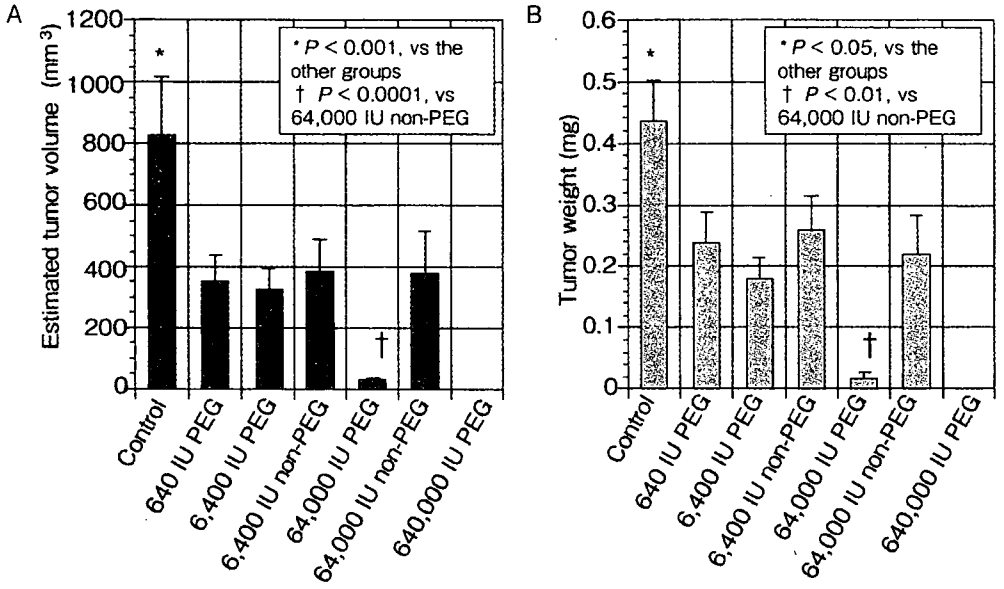

図5　PEG-IFN-α 2bとIFN-α 2bの*in vivo*における抗腫瘍効果の比較

ヌードマウス皮下にヒト肝癌腫瘍を作成し，640，6,400，64,000，640,000 IUのPEG-IFN-α 2b，6,400 IU，64,000 IUのIFN-α 2bあるいは培養液(コントロール)を1週間に2回，合計4回ヌードマウスの皮下に接種し，腫瘍の腫瘍体積(A)および腫瘍重量(B)をそれぞれIFN接種開始から14日目と15日目に比較した．同じ活性のPEG-IFN-α 2bとIFN-α 2b投与では，PEG-IFN-α 2b投与の方が腫瘍の体積および重量ともに低い値を呈した．640 IU PEG-IFN-α 2bのヌードマウスへの投与は，C型慢性肝炎の治療にヒトに使用する量に相当．PEGは，PEG-IFN-α 2b，non-PEGは，IFN-α 2bの略．値は，平均値±標準誤差を示す．

れた．このように，これらIFN-α製剤は臨床量で肝癌の増殖を抑制し，その増殖抑制機序としてアポトーシスの誘導と血管新生抑制が示唆された[17]．

次に，PEG-IFN-α 2bについて検討を行った．PEG-IFN-α 2bは，PEG化により吸収・排泄速度が低下し，通常のIFNに比べ生物学的半減期が数倍延長する結果，長時間IFN-α 2bの血液濃度が維持されるという特徴を有する．臨床量の1/3量(640 IU/mouse, 3.2×10^4 IU/kg)，その10倍，100倍，1,000倍量を1週間に2回，合計4回皮下に投与し腫瘍の経時的な推定体積や，15日目に摘出された腫瘍の重量や組織像を比較した．その結果，臨床量の1/3量の投与でコントロールに比べ約50％前後，腫瘍の体積および重量が減少した(図5)．増殖抑制機序としては，アポトーシスの誘導を認めたが，血管新生抑制は確認できなかった．PEG-IFN-α 2bと同じ活性(IU)のIFN-α 2bを同様の方法で投与し抗腫瘍作用をPEG-IFN-α 2bを投与した場合と比較すると，PEG-IFN-α 2bを投与した方が，腫瘍のアポトーシス誘導は高度であり，有意により強い抗腫瘍作用を認めた(図5，図6)．*in vitro*では，PEG-IFN-α 2bはIFN-α 2bと同程度に増殖抑制効果が最も低いIFN-α製剤であったが，PEG化により長時間血中IFN-α 2bの濃度が維持されたことにより，肝癌細胞に持続的に作用し，非PEG化IFN-α 2bや他のIFN-α製剤より強い増殖抑制作用を発揮したと推察される[15]．

