非腫瘍部でおのおの発現しているSnail遺伝子の比と癌細胞の浸潤能とに相関が認められたと報告している[24]．Giannelliらの検討によると肝細胞癌組織においてラミニン-5，Snail, Slugの発現が亢進しており，E-カドヘリンの発現は低下し，β-カテニンは核内へ移行していた．*in vitro*における検討にて，肝癌細胞株の中でE-カドヘリンの発現の少ない浸潤型の細胞はラミニン-5上で培養するとSnail, Slugの発現が亢進し（表1），E-カドヘリンの発現は低下した．β-カテニンは核内へ移行し細胞の形態も紡錘形に近くなり，細胞間接着が乏しくおのおのの細胞が分散するようになりEMTの所見を呈していた．しかし，このような変化はインテグリン$\alpha3$の機能を抑制することで元の状態に回帰した．一方，E-カドヘリンの発現が多い非浸潤型の肝癌細胞ではラミニン-5上で培養してもSnail, Slugの発現亢進，E-カドヘリンの発現低下は認めるが細胞の分散傾向はみられなかった．しかし，ラミニン-5とともにTGF-$\beta1$を添加するとEMTを呈した．この場合も，インテグリン$\alpha3$の機能を抑制することで元の状態に回帰した[25]．このようにラミニン-5はEMTという肝癌細胞の形態・機能変化を促進することで転移・浸潤にも関与していると考えられる．

7 おわりに

肝細胞癌における細胞外基質，とりわけ基底膜成分であるラミニン-5は癌細胞特有の機能である転移・浸潤能を亢進させ，近年注目されている肝癌細胞の上皮・間葉移行をも促進する．肝癌細胞の機能・形態的変化に肝癌細胞が発現する各種インテグリンは，細胞外基質と肝癌細胞間の情報伝達に関与し肝癌細胞の機能・形態的変化を促している．今後，細胞外基質を介した肝癌細胞の転移・浸潤機序がより明確になり，肝癌細胞の転移・浸潤が抑制可能となることが期待される．

文　献

1) Bedossa P, Paradis V : Liver extracellular matrix in health and disease. J Pathol 200 : 504–515, 2003
2) Liotta LA : Tumor invasion and metastasis-role of the extracellular matrix: Rhoads Memorial Award Lecture. Cancer Res 46 : 1–7, 1986
3) Torimura T, Ueno T, Tanikawa K et al : The extracellular matrix in hepatocellular carcinoma shows different localization patterns depending on the

differentiation and the histological pattern of tumors: immunohistochemical analysis. J Hepatol 21 : 37–46, 1994
4) Kin M, Torimura T, Tanikawa K et al : Sinusoidal capillarization in small hepatocellular carcinoma. Pathol Int 44 : 771–778, 1994
5) Torimura T, Ueno T, Tanikawa K et al : Mechanism of fibrous capsule formation surrounding hepatocellular carcinoma. Arch Pathol Lab Med 115 : 365–371, 1991
6) Miyazaki K : Laminin-5 (laminin-332) : Unique biological activity and role in tumor growth and invasion. Cancer Sci 97 : 91–98, 2006
7) Giannelli G, Fransvea E, Antonaci S et al : Laminin-5 chains are expressed differentially in metastatic and nonmetastatic hepatocellular carcinoma. Clinical Cancer Research 9 : 3684–3691, 2003
8) Guo W, Giancotti FG : Integrin signaling during tumour progression. Nat Rev Mol Cell Biol 5 : 816–826, 2004
9) Torimura T, Ueno T, Tanikawa K et al : Coordinated expression of integrin α6β1 and la-minin in hepatocellular carcinoma. Hum Pathol 28 : 1131–1138, 1997
10) Ogata R : Type Ⅳ collagen and laminin enhance the motility, adhesion, and proliferation of hepatoma cells. Kurume Med J 45 : 11–20, 1998
11) Torimura T, Ueno T, Sata M et al : Autocrine motility factor enhances hepatoma cell invasion across the basement membrane through activation of β1integrins. Hepatology 34 : 62–71, 2001
12) Yang C, Zeisberg M, Kalluri R et al : Integrin α1β1 and α2β1 are the key regulators of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment. Cancer Res 63 : 8312–8317, 2003
13) Carloni V, Mazzocca A, Gentilini P et al : The integrin α6β1 is necessary for the matrix-dependent activation of FAK and MAP kinase and the migration of human hepatocarcinoma cells. Hepatology 34 : 42–49, 2001
14) Giannelli G, Fransvea E, Antonaci S et al : Transforming growth factor-β1 triggers hepatocellular carcinoma invasiveness via α3β1 integrin. Am J Pathol 161 : 183–193, 2002
15) Springman EB, Angleton EL, Birkedahl-Hansen H et al : Multiple modes of activation of latent human fibroblast collagenase: Evidence for the role of a cys-73 active site zinc complex in latency and a cysteine switch mechanism for activation. Proc Natl Acad Sci USA 87 : 364–368, 1990
16) Sato H, Takino T, Okada T et al : A matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature 370 : 61–65, 1994
17) Ogata R, Torimura T, Kin M et al : Increased expression of membrane type 1 matrix metalloproteinase and matrix metalloproteinase-2 with tumor dedifferentiation in hepatocellular carcinomas. Hum Pathol 30 : 443–450, 1999
18) Arai I, Nagano H, Monden M et al : overexpresion of MT3-MMP in hepatocellular carcinoma correlates with capsular invasion. Hepatogastroenterology 54 : 167–171, 2007
19) Harada T, Arii S, Imamura M et al : Membrane-type matrix metalloproteinase-1 (MT1-MMP) gene is overexpressed in highly invasive hepatocellular carcinoma. J Hepatol 28 : 231–239, 1998
20) Théret N, Musso O, Clement B et al : Increased extracellular matrix remodeling is associated with tumor progression in human hepatocellular carcinomas. Hepatology 34 : 82–88, 2001
21) Arii S, Mise M, Imamura M et al : Overexpresion of matrix metalloproteinase 9 gene in hepatocellular carcinoma with invasive potential. Hepatology 24 : 316–322, 1996
22) Bergamini C, Sgarra C, Giannelli G et al : Laminin-5 stimulates hepatocellular carcinoma growth through a different function of α6β4 and α3β1 integrins. Hepatology 46 : 1801–1809, 2007
23) He TC, Sparks AB, Rago C et al : Identification of c-MYC as a target of the ABC pathway. Science 281 : 1509–1512, 1998
24) Sugimachi K, Tanaka S, Tsuneyoshi M et al : Transcriptional repressor Snail and progression of human hepatocellular carcinoma. Clin Cancer Res 9 : 2657–2664, 2003
25) Giannelli G, Fransvea E, Antonaci S et al : Laminin-5 with transforming growth factor-β1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. Gastroenterology 129 : 1375–1383, 2005

INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 24: 17-22, 2009

Epigallocatechin-3-gallate improves nonalcoholic steatohepatitis model mice expressing nuclear sterol regulatory element binding protein-1c in adipose tissue

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Abstract. We examined whether or not epigallocatechin-3-gallate (EGCG) improves liver injury of nonalcoholic steatohepatitis (NASH) model mice expressing nuclear sterol regulatory element-binding protein 1c (nSREBP-1c) in adipose tissue. nSREBP-1c transgenic C57BL6 mice aged 30 weeks were divided into group 1 (no treatment), group 2 (ascorbic acid alone), group 3 (ascorbic acid and 0.05% EGCG), and group 4 (ascorbic acid and 0.1% EGCG). At 42 weeks, we performed measurement of liver weight to body weight, biochemical assays, morphometry of liver specimens, immunohistochemistry for 8-hydro-2'-deoxyguanosine (8-OhdG), and Western blotting for insulin and TNF-α signalings. Ratio of liver weight to body weight in the high dose EGCG-treated group (group 4) was significantly lower than those of groups 1 and 2 ($p<0.05$ and <0.01, respectively). Blood ALT, glucose, total cholesterol, and triglyceride levels of group 4 were significantly low compared with those of the EGCG-non-treated group (groups 1 and 2) ($p<0.05$, respectively). The degrees of steatosis, inflammation, ballooning hepatocytes and Mallory-Denk bodies in group 4 significantly improved compared with those in other groups ($p<0.05$, respectively). The 8-OhdG immunolocalization in liver tissues of the group 4 obviously decreased compared with those of groups 2 and 3. For Western blotting, the expressions of insulin receptor substrate-1 (IRS-1) and phosphorylated IRS-1 (pIRS-1) in liver tissues of group 4 increased compared with those of groups 2 and 3. On the other hand, the expressions of pAkt, pIKKß and pNF-κB decreased compared with those of groups 2 and 3. From these results, EGCG reduces inflammation, insulin resistance and oxidative stress, and suppresses liver injury in nSREBP-1c transgenic mice.

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Abbreviations: epigallocatechin-3-gallate, EGCG; nonalcoholic steatohepatitis, NASH; nuclear sterol regulatory element-binding protein 1c, nSREBP-1c; 8-hydro-2'-deoxyguanosine, 8-OhdG; insulin receptor substrate-1, IRS-1; phosphorylated IRS-1, pIRS-1; nonalcoholic fatty liver disease, NAFLD; nonalcoholic steatohepatitis, NASH; aspartate aminotransferase, AST; alanine aminotransferase, ALT; glycogen synthase kinase, GSK; hepatic stellate cells, HSC

Key words: nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, nuclear sterol regulatory element-binding protein 1c, green tea polyphenol

Introduction

Although the obesity epidemic is a worldwide phenomenon, the severity of the epidemic differs greatly from region to region. The prevalence of obesity among Japanese adults is 3.4% in males and 3.8% in females, and is around one tenth of that in the USA (1). In addition, currently in Japan almost 30% of adult males and females over 50 years old are obese. This rising incidence of obesity parallels the dramatic increase in fatty liver in these age groups. Based on annual health checks, the prevalence of fatty liver diagnosed by ultrasonography increased from 10% in 1980 to 20-40% in 2000 among adults. Thus, nonalcoholic fatty liver disease (NAFLD) is now emerging as the most common liver disease in Japan (2,3). The recent worldwide rise in the number of patients with nonalcoholic steatohepatitis (NASH) tends to parallel the increase in metabolic syndrome, which includes obesity, type 2 diabetes and hyperlipidemia (4).

NAFLD mainly comprises of simple steatosis that is considered benign, however some patients have nonalcoholic steatohepatitis (NASH), which is a clinicopathological entity characterized by the development of hepatic histological changes resembling those induced by excessive alcohol intake that occur in the absence of alcohol abuse (5). Some patients with NASH progress to end-stage liver disease, such as cirrhosis and hepatocellular carcinoma in as little as a decade, and treatment of NASH is therefore very important. However, no single agent improves the histological end points and long-term outcomes (6-8).

Lipodystrophic mice expressing nuclear sterol regulatory element-binding protein 1c (nSREBP-1c) in the adipose tissues show severe insulin resistance, and develop NASH. These animals have marked fatty liver accompanied by hyperlipidemia, hypoleptinemia, and hypoadiponectinemia (9). Immunoreactive 8-hydroxy-2'-deoxyguanosine was observed in the livers of these model mice, suggesting that in addition to insulin resistance, oxidative stress is involved in the development of the NASH-like lesions.

On the other hand, epigallocatechin-3-gallate (EGCG), a type of green tea polyphenol, is a major component of green tea extract, and has the effects of body-fat reduction (10) and antihyperlipidemia (11,12). Therefore, EGCG is useful for NASH patients with obesity and/or hyperlipidemia, but there are no reports indicating that EGCG shows improvement of NASH. In the present study, we examined whether or not catechins improve liver injury of NASH model mice expressing nSREBP-1c in adipose tissue.

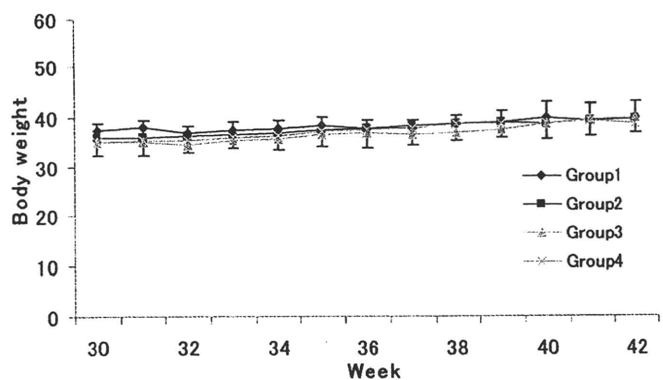

Figure 1. Changes of body weight in mice in the four groups. Body weight of mice in the four groups gradually increased throughout the course of the experiment period, but there is no significant difference among each group.

Materials and methods

Animals and treatment. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine. Transgenic C57BL6 mice (Jackson Lab, ME) expressing nSREBP-1c in adipose tissue were purchased from The Jackson Laboratory (Bar Harbor, ME) (13). We identified nSREBP-1c transgenic mice by amplifying genomic DNA isolated from tails by polymerase chain reaction using a forward primer 5'-CTACATTCGCTTTCTGCAAC-3', and used heterozygous transgenic mice in the following studies. They were bred in our laboratory, mating with wild-type C57BL6 mice (Jackson Lab), in plastic cages with wood chip bedding at a temperature of 18-22˚C, moisture of 40-60%, and on a 12-h light/dark cycle. They were supplied with regular mouse chow (1450 kj/100 g, protein; 24.9 g/100 g, fat; 4.6 g/100 g, Nippon CLEA; Shizuoka, Japan) and water *ad libitum*. nSREBP-1c transgenic C57BL6 male mice aged 30 weeks, which show the typical NASH in liver histology at this age (9), were prepared and used in this study. These mice were divided into four groups [group 1, mice given distilled water alone (n=6); group 2, mice given distilled water containing 0.005% ascorbic acid, which is used to block the oxidation of EGCG (n=6); group 3, mice given distilled water containing 0.005% ascorbic acid and low dose EGCG (0.05%) (n=6); group 4, mice given distilled water containing 0.005% ascorbic acid and high dose EGCG (0.1%) (n=6)], and body weight was measured weekly for 12 weeks. After mice were anethetized with ether at week 43, body weight was measured, blood sample was collected from inferior vena cava, and these mice were examined as follows.

Measurement of body weight and liver weight at week 43. The body and liver weights of mice in each group after sacrifice were recorded and the percentage of expression of liver weight to body weight was measured.

Biochemical assays containing serum biological markers. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, total cholesterol, triglyceride, phospholipid and free fatty acid levels were measured according to the manufacturer's instructions.

Table I. Ratio of liver weight to body weight and blood biochemical assay.

Group	1	2	3	4
Liver weight/ body weight x100 (%)	12.8±2[a]	15.2±1.8[b]	12.4±5.6	7.2±4.8
AST	200±132	183±8	248±153	187±57
ALT	321±260[a]	309±47[a]	286±257	151±67
Glucose	230±35	274±23	272±45	315±54
Triglyceride	50±23[a]	56±19[a]	35±25	30±7
Total cholesterol	156±26[a]	176±17[a]	132±54	107±42
Free fatty acid	508±171	498±68	466±116	496±126
Phospholipid	288±47[a]	309±41[a]	240±101	199±57

Group 1, mice given distilled water alone (n=6); group 2, mice given distilled water containing 0.005% ascorbic acid alone (n=6); group 3, mice given distilled water containing 0.005% ascorbic acid and low dose EGCG (0.05%) (n=6); group 4, mice given distilled water containing 0.005% ascorbic acid and high dose EGCG (0.1%) (n=6). EGCG, epigallocatechin-3-gallate; [a]$p<0.05$, [b]$p<0.01$ compared with group 4.

Histological diagnosis and morphometry of liver specimens. Paraffin-embedded sections of the liver were stained with either hematoxylin-eosin for standard microscopy and Azan-Mallory stain to observe the localization of extracellular matrix. The specimens were reviewed by two independent pathologists. Each specimen was assigned to one of the following histological subgroups for the purpose of comparative analysis, type 1, simple steatosis affecting >33% of the lobules; type 2, steatosis and lobular inflammation; type 3, steatosis and ballooning; type 4, steatosis, ballooning hepatocytes and Mallory-Denk bodies or fibrosis. We dealt with types 3 and 4 as NASH, as described previously (14). In addition, we performed morphometry of liver specimens using the histological scoring system of Kleiner *et al* (15).