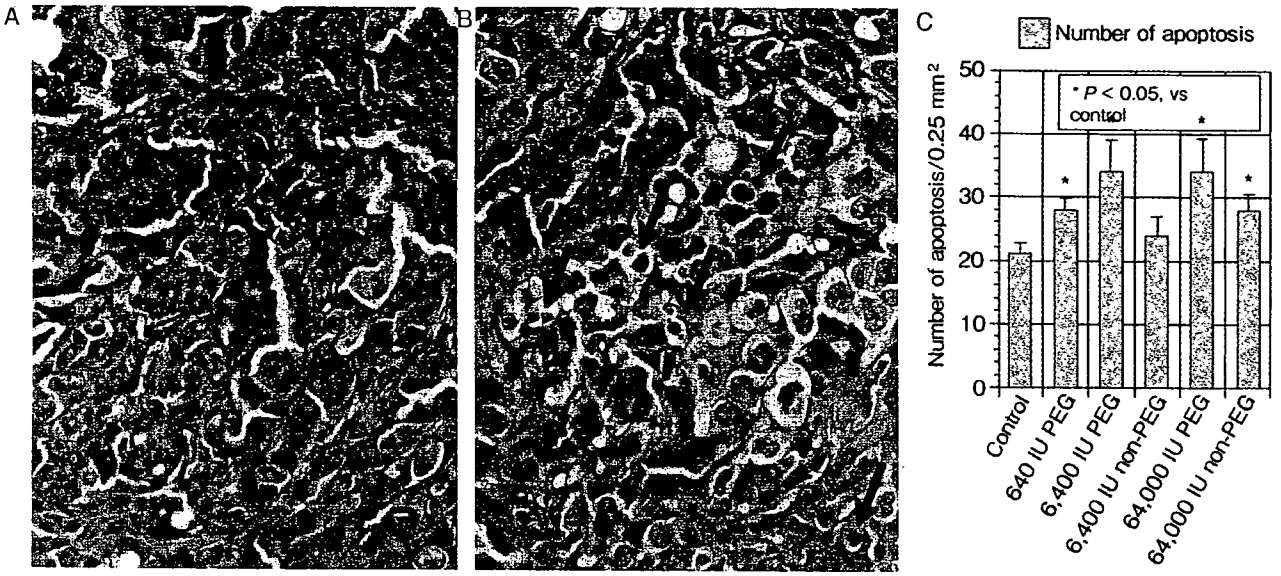

図6　PEG-IFN-α 2bあるいはIFN-α 2bのヌードマウス皮下移植ヒト肝癌腫瘍組織に対する作用

ヌードマウス皮下にヒト肝癌腫瘍を作成し，640，6,400，64,000，640,000 IUのPEG-IFN-α 2b，6,400 IU，64,000 IUのIFN-α 2bあるいは培養液(コントロール)を1週間に2回，合計4回ヌードマウスの皮下に接種し，15日目に切除した腫瘍からHE染色標本を作製し，腫瘍細胞のアポトーシスを測定した．A, Bに，腫瘍の組織像(200倍，ヘマトキシリン・エオジン染色)を示す．コントロール(A)に比べ，6,400 IU PEG-IFN-α 2b投与マウス(B)の腫瘍組織にアポトーシスの出現が目立つ．Cには，各種活性のPEG-IFN-α 2bあるいはIFN-α 2bを投与されたマウスおよびコントロールマウスの皮下腫瘍の癌細胞のアポトーシス数の測定結果を示す．PEG-IFN-α 2bあるいはIFN-α 2bの投与量が増えるとアポトーシス数は増加し，同じ活性では，IFN-α 2bに比べPEG-IFN-α 2bの方がアポトーシス数は増加している．640,000 IU PEG-IFN-α 2b投与マウスは，腫瘍が消失したため測定値なし．値は，平均値±標準誤差を示す．

6 おわりに

これまでのわれわれの検討により，肝癌細胞がIFN-αの作用発現に重要なⅠ型IFNのレセプターを発現していること，IFN-αが，*in vitro*では，肝癌細胞株に対しアポトーシス・細胞周期の進行停止などを誘導し直接的に増殖を抑制すること，*in vivo*では，臨床量のIFN投与でも，アポトーシス誘導などにより抗腫瘍作用がもたらされることが明らかとなった．ヒトの肝細胞癌および非癌部組織は，比較的高頻度にIFNAR-2鎖を発現しており[16]，今回の実験結果から，IFN投与による肝発癌・再発抑制機序の一つとしてIFNによる直接的抗腫瘍作用が考えられる．また，血清AFP持続高値のC型慢性肝炎患者がIFN投与後に血清AFP値が低下する機序が，臨床的に不顕性な初期の段階の肝癌細胞のIFNによる直接的排除である可能性も推察される．

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1. C型肝硬変と抗ウイルス療法

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成　　績

1. C型慢性肝炎，肝硬変に対するペグインターフェロン（PegIFN）とリバビリン（RBV）併用療法治療成績

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）肝硬変に対する治療に関する研究班（主任研究者：八橋 弘：長崎医療センター）ではPegIFNとRBV併用療法の治療成績，治療効果に関わる因子，治療効果予測に関する解析を行っている。本研究班登録症例の現時点での成績を紹介する。

2004年12月から2006年12月までの期間，26の国立病院機構肝疾患専門医療施設内にて1,098例（HCV Ⅰ型 822例，Ⅱ型 261例）のC型慢性肝炎症例（肝硬変例を含む）にPegIFNとRBV併用療法が導入された（図1）。Ⅰ型822例の平均年齢は56.6歳（17～79歳），性別では男性456例（55%），女性366例（45%）。治療前に肝生検が施行された618例中，F0-2は計426例（69%），F3-4は192例（31%）であった。HCV Ⅱ型 261例の平均年齢は52.2歳（22～77歳），性別では男性127例

施設名	合計
大阪医療	228
長崎医療	111
九州医療	99
呉医療	66
京都医療	64
小倉	51
大阪南医療	46
東京	45
金沢医療	43
大分医療	42
災害医療	35
国際医療	35
横浜医療	34
熊本医療	31
仙台医療	26
西埼玉中央	26
名古屋医療	26
相模原	20
中信松本	18
東京医療	16
嬉野医療	11
岡山医療	7
米子医療	6
西札幌	4
善通寺	4
別府医療	4
合計	1,098

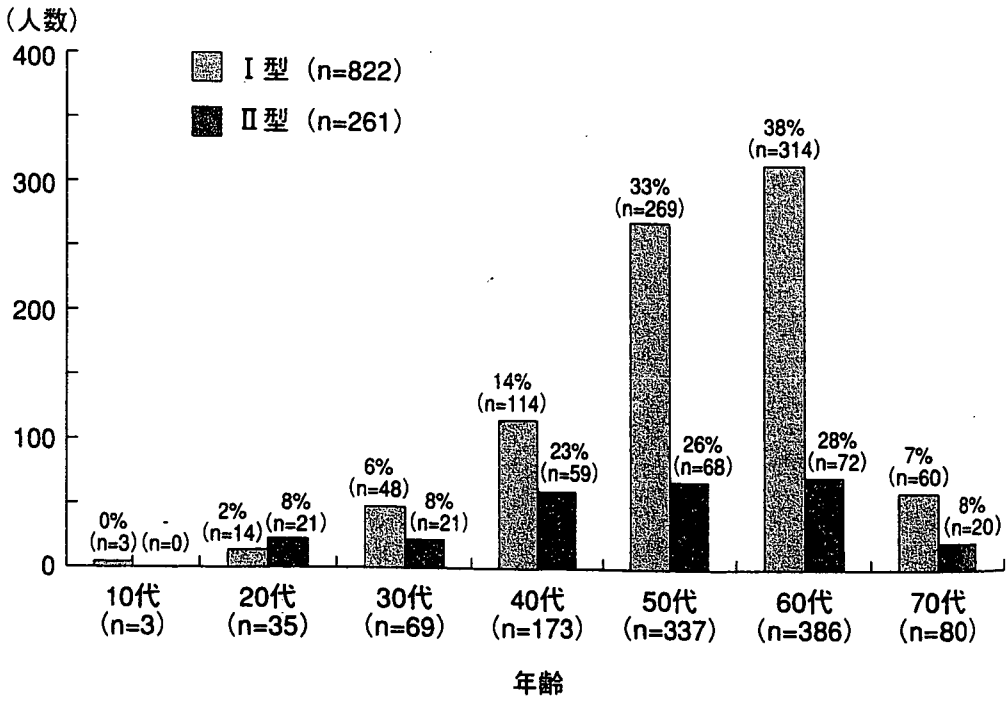
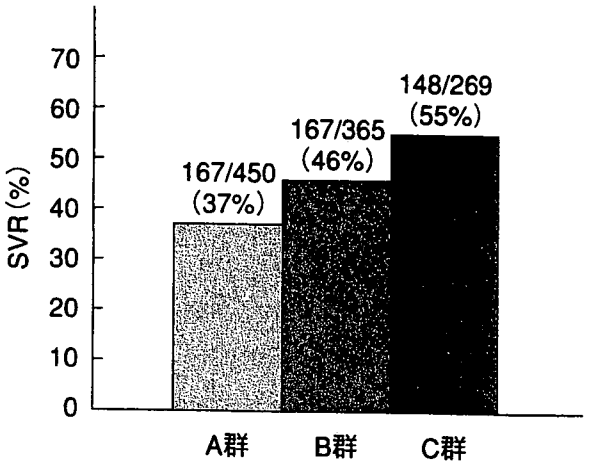