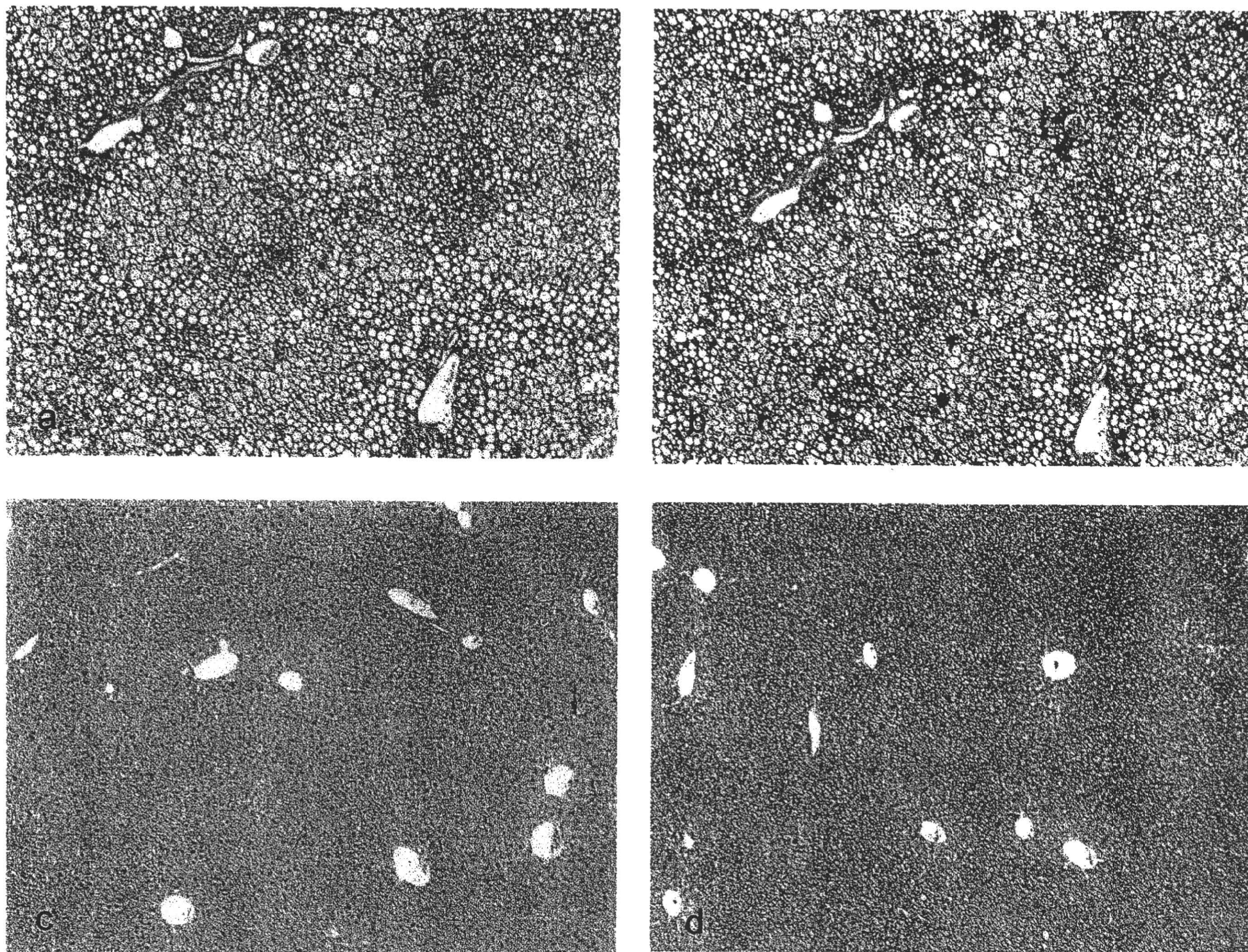

Figure 2. Histological futures of liver specimens. (a) Mouse treated with 0.005% ascorbic acid for 12 weeks. Hematoxylin-eosin stain, x100. (b) Mouse treated with 0.005% ascorbic acid for 12 weeks; Azan-Mallory stain, x100. (c) Mouse treated with 0.005% ascorbic acid and 0.1% EGCG for 12 weeks; Hematoxylin-eosin stain, x100. (d) Mouse treated with 0.005% ascorbic acid and 0.1% EGCG for 12 weeks; Azan-Mallory stain, x100. Immunoreactive products for anti-8-OhdG antibody localized in the cytoplasm of hepatocytes, especially deformed hepatocytes with fat droplets. Liver tissues of mice treated with 0.005% ascorbic acid for 12 weeks show histological features of typical NASH. However, those of mice with 0.005% ascorbic acid and 0.1% EGCG are similar to normal liver.

Immunohistochemistry. Immunoreactive products of 8-hydro-2'-deoxyguanosine (8-OhdG), a marker of oxidative DNA damage, in the liver were examined. Paraffin sections were incubated overnight with mouse monoclonal anti-8-OhgG antibody (Japan Institute for the Control of Aging, Fukuroi, Japan), followed by incubation with alkaline phosphatase-labeled horse antimouse IgG (Vector, Burlingame, CA) and visualization by diamnobenzidine. The degree of 8-OhdG immunolocalization in the liver tissues was categorized as 0, 1, 2 or 3. That is, 0, none in liver tissue; 1, <1/3 in the intrahepatic lobules; 2, 1/3-2/3 in the intrahepatic lobules; and 3, >2/3 in the intrahepatic lobules.

Western blotting. Western blotting was performed using anti-insulin receptor (IR), anti-insulin receptor substrate (IRS)-1 and anti-phosphorylated IRS-1 (pIRS-1), anti-pGSK3α/ß antibodies for insulin signaling, and using anti-Akt, -pAkt, -IκBα, -pIκBα, -NF-κB and -pNF-κB antibodies for TNF-α signaling in liver tissues. Whole extracts were prepared from liver tissues using Triton lysis buffer-containing protease and phosphatase inhibitors. Protein concentration of the extracts was determined, and 40 μg of protein was electrophorased on 10% SDS-polyacrylamide gels. The gels were then blotted onto the nitrocellulose membrane.

Statistical analysis. Numerical data were expressed as means ±SD. Student's t test was performed to assess statistical significance among each group. P-values <0.05 were considered significant.

Results

Changes of body weight of mice in the four groups. Body weight of mice in the four groups gradually increased throughout the course of the experiment period as shown in Fig. 1, but there was no significant difference among each group.

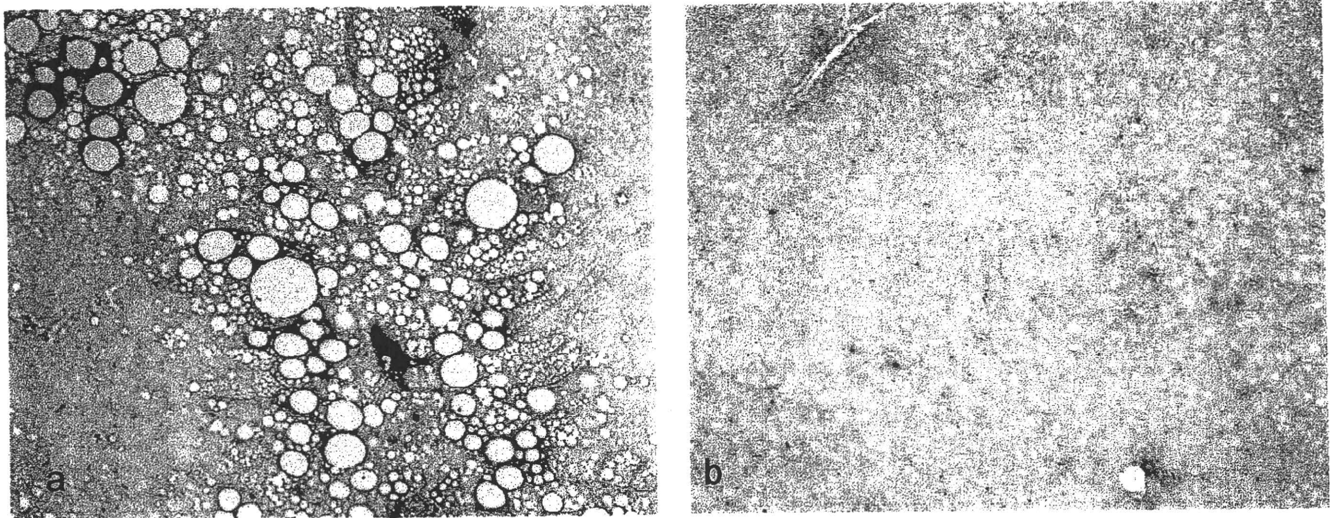

Figure 3. 8-OhdG immunolocalization in liver tissues. (a) 8-OhdG immunolocalization in the liver tissues of the 0.005% ascorbic acid-treated group is at grade 3, x100. (b) 8-OhdG immunolocalization in the liver tissues of the 0.005% ascorbic acid and 0.1% EGCG-treated group is at grade 1, x100. The degree of 8-OhdG immunolocalization in the liver tissues of the 0.005% ascorbic acid and 0.1% EGCG-treated group is low compared with that of other groups.

Ratio of liver weight to body weight at week 43. Ratio (percentage of expression) of liver weight to body weight in the high dose EGCG-treated group (group 4) was lowest compared with that of other groups (group 1, 12.8±2; group 2, 15.2±1.8; group 3, 12.4±5.6; and group 4, 7.2±4.8), and the difference between group 4 and groups 1 and 2 was significant ($p<0.05$ and <0.01, respectively) (Table I).

Biochemical assays containing serum biological markers. Blood ALT, total cholesterol, triglyceride and phospholipid levels of group 4 were significantly low compared with those of groups 1 and 2 ($p<0.05$, respectively) (Table I). The elevation of serum ALT, AST levels by EGCG treatment was not recognized. In addition, there were no significant differences between AST, free fatty acid, and glucose levels between any of the groups.

Morphometry of liver specimens. The degrees of steatosis, intralobular fibrosis, ballooning hepatocyte appearance and Mallory-Denk body appearance in group 4 significantly decreased compared with those in other groups ($p<0.05$) (Table II, Fig. 2).

Immunohistochemistry. Immunoreactive products for anti-8-OhdG antibody localized in the cytoplasm of hepatocytes, especially deformed hepatocytes with fat droplets (Fig. 3). The 8-OhdG immunolocalization in liver tissues of group 4 showed an obvious decrease compared with those of other groups, and the difference between the degree of 8-OhdG immunolocalization in group 4 and that of groups 2 and 3 was significant ($p<0.05$) (Table II, Fig. 3).

Western blotting. In Western blotting, the expressions of IR and pIRS-1 in liver tissues of group 4 increased compared with those of other groups. On the other hand, the expressions of pAkt, pIKKß and pNF-κB in liver tissues of group 4 decreased compared with those of other groups (Fig. 4).

Table II. Morphometry of liver tissues and degree of 8-OhdG immunolocalization in each group.

Group	1	2	3	4
Steatosis	2.9±0.4[a]	3.0±0[a]	2.4±0.5[a]	1.0±1.0
Ballooning hepatocyte	1.3±0.5[a]	2.0±0[a]	1.0±0[a]	0.4±0.5
Mallory-Denk body	0.9±0.4[a]	1.8±0.4[a]	0.8±0.4[a]	0.4±0.5
Fibrosis	1.3±0.8	1.0±0.7	1.2±0.4	0.8±1.1
8-OhdG localization	1.4±0.5	2.2±1.1[a]	1.8±1.3[a]	1.2±0.4

Group 1, mice given distilled water alone (n=6); group 2, mice given distilled water containing 0.005% ascorbic acid alone (n=6); group 3, mice given distilled water containing 0.005% ascorbic acid and low dose EGCG (0.05%) (n=6); group 4, mice given distilled water containing 0.005% ascorbic acid and high dose EGCG (0.1%) (n=6). EGCG, epigallocatechin-3-gallate: [a]$p<0.05$ compared with group 4.

Discussion

Nonalcoholic fatty liver disease (NAFLD) is associated with metabolic syndrome. The metabolic syndrome is characterized by insulin resistance, which is produced by a complex interaction between genetic factors, macronutrient intake and lifestyle that alters the cytokine profile, cell biology and biochemical milieu of the liver, adipose tissue and striated muscle. The resultant disequilibrium in lipid homeostasis causes triglycerides to accumulate in the liver (16). An increase in oxidative stress, due to the generation of reactive oxygen species as a result of mitochondrial abnormalities and induction of the cytochrome P-450 system is one mechanism by which the nonalcoholic fatty liver develops into NASH (4). The pathogenesis of cytologic ballooning and Mallory-Denk body formation and their role in NAFLD remain to be defined. In addition, inflammation and fibrosis are likely to be secondary to cirrhosis, hepatocellular carcinoma and death (2,3,17).

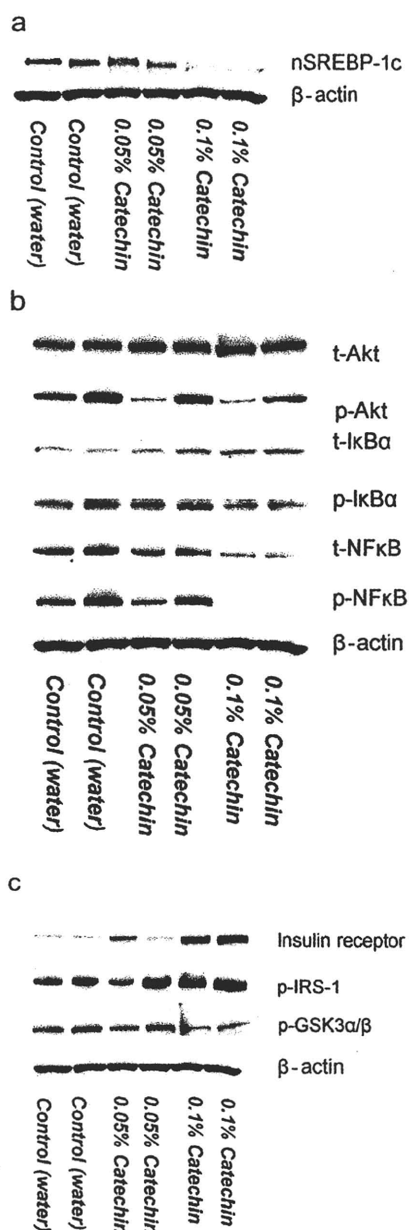

Figure 4. Western blotting. IκB, Inhibitor of κB; NF-κB: nuclear factor κB; t, total; p, phospholylated; IRS, insulin receptor substrate; GSK, glycogen synthase kinase. For Western blotting, the expressions of IR and pIRS-1 in liver tissues of group 4 increased compared with those of other groups. On the other hand, the expressions of pAkt, pIKKß and pNF-κB in group 4 decrease compared with those of other groups.

EGCG is an antioxidant and chemopreventional polyphenol that is found in green tea. It blocks activation of Ap-1 or NF-κB (18). EGCG has shown the inhibition of activation of IKKα, phosphorylation and subsequent degradation of IκBα (18). In addition, EGCG suppresses the proliferation of hepatic stellate cells and production of extracellular matrix in the hepatic fibrosis (19-21). Yumei *et al* reported that EGCG induced the *de novo* synthesis of glutathione and antifibrogenic effects in passaged rat hepatic stellate cells (22,23).

In the present study, green tea polyphenols containing EGCG showed effects of reducing inflammation, insulin resistance and oxidative stress (24-26), and improved the liver injury of transgenic mice expressing nSREBP-1c in the adipose tissue. EGCG inhibits nSREBP-1c expression in adipose tissues and Akt, IκBα and NF-κB expressions of liver tissues, and improves the insulin resistance of the liver tissues by promoting the functional recovery of the insulin receptor, insulin receptor substrate-1 (IRS-1) and glycogen synthase kinase (GSK) in the nSREBP-1c transgenic NASH model mice. The direct effect of EGCG to this model mouse is unclear. Its mechanism is likely due to the antioxidant and chemoprevention effects of EGCG (18).