図1　Peg-IFN＋RBV治療例の年齢分布（genotype別）（2004年12月～2006年12月導入症例，n=1,083）

	SVR率	24週未満で治療中止		24週以上48週未満で治療中止		48週以上の治療
A群：全症例に対して	167/450 (37%)	0/85 (0%)	＋	19/96 (20%)	＋	148/269 (55%)
B群：24週以上投与例に対して	167/365 (46%)			19/96 (20%)	＋	148/269 (55%)
C群：48週以上投与例に対して	148/269 (55%)					148/269 (55%)



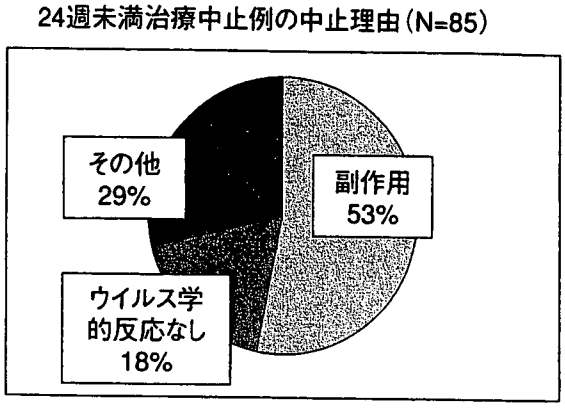


図2　I型・high Peg-IFN＋PBV治療例のSVR率（PP解析）と24未満治療中止例での中止理由

（49％），女性134例（51％）。治療前に肝生検が施行された167例中，F0-2は計123例（74％），F3-4は44例（26％）であった。

I型822症例中，治療前ウイルス量が100 KIU/ml以上の高ウイルス症例で，なおかつ2007年1月の時点で治療効果判定が可能であった450例で解析を行った。450例中，24週間未満に治療を中断した85例ではSustained Virological Response（SVR）は1例もなく，24週間以上48週間未満に治療を中止した96例中19例（20％）がSVRと，ともに低いSVR率であったが，48週間以上治療を行った269例中148例（55％）がSVRとなった。

治療終了後24週経過した450例でのSVR（Sustained Viral Response）率を計算すると，450例全体（A群）ではその中の167例（37％）がSVRに，450例のうち24週間未満に治療を中断した85例を除いた365例（B群）中167例（46％）がSVRに，450例から24週間未満に治療を中断した85例と24週間以上48週間未満に治療を中止した96例を合わせて除外した48週以上投与した269例（C群）中148例（55％）がSVRとなった(**図2**)。24週間未満に治療を中断した8例での治療中断の理由は，副作用が53％，ウイルス学的な反応がない18％，その他の理由29％であった。

代表的な治療効果予測因子と治療効果（SVR）との関連(**図3, 4**)に関する検討では，性差，治療前HCV Core抗原量ではSVR率に差は認められなかったが，年齢，治療中の効果予測因子であるEVR（Early Viral Response：治療12週目の血中HCV-RNA陰性化）の有り無し，肝線維化の程度ではSVR率で有意差を認めた。

SVRに関与する因子の検討では，宿主因子（年齢，性別，体重，白血球数，好中球数，Hb値，血小板数，ALT値，初回再治療の有無，肝硬変の有無），ウイルス因子（HCV-RNA量：アンプコアハイレンジ法），薬剤因子（治療期間，Peg-IFN α2b（m/kg）の開始量，Ribavirin（mg/kg）の開始量）を変数として用いて単変量，多変量解析を行ったところ，単変量解析では，年齢，白血球

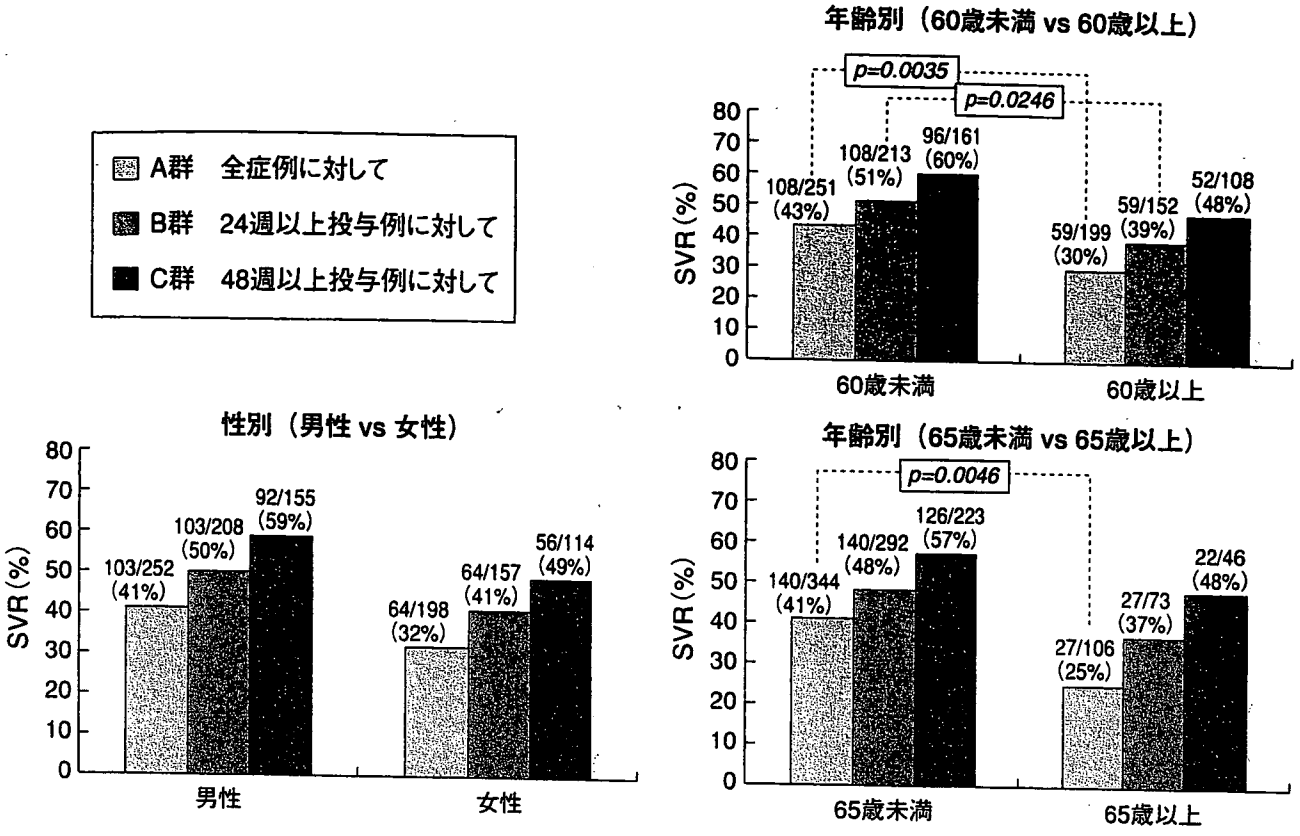


図3　I型・high Peg-IFN＋RBV治療例SVR率（PP解析）

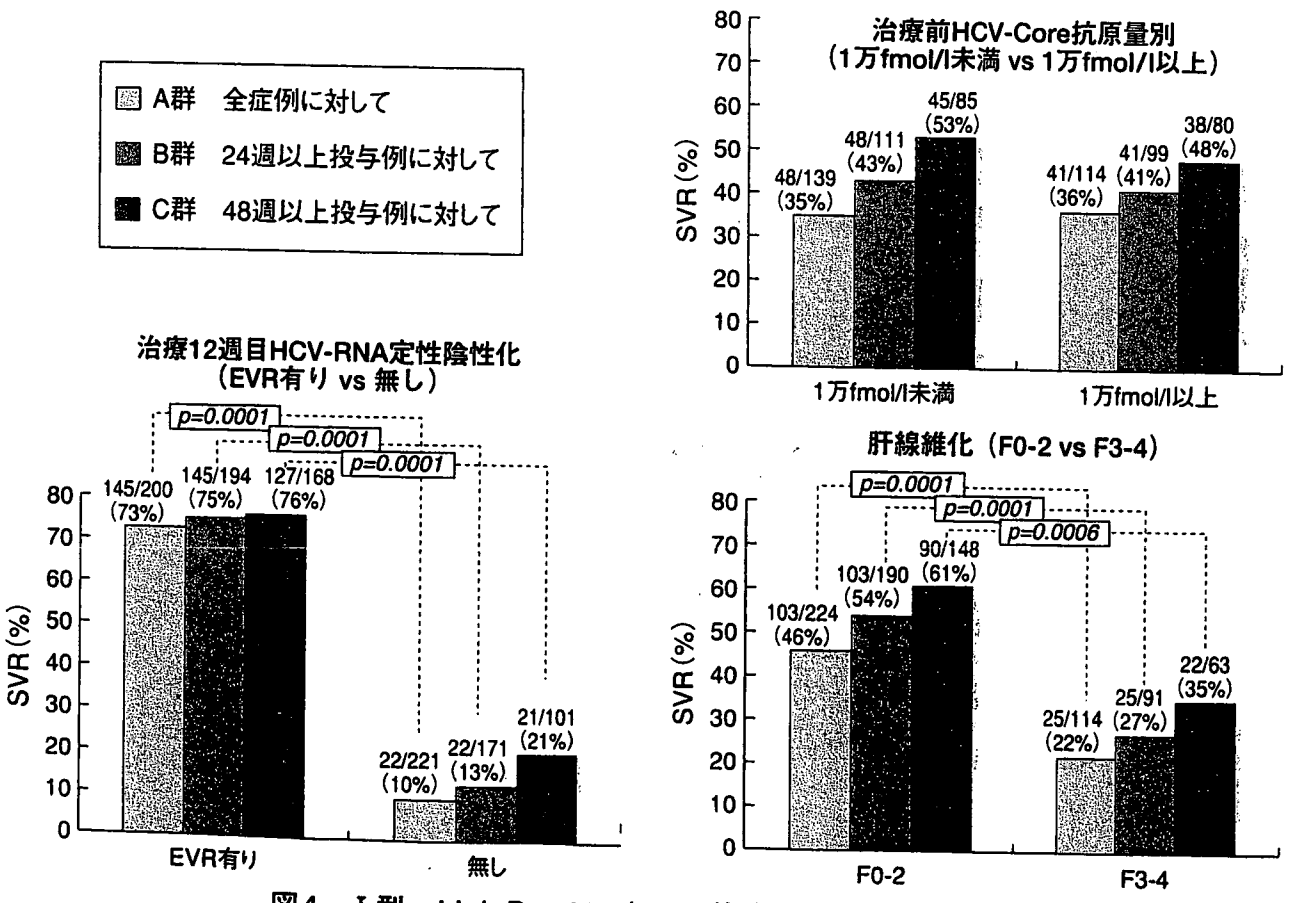


図4　I型・high Peg-IFN＋RBV治療例SVR率（PP解析）

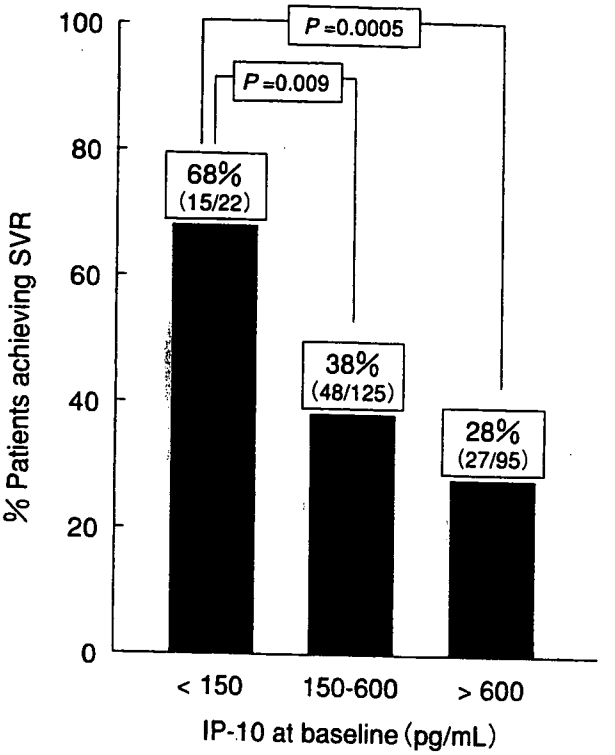


図5　治療前血中IP-10値と治療効果との関係
肝硬変に対する治療研究班登録症例　Peg-IFN/RBV併用療法を施行した242例

数，血小板数，肝硬変の有無，治療期間，Peg-IFNα2b（m/kg）の開始量，Ribavirin（mg/kg）の開始量，の7因子が有意となった。さらに，その7因子で多変量解析を行ったところ，治療期間，血小板数，肝硬変の有無の3つが有意な因子として抽出された。

以上まとめると，HCVⅠ型高ウイルス例でのPeg-IFNとRBV併用療法の治療成績には，1）治療期間，2）血小板数，3）肝硬変の有無が大きく関与していると考えられる。血小板数と肝硬変の因子は，ともに肝線維化が高度に進展していることを意味している。HCVⅠ型高ウイルス症例に対するPeg-IFNとRBV併用療法においても肝線維化進展例は難治例と考えられた。また，治療期間によって，SVR率の歴然とした差を認めたことから，HCVⅠ型高ウイルス症例に対する本治療では，十分な治療期間が必要であることをあらためて再確認した。