Ingestion of tea rich catechins leads to a reduction in body fat and malondialdehyde-modified LDL in men (27). In obese individuals, body fat mainly accumulates in submucosal and visceral adipose tissue. Obesity alters both the cellular composition and function of adipose tissue. Adipose tissue of obese individuals contains an increased number of macrophages. Macrophages, adipocytes, and other cellular components of adipose tissue produce numerous circulating inflammatory markers including pro- and anti-inflammatory factors, chemokines, growth factors, and proteases that include a systemic inflammatory state and insulin resistance seen in individuals with increased body mass index (28). In this study, there is no significant difference among the four groups for body weight. Probably, the visceral adipose tissue of nSREBP-1c transgenic mice is less than that of wild type C57BL6 mice, and the significant difference is not recognized among each group.

The liver component of this metabolic disorder is NAFLD, which includes a spectrum of liver pathology ranging from steatosis to cirrhosis. Steatosis is often seen in obese individuals, and both presence and severity of steatosis correlate positively with adiposity. Increased hepatic free fatty acid oxidation that occurs in steatotic livers increases the generation of reactive oxygen species. Increased hepatocyte exposure to reactive oxygen species generates a state of oxidant stress and mitochondrial dysfunction, including hepatocellular injury and activation of hepatic stellate cells (HSC). In NAFLD, intestine-originated endotoxin accumulates the substance in the liver rather than to escape from liver. Increased levels of glucose and insulin up-regulate the synthesis of transforming growth factor-ß, angiotensin II, leptin, adiponectin and so on by HSC (29-31), and develop to the hepatic fibrosis.

In this study, there was no evidence of side effects by EGCG treatment. It will be important to clearly determine whether EGCG or green tea consumption can be used as a tool to prevent the development of NASH.

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References

1. Yoshiike N and Lwin H: Epidemiological aspects of obesity and NASH/NAFLD in Japan. Hepatol Res 33: 77-82, 2005.
2. Hashimoto E, Yatsuji S, Kaneda H, Yoshioka Y, Taniai M, Tokushige K and Shiratori K: The characteristics and natural history of Japanese patients with nonalcoholic fatty liver diseases. Hepatol Res 33: 72-76, 2005.

3. Ono M and Saibara T: Clinical features of nonalcoholic steatohepatitis in Japan: evidence from the literature. J Gastroenterol 41: 725-732, 2006.
4. Harrison SA, Kadakia S, Lang KA and Schenker S: Nonalcoholic steatohepatits: what we know in the new millennium. Am J Gastroenterol 97: 2714-2724, 2002.
5. Ludwig J, Viggiano TR, McGill DB and Ott BJ: Nonalcoholic steatohepatitis Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 55: 434-438, 1980.
6. Powel EE, Cooksley WG, Hanson R, Searle J, Halliday JW and Powell JW: The natural history of steatohepatitis: a follow-up study of forty-two patients for up to 21 years. Hepatology 11: 74-80, 1990.
7. Yoshioka Y, Hashimoto E, Yatsuji S, Kaneda H, Taniai M, Tokushige K and Shiratori K: Nonalcoholic steatohepatitis: cirrhosis, hepatocellular carcinoma and burnt-out NASH. J Gastroenterol 39: 1215-1218, 2004.
8. Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M and Rizzetto M: Expanding the natural history of NASH: from cryptogenic cirrhosis to hepatocellular carcinoma. Gastroenterology 123: 134-140, 2002.
9. Nakayama H, Otabe S, Ueno T, Hirota N, Yuan X, Fukutani T, Hashinaga T, Wada N and Yamada K: Transgenic mice expressing nuclear sterol regulatory element-binding protein 1c in adipose tissue exhibit liver histology similar to nonalcoholic steatohepatitis. Metabolism 56: 470-475, 2007.
10. Bose M, Lambert JD, Ju J, Reuhl KR and Shapses SA: The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. J Nutr 138: 1677-1683, 2008.
11. Lee SM, Kim CW, Kim JK, Shin HJ and Baik JH: GCG-rich tea catechins are effective in lowering cholesterol and triglyceride concentrations in hyperlipidemic rats. Lipids 43: 419-429, 2008.
12. Murase T, Nagasawa A, Suzuki J, Hase T and Tokimitsu I: Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. Int J Obes 26: 1459-1464, 2002.
13. Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL and Brown MS: Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev 12: 3182-3194, 1998.
14. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC and McCullough AJ: Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology 116: 1413-1419, 1999.
15. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ and Sanyal AJ: Nonalcoholic Steatohepatitis Clinical Research Network. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41: 1313-1321, 2005.
16. Korenblat KM, Fabbrini E, Mohammed BS and Klein A: Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. Gastroenterology 134: 1369-1375, 2008.
17. Sanyal AJ: Mechanisms of disease: pathogenesis of nonalcoholic fatty liver disease. Nat Clin Pra Gastroenterol Hepatol 2: 46-53, 2005.
18. Surh YJ: Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 3: 768-780, 2003.
19. Sakata R, Ueno T, Nakamura T, Sakamoto M, Torimura T and Sata M: Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90. J Hepatol 40: 52-59, 2004.
20. Chen A, Zhang L, Xu J and Tang J: The antioxidant (-)-epigallocatechin-3-gallate inhibits activated hepatic stellate cell growth and suppresses acetaldehyde-induced gene expression. Biochem J 368: 695-704, 2002.
21. Zhen M, Wang Q, Huang X, Cao L, Chen X, Sun K, Liu YJ, Li W and Zhang LJ: Green tea polyphenol epigallocatechin-3-gallate inhibits oxidative damage and preventive effects on carbon tetrachloride-induced hepatic fibrosis. J Nutr Biochem 18: 795-805, 2007.
22. Yumei F, Zhou Y, Zheng S and Chen A: The antifibrogenic effect of (-)-epigallocatechin gallate results from the induction of de novo synthesis of glutathione in passaged rat hepatic stellate cells. Lab Invest 86: 697-709, 2006.
23. Fu Y, Zheng S, Lu SC and Chen A: Epigallocatechin-3-gallate inhibits growth of activated hepatic stellate cells by enhancing the capacity of glutathione synthesis. Mol Pharmacol 73: 1465-1473, 2008.
24. Csala M, Margitti E, Senesi S, Gamberucci A, Banhegyi G, Mandl J, *et al*: Inhibition of hepatic glucose 6-phosphatase system by the green tea flavanol epigallocatechin gallate. FEBS Lett 581: 1693-1698, 2007.
25. Collins QF, Liu H, Pi J, Liu Z, Quon MJ and Cao W: Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated protein kinase. J Biol Chem 282: 30143-30149, 2007.
26. Igarashi K, Honma K, Yoshinari O, Nanjo F and Hara Y: Effects of dietary catechins on glucose tolerance, blood pressure and oxidative status in Goto-Kakizaki rats. J Nutr Sci Vitaminol 53: 496-500, 2007.
27. Nagao T, Komine Y, Soga S, Meguro S, Hase T, Tanaka Y and Tokimitsu I: Ingestion of a tea rich in catechins leads to a reduction in body fat and malondialdehyde-modified LDL in men. Am J Clin Nutr 81: 122-129, 2005.
28. Angulo P: NAFLD, obesity, and bariatric surgery. Gastroenterology 130: 1848-1852, 2006 .
29. Bataller R, Sancho-Bru P, Gines P and Brenner DA: Liver fibrogenesis: a new role for the rennin-angiotensin system. Antioxid Redox Signal 7: 1346-1355, 2005.
30. Ding X, Saxena NK, Lin S, Xu A, Srinivasan S and Ananina FA: The roles of leptin and adiponectin: a novel paradigm in adipocytokine regulation of liver fibrosis and stellate cell biology. Am J Pathol 166: 1655-1669, 2005.
31. Oben JA, Roskams T, Yang S, Lin H, Sinelli N, Torbenson M, Smedh U, Moran TH, Li Z, Huang J, Thomas SA and Diehl AM: Hepatic fibrogenesis requires sympathetic neurotransmitters. Gut 53: 438-445, 2004.