2. HCVⅠ型の高ウイルス症例に対するPeg-IFNとRBV併用療法の治療効果と血中IP-10値に関する検討－IFNシグナルとの関連－

IP-10は，IFNγによって誘導されるケモカイン（CXCL10）であり，健常者に比較してC型慢性肝炎患者において有意に高いことが知られている。2006年，C型慢性肝炎に対するPeg-IFNとRBV併用療法の治療効果と血中IP-10値には密接な関係があることが欧米で相次いで報告され注目されている[1～3]。

今回，日本人を対象として，血中IP-10（CXCL10）濃度とPeg-IFNとRBV併用療法の治療効果との関連について検討した。健常者40名とHCV 1b感染者92名でのIP-10値の平均値（±SD）は，前者が97.9（±24.9）pg/ml，後者では，543.9（±297.4）pg/mlであり，HCV 1b感染者では非感染者に比較して有意に高値であった（$p<0.0001$）。Peg-IFNとRBV併用療法の治療効果と血中IP-10値との関連について242例（SVR 90例，Non-SVR 152例）で検討した。SVR例での中央値は，417（197～752）pg/ml，Non-SVR例では565（293～565）pg/mlと，SVR例ではNon-SVR例に比して有意に低い値（$p=0.018$）であっ

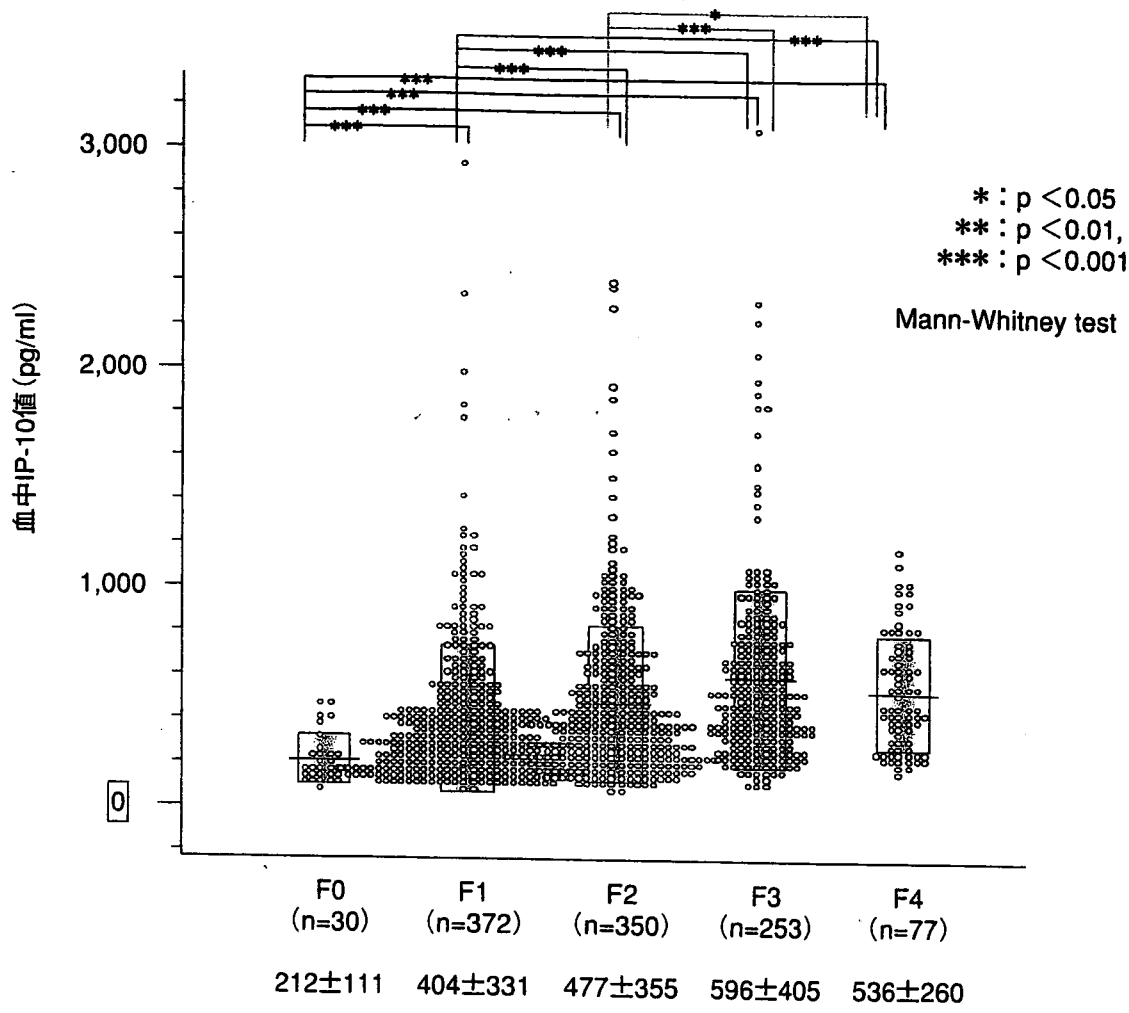


図6　治療前血中IP-10値と肝線維化（F0-4）との関係
肝硬変に対する治療研究班登録症例，肝生検後にIFN治療を施行した1,082例

た。血中IP-10濃度の層別解析では，血中IP-10濃度が150 pg/ml未満では68％，150～600 pg/mlでは38％，600 pg/ml以上では28％のSVR率であり，600 pg/ml以上では有意に低いSVR率（68％ vs. 28％：p=0.0005）を示した（**図5**）。さらに肝線維化の進展度とIP-10値との関係では，肝線維化の進展とともに，IP-10値は上昇した（**図6**）。

Peg-IFNとRBV併用療法中のIP-10値の推移では，Non-SVR例では，治療初期にはその値は一度上昇したあとに低下するのに対し，SVR例では上昇することなく持続的に低下する例が多く，治療効果とIP10の推移には差が認められた。

Peg-IFNとRBV併用療法中のIFN誘導関連遺伝子（IP-10，IFNAR2，IRF1，IRF，PKR，MxA）の動きを，PBMCを用いてReal-time PCR法で定量を行ったところ，Peg-IFNとRBV投与によってmRNAレベルでIFNAR2，IRF1，IRF2の値は，治療前に比して有意にその発現が低下するも，MxA，PKRは有意にその発現が増強していた。治療効果との関連では，治療前のmRNAレベルのIP-10，IFNAR2，IRF1，IRF，PKR，MxA因子の発現量に関してはNon-EVR群，EVR群との間に差は見いだせなかったが，Peg-IFNとRBV投与後の推移に関しては，治療2週目の時点で，IP10とPKRの発現量は，Non-EVR群ではEVR群に比して，有意に上昇した。今回のPeg-IFNとRBV投与後のIFN誘導関連遺伝子の検討からは，治療抵抗例においてIFN誘導関連遺伝子発現が低下している，IFN刺激に反応しない，ないし反応不良といった現象は確認されず，むしろ治療初期の反応は