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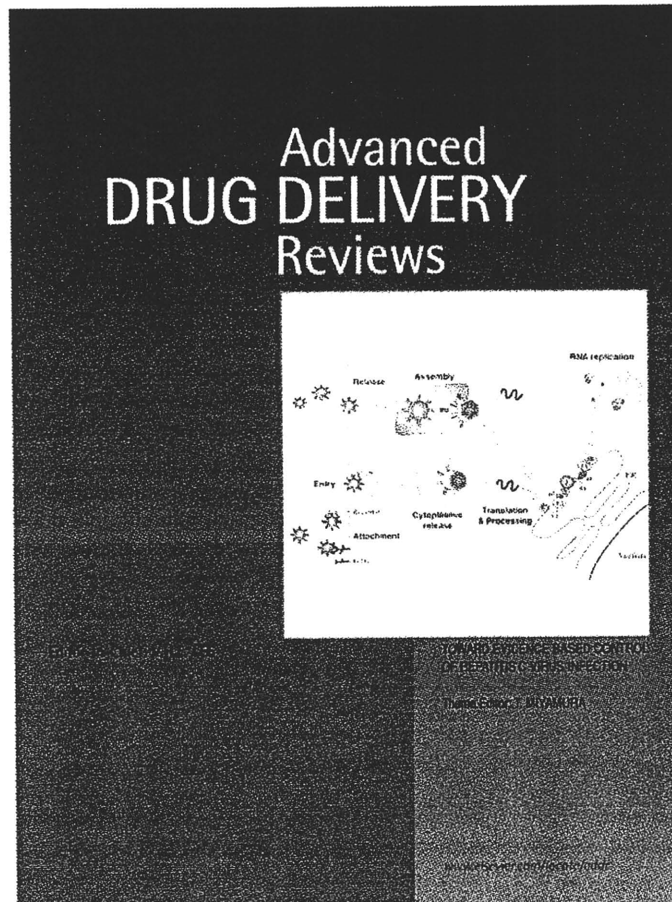

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Hepatitis C viral life cycle ☆

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Abstract

Hepatitis C virus (HCV) has been recognized as a major cause of chronic liver diseases worldwide. Molecular studies of the virus became possible with the successful cloning of its genome in 1989. Although much work remains to be done regarding early and late stages of the HCV life cycle, significant progress has been made with respect to the molecular biology of HCV, especially the viral protein processing and the genome replication. This review summarizes our current understanding of genomic organization of HCV, features of the viral protein characteristics, and the viral life cycle.
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Keywords: Hepatitis C virus; Translation; Polyprotein processing; RNA replication; Viral assembly

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1. Introduction

Since its discovery in 1989, representing a turning-point in the search for infectious agents associated with post-transfusion non-A, non-B hepatitis, hepatitis C virus (HCV) has been recognized as a major cause of chronic liver disease and affects approximately 200 million people worldwide at the present time [1–3]. Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma [3–8]. In general, people with chronic hepatitis C are relatively asymptomatic and have few, if any, clinical manifestations prior to the development of cirrhosis.

HCV is a small, enveloped RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family, which also includes several classical flaviviruses, including dengue virus and yellow fever virus, as well as pestiviruses, such as bovine viral diarrhea virus and the unassigned GB viruses [9,10]. This review summarizes our current understanding of genomic organization of HCV, as well as features of the viral protein characteristics, and the viral life cycle.

2. Genomic organization

The HCV genome consists of a single-stranded positive-sense RNA of approximately 9.6 kb, which contains an open reading frame (ORF) encoding a polyprotein precursor of approximately 3000 residues flanked by untranslated regions (UTRs) at both ends [11]. The precursor is cleaved into at least 10 different proteins: the structural proteins Core, E1, E2 and p7, as well as the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 1).

An important feature of the HCV genome is its high degree of genetic variability [12,13]. Mutation rates, however, vary in different regions. The E1 and E2 regions are the most variable, while the 5′UTR and terminal segment of the 3′UTR have the highest degree of sequence conservation among various isolates. The 5′ UTR, which is ~341 nucleotide (nt) in length, contains an internal ribosomal entry site (IRES), which is essential for cap-independent translation of viral RNA, from which four highly structured domains (domains I-IV) are produced (Fig. 1) [14–19]. These are largely conserved among HCV and related viruses [15,16]. As with other RNA viruses with IRES-mediated expression, the HCV 5′NTR is thought to contain determinants for translation, as well as cis-acting elements for RNA replication. It has been shown that (i) the sequence upstream of the IRES is essential for viral RNA replication, (ii) sequences within the IRES are required for high-level HCV replication, and (iii) the stem–loop domain II of the IRES is crucial for replication [20]. A recent study has revealed that the 5′UTR is capable of binding to a liver-specific microRNA, miR-122, resulting in enhanced HCV RNA replication [21]. (Fig. 2).

The 3′UTR varies between 200 and 235 nt in length, including a short variable region, a poly(U/UC) tract with an average length of 80 nt, and a virtually invariant 98-nt X-tail region [22–24] The X region forms three stable stem–loop structures that are highly conserved among all genotypes and, as a result, the HCV genome likely ends with a double-strand stem structure. It appears that the 3′X region, as well as the 52 nt upstream of the poly(U/C) tract, are crucial for RNA replication, while the remainder of the 3′UTR plays a role in enhancement of replication [25,26].

To date, hepaciviruses are divided into six principal genotypes of HCV that differ in their nucleotide sequences by 31–34%, and in their amino acid sequences by ~30%. HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies [12]. HCV heterogeneity is primarily due to a high error rate of the RNA-dependent RNA polymerase encoded by the NS5B gene. The existence of different quasispecies of the HCV genome appears to contribute to viral persistence. It has been shown that patients with chronic hepatitis C have greater genetic complexity in terms of the population of quasispecies they possess than patients with spontaneous clearance [13]. During the course of chronic infection, random genetic drift steadily induces the development of quasispecies primarily due to changes in the

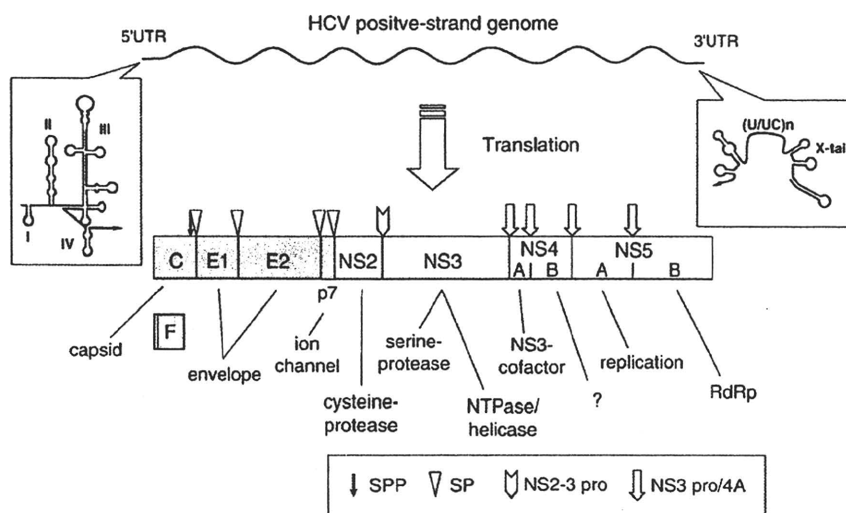

Fig. 1.

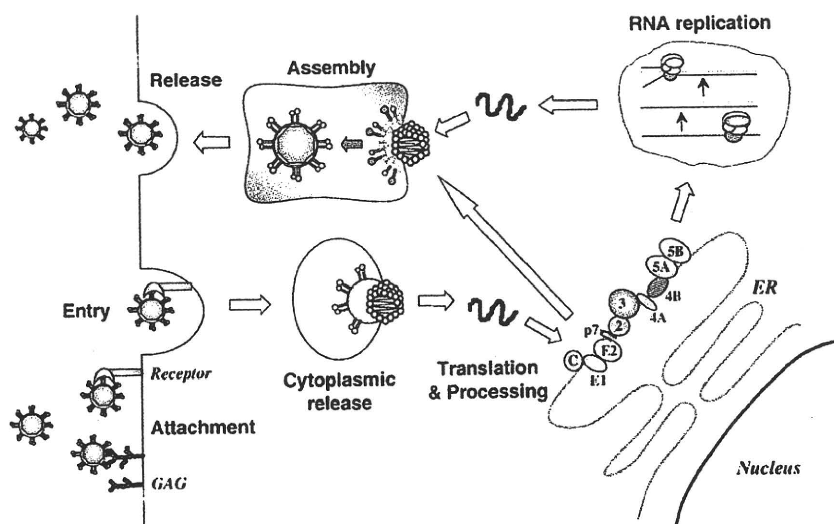

Fig. 2.

hypervariable region 1, involving the 27 N-terminal 27 residues of the E2 envelope protein [27–29].

3. Features of the viral proteins

3.1. Core protein

The HCV core protein, which is derived from the N-terminus of the polyprotein, most likely forms the viral nucleocapsid given similarities between its position and that of sequences encoding viral nucleocapsids in other flavivirus genomes. The amino acid sequence of the core protein is highly conserved among different HCV strains, compared with other HCV proteins. HCV core protein has been extensively used in a number of serologic assays since anti-core antibodies are highly prevalent among HCV-infected individuals. Although several core proteins of varying molecular weights have been identified [30–33], the core protein is released as a 191-residue precursor of 23 kDa and further processing yields the predominant form of 21 kDa. The N-terminal domain of the core protein is highly basic, while its C-terminus is hydrophobic. Several groups have reported a complex intracellular localization of the core protein [30,33–42]. The core protein is primarily detected in the cytoplasm, in association with the endoplasmic reticulum (ER), lipid droplets, and mitochondria. In some studies, a fraction of the core protein has also been found in the nucleus.

The ubiquitin–proteasome pathway, a major route by which selective protein degradation occurs in eukaryotic cells, is involved in post-translational modification of the core protein [32,43–45]. An initial report indicated that processing at the carboxyl-terminal hydrophobic domain of the core protein produced efficient polyubiquitylation and proteasomal degradation [32]. Recently, ubiquitin ligase E6AP has been identified as an HCV core-binding protein that enhances ubiquitylation and degradation of mature, as well as carboxyl-terminus truncated-core protein, and it has been suggested that E6AP-dependent degradation of the core protein is common to a variety of HCV isolates and plays a critical role in the HCV life cycle [45].

The core protein is likely multifunctional and essential for viral replication, maturation, and pathogenesis. It is involved not only in formation of the HCV virion, but also has a number of regulatory functions, including modulation of signaling pathways, cellular and viral gene expression, cell transformation, apoptosis, and lipid metabolism [reviewed in 46].

3.2. E1 and E2 envelope proteins

The E1 and E2 proteins are essential components of the virion envelope and are necessary for viral entry. These glycosylated proteins extend from aa 192–383 (E1) and from aa 384–746 (E2) of the polyprotein, and have molecular weights of 33–35 and 70–72 kDa, respectively [47]. Along the precursor polyprotein, it has been suggested that the C-terminal transmembrane domains of E1 and E2 form hairpin structures that pass through the membrane twice, thereby allowing processing by a signal peptide in the ER lumen [48]. Upon signal peptidase cleavage, the C-termini are thought to translocate into the cytoplasm in order to generate the type I membrane topology of mature E1 and E2. Mature E1 and E2 remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediates retention of the E1–E2 complex in the ER. It has recently been demonstrated that, in addition to this conventional type I membrane topology, E1 protein also adopts a polytopic topology, in which the protein twice spans the ER membrane with an intervening cytoplasmic loop spanning aa 288–360 [49].

3.3. p7 protein

The p7 protein is a small (63 aa) hydrophobic polypeptide that adopts a double membrane-spanning topology. This protein is essential for the production of infectious virions in vivo [50] and may belong to a small protein family of viroporins, which are known to enhance membrane permeability. It has been revealed