表1　C型肝炎IFN治療効果に影響を及ぼす因子（J. Heatecote 2007）

Viral Factors that Influence Response to Therapy	Host Factors that Influence Response to Therapy
・Genotype 2>3>4>1 ・Baseline viral load (HVL v LVL) ・Viral load decline (RVR v SVR) ・Co-infection (HIV/HBV) ・Quasispecies development ・Virus interferes with function of crucial genes in several antiviral pathways 　- ? Host dependent	・Ethnicity (Blacks<Caucasians<Asians) (genes governing immune response) 　- eg: IP-10 level 　- IL polymorphisms 　- MHC ・Steatosis/ high BMI / insulin resistance ・Cirrhosis ・'Gene signature' - pre-treatment interferon pathway already upregulated (liver tissue)

逆で，Non-EVR群ではEVR群よりもIFNによる反応は良好であった。

欧米[4～6]からも，治療抵抗例でこそ，よりIFN誘導関連遺伝子が過剰発現しているという報告が，いくつか散見される（表1）。われわれの検討結果からは，Peg-IFNとRBV併用療法治療抵抗例では，IFNシグナルのある部分が障害されて伝達障害が発生しているというメカニズムよりも，むしろC型肝炎の炎症や線維化に伴い内因性のIFN関連シグナルが過剰発現することで，何らかのメカニズムでフィードバック機構が作動し，その結果，外因性のIFN関連シグナルが抑制されるというメカニズムが想定された。

結　論

1. 肝硬変であること，肝線維化が進展していることは，Peg-IFNとRBV併用療法での重要な治療抵抗性因子である。
2. 治療抵抗例では，IFNシグナルの発現低下や反応性の低下は認められず，むしろ過剰発現していた。
3. 肝硬変症例の多くの例では，現行の治療法を駆使しても確実にSVRとすることが困難であることから，Peg-IFNとRBV併用療法途中でSVRが困難と判断した時点，もしくは治療開始時点から，ウイルス駆除を目指さないIFN少量長期投与でのALT値の安定化ないしAFPの低下を治療目標とした発癌抑制療法を選択すべきである。

平成18年度　厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）肝硬変に対する治療に関する研究班

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Hepatology Research 2007; 37: 661–666 doi: 10.1111/j.1872-034X.2007.00094.x

Short Communication

Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy

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Aim: The clinical significance of hepatitis B virus (HBV) core-related antigen (HBcrAg) in predicting the reactivation of hepatitis after halting lamivudine administration was analyzed.

Methods: A total of 34 patients with chronic hepatitis B were enrolled. Lamivudine was administered for at least 6 months before cessation, and reactivation of hepatitis was defined as elevation of alanine aminotransferase levels to more than 80 IU/L within 12 months of cessation.

Results: In total, 20 (59%) patients experienced hepatitis reactivation. Although concentrations of HBV DNA and HBcrAg in serum did not differ between the two groups of patients at the onset of lamivudine administration, HBcrAg serum levels were significantly higher ($P = 0.009$) in the reactivation patients (median 4.9, 25–75% range 4.7–5.9 log unit/mL) than the non-reactivation patients (median 3.2, 25–75% range <3.0–4.5 log unit/mL) post-lamivudine treatment. The concentration of HBV DNA did not differ between the two groups (median <3.7, 25–75% range <3.7–<3.7 log copy/mL in the reactivation group vs. median <3.7, 25–75% range <3.7–<3.7 log copy/mL in the non-reactivation group). Receiver operating characteristic analysis of HBcrAg concentration showed an area under the curve of 0.764 in predicting patients without reactivation of hepatitis.

Conclusion: HBcrAg can be a useful marker to identify patients who are not at risk of reactivation of severe hepatitis after discontinuation of lamivudine administration.

Key words: chronic hepatitis B, hepatitis B virus core-related antigen, hepatitis B virus DNA, hepatitis reactivation, lamivudine

INTRODUCTION

LAMIVUDINE, A NUCLEOSIDE analog that inhibits reverse transcriptase, has been found to inhibit the replication of hepatitis B virus (HBV), reduce hepatitis, and improve histological findings of the liver in long-term treatment.[1,2] Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B.[3,4] However, there are a number of problems with lamivudine therapy, including hepatitis relapse due to the appearance of YMDD mutant viruses and the reactivation of hepatitis after its discontinuation.[5,6]

During lamivudine administration, the concentration of serum HBV DNA decreases, and usually becomes undetectable to even high sensitivity HBV DNA assays. However, this undetectable level is an inadequate indicator for safely discontinuing lamivudine

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Received 17 September 2006; revision 20 February 2007; accepted 25 February 2007.

administration as active hepatitis often recurs in patients post-treatment.

Previously, a chemiluminescence enzyme immunoassay (CLEIA) was developed by our laboratory to detect of hepatitis B core-related antigen (HBcrAg).[7,8] This HBcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens using monoclonal antibodies, which recognize common epitopes of these two denatured antigens because both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical.[9–11] Although this assay reflects the viral load of HBV in a similar manner to HBV DNA assays during disease progression, HBcrAg CLEIA shows characteristics different from HBV DNA assays under lamivudine administration since HBcrAg levels decrease more slowly than HBV DNA after treatment begins.[12] In the present study, we analyzed the clinical significance of the HBcrAg assay in predicting the likelihood of non-reactivation of hepatitis after discontinuing lamivudine administration in HBV treatment.

METHODS

Patients

A TOTAL OF 34 patients with chronic hepatitis B who were treated with lamivudine for at least 6 months were enrolled in the present study. The patients comprised 20 men and 14 women with a median age of 46 years (range 23–65 years), and were selected retrospectively from five medical institutions in Japan (Shinshu University Hospital, Kyoto Prefectural University Hospital, National Nagasaki Medical Center, Toranomon Hospital, and Hiroshima University Hospital). Written informed consent was obtained from each patient.

Of the 27 patients whose HBV genotype was determined, 25 (93%) were genotype C and the remaining two (7%) were genotype B. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 16 (47%) of the 34 patients before lamivudine administration.

For treatment of HBV infection, daily doses of 100 mg lamivudine were administered for at least 6 months. Lamivudine administration was stopped when alanine aminotransferase (ALT) levels were reduced to 40 IU/L or less in at least three separate tests. Serum samples were taken at several time points during and after lamivudine administration, and patients were seen at least once a month for at least 12 months after cessation of lamivudine. Estimated duration of HBV DNA level <3.7 log copy/mL before stopping lamivudine was a median 10 months (range 0–29 months).

Reactivation of hepatitis was defined as elevation of ALT to more than 80 IU/L within 12 months of stopping lamivudine treatment.

Serological markers for HBV

Serum hepatitis B surface antigen, HBe antigen, and anti-HBe antibody were measured by commercially available CLEIA kits (Fujirebio, Tokyo, Japan). Six major genotypes (A–F) of HBV are detectable using the method reported by Mizokami *et al.*[13] in which the surface gene sequence is amplified by polymerase chain reaction (PCR) and analyzed by restriction fragment length polymorphism. Serum concentration of HBV DNA was determined using a transcription mediated amplification (TMA) assay kit (Chugai Diagnostics Science, Tokyo, Japan) which has a quantitative range of 3.7–8.7 log copy/mL.

Serum concentration of HBcrAg was measured using a CLEIA developed by Fujirebio, as described previously.[7] Briefly, 150 μL of serum was incubated with 150 μL of pretreatment solution containing 15% sodium dodecylsulfate at 60°C for 30 min. After incubation, 120 μL of pretreated specimen was added to a ferrite microparticle solution in an assay tube. Ferrite microparticles were coated with monoclonal antibodies (HB44, HB61, HB114) against denatured HBc and HBe antigens. After washing, two other monoclonal antibodies against denatured HBcAg and HBeAg (HB91 and HB110) labeled with alkaline phosphatase were added as secondary antibodies. After further washing, 200 μL of AMPPD (3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt; Applied Biosystems, Bedford, MA) solution was added as substrate, and the assay tube was incubated for 5 min at 37°C.

From this, the relative chemiluminescence intensity was measured, and HBcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBcrAg concentration was expressed as units/mL (U/mL) and a immunoreactivity of recombinant pro-HBe antigen of 10 fg/mL was defined as 1 U/mL. In the present study, the cutoff value of HBcrAg concentration was set at 3.0 log U/mL.

Statistical analysis

The Mann–Whitney *U*-test was used to analyze quantitative data, and Fisher's exact test was used for

qualitative data. Receiver operating characteristic (ROC) curve analysis was used to analyze cut-off levels of HBcrAg concentration for prospective recurrence of hepatitis. Statistical analyses were performed using the SPSS 14.0 J statistical software package (SPSS, Chicago, IL, USA), and a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TWENTY (59%) OF the 34 patients enrolled in the present study showed reactivation of hepatitis within 12 months after discontinuing lamivudine administration, with 15 (75%) showing reactivation within 6 months. The peak serum ALT levels in the 20 reactivation patients ranged from 103 to 1019 IU/L, with a median of 308 IU/L. After lamivudine cessation, the maximum serum HBV DNA was significantly higher ($P < 0.001$) in the reactivation patients (median 7.8, 25–75% range 7.4–8.1 log copy/mL) than in the non-reactivation patients (median 4.8, 25–75% range 4.1–5.9 log copy/mL).

Table 1 shows a comparison of the clinical backgrounds at the onset and completion of lamivudine administration between the two groups of patients. Although backgrounds were similar between the two groups just prior to lamivudine administration, HBcrAg levels were significantly higher in the reactivation patients after treatment. Both HBV DNA levels and positive rates of HBe antigen were similarly low between the two groups. The duration of undetectable HBV DNA before stopping lamivudine administration was also similar ($P > 0.2$) between the two groups (reactivation patients, median 11 months, 25–75% range 8–13 months vs. non-reactivation patients, median 6 months, 25–75% range 5–13 months).

In 23 patients who were negative for HBe antigen after treatment, HBcrAg levels were significantly higher ($P = 0.011$) in the reactivation patients ($n = 12$, median 4.8 log U/mL, 25–75% range 4.0–5.0 log U/mL) than in non-reactivation patients ($n = 11$, median 3.0 log U/mL, 25–75% range 2.5–4.4 log U/mL). In contrast, levels were similar ($P > 0.2$) between the two groups in 11 patients who were positive for HBe antigen after treatment (reactivation patients $n = 8$, median 5.9 log U/mL, 25–75% range 5.1–6.1 log U/mL vs. non-reactivation patients $n = 3$, median 5.6 log U/mL, 25–75% range 2.5–8.0 log U/mL).

The ability of HBcrAg concentration to predict non-recurrence of hepatitis was analyzed using a ROC curve (Fig. 1), and the area under the curve was as wide as 0.764. The point at which specificity was 0.8 and sensi-

Table 1 Comparison of clinical characteristics at the onset and cessation of lamivudine administration between patients with and without reactivation of hepatitis

Characteristics	Reactivation of hepatitis		*P*-value†
	Positive ($n = 20$)	Negative ($n = 14$)	
Demographics			
Age (years)	44 (38–51)	50 (35–59)	NS
Sex (male/female)	13/7	7/7	NS
HBV genotype (B/C)	0/16	2/9	NS
At onset of lamivudine administration			
ALT (IU/mL)	103 (57–234)	211 (76–515)	NS
HBeAg (positive)	12 (60%)	4 (29%)	NS
HBV DNA (log copy/mL)	7.1 (6.1–8.1)	6.0 (5.3–7.4)	NS
HBcrAg (log unit/mL)	6.2 (5.6–7.7)	6.4 (5.0–6.6)	NS
At cessation of lamivudine administration			
Duration of lamivudine (months)	12.7 (10.4–16.3)	10.3 (6.4–17)	NS
ALT (IU/mL)	30 (15–36)	21 (15–24)	NS
HBeAg (positive)	8 (40%)	3 (21%)	NS
HBV DNA (log copy/mL)	<3.7 (<3.7–<3.7)	<3.7 (<3.7–<3.7)	NS
HBcrAg (log unit/mL)	4.9 (4.7–5.9)	3.2 (<3.0–4.5)	0.009

†Analysis of continuous variables performed using Mann–Whitney *U*-test; analysis of dichotomous variables performed using Fisher's exact test. Values shown as median (25–75% range) or *n* (%).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

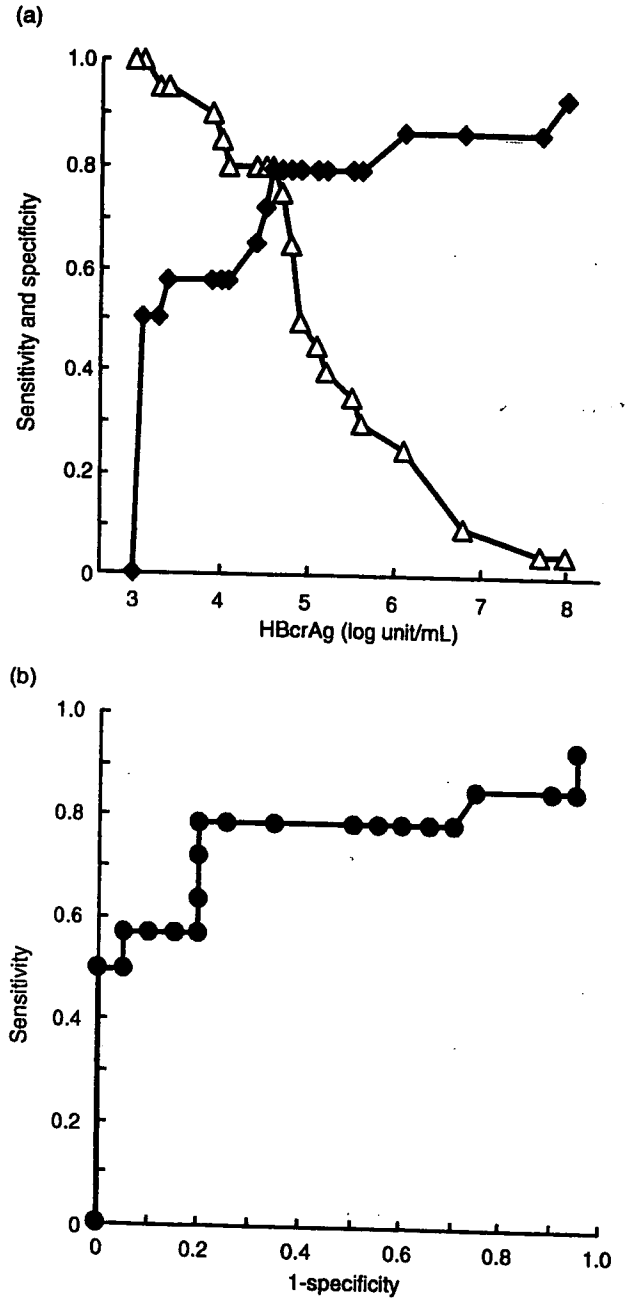

Figure 1 Receiver-operator characteristic (ROC) analysis of hepatitis B core-related antigen (HBcrAg) concentration for predicting patients without risk of reactivation of hepatitis within 12 months after halting lamivudine administration. (a) Sensitivity (■) and specificity (Δ) curves according to concentration of HBcrAg. (b) The ROC curve with the area under curve of 0.764.

tivity approximately 0.8 was deemed best for halting treatment without the risk of hepatitis recurrence. This point corresponds to an HBcrAg concentration of 4.1–4.6 log unit/mL.

DISCUSSION

THE REACTIVATION OF hepatitis following lamivudine administration was defined in the present study as an elevation of serum ALT level to more than 80 IU/L because we sought to find a more reliable indicator for safer discontinuation of lamivudine administration. Under these conditions, the majority (20/34) of patients showed reactivation of hepatitis within 12 months, as has been previously reported.[5,6] HBV DNA levels at the time of discontinuing lamivudine were similarly low between the two groups of patients, which is understandable as an undetectable reading typically indicates HBV remission following lamivudine therapy. However, HBcrAg levels were significantly higher in reactivation patients, implying that HBcrAg level is a better marker than HBV DNA level for predicting non-reactivation of hepatitis after discontinuing lamivudine administration especially in patients without HBe antigen.

In this study, ROC curve analyses showed a wide area under the curve of 0.764 in predicting the non-reactivation of HBV with HBcrAg level. If the corresponding cutoff is set at 4.5 logU/mL, then both specificity and sensitivity are as high as approximately 0.8. To obtain a higher specificity of 0.9, the cutoff value of HBcrAg concentration should be set at 4.0 log unit/mL. In this case, the sensitivity would still be nearly 0.6. The cutoff value of HBcrAg for predicting the non-relapse of hepatitis in our study is a little higher than that reported by Shinkai *et al.* (3.4 logU/mL).[14] Because numbers of patients analyzed were small in both studies, further studies are required to confirm the most appropriate cutoff value. It is noteworthy that this cutoff value may also differ among genotypes, which have been reported to be correlated with outcome of chronic HBV infection.[15] However, as over 90% of the patients had genotype C in this study, reactivation could not be analyzed in relation to HBV genotypes.

The HBV is an enveloped DNA virus containing a relaxed circular DNA genome which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells and serves as transcriptional template for the production of viral RNA.[11,16,17] Reverse transcription of pregenomic RNA and second-strand DNA synthesis then occur in the cytoplasm within viral

capsids formed by the HBV core protein. Because lamivudine inhibits reverse transcription of pregenomic RNA, it directly suppresses production of HBV virions, and serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. However, the production of viral proteins is not suppressed by lamivudine as this process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which also serves as a template for mRNAs, decreases quite slowly after commencement of administration of nucleoside analogs.[18,19] Thus, it is possible that serum HBcrAg levels reflect the cccDNA level in hepatocytes more accurately than serum HBV DNA. High levels of cccDNA are considered to be associated with hepatitis reactivation because they preceed reactivation of viral replication and consequent elevation of HBV DNA level in serum.

Lamivudine has already been eliminated from first line therapy in naïve chronic hepatitis B patients due to a higher incidence of developing resistant mutations than new antiviral agents, such as adefovir dipivoxil and entecavir.[20] However, the distinct characteristic of the HBcrAg assay under lamivudine therapy that is different from other HBV DNA assays is that lamivudine suppresses production of HBV virions by inhibiting reverse transcription of pregenomic RNA, but does not suppress the production of viral proteins, in which reverse transcription is unnecessary. Thus, it is possible that the HBcrAg assay may also useful for patients undergoing entecavir or adefovir dipivoxil administration because the main mechanism of suppressing HBV replication is similar between lamivudine and other antiviral agents. As a considerable number of patients who started lamivudine administration in the past are still taking this treatment now, the present study may be valuable for such patients when they consider changing therapies in the future. Additionally, further studies are required to determine whether the HBcrAg assay is indeed applicable to antiviral agents other than lamivudine.

In conclusion, significant markers that can predict reactivation of hepatitis after discontinuing lamivudine administration are clinically valuable because the reactivation of hepatitis is a fundamental problem in lamivudine therapy. Our results suggest that patients with an HBcrAg level of less than 4.5 log unit/mL may stop lamivudine administration with a lower risk of reactivation. The present study is a preliminary one because the patients enrolled were selected retrospectively without standardized criteria for stopping lamivudine and the number of patients enrolled was not large; however, the results may be valuable for patients with hepatitis B undergoing lamivudine therapy as such a diagnostic marker has rarely been reported. Further studies are required to establish the clinical significance of the HBcrAg assay in the treatment of hepatitis B.

ACKNOWLEDGMENT

THIS RESEARCH WAS supported in part by a Grant-in Aid from Research on hepatitis, the Ministry of Health, Labour and Welfare of Japan.

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Hepatology Research 2007; 37: 425–432 doi: 10.1111/j.1872-034X.2007.00050.x

Original Article

Pathological analysis of oxyphilic granular hepatocytes and hepatocellular mitochondria in chronic hepatitis C

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Aim: Oxyphilic granular hepatocyte (OGH) results from hepatocellular changes associated with chronic hepatitis. The histopathological significance of OGH has not been clarified.

Methods: The subjects consisted of two groups of patients with hepatitis C: one group of patients who had undergone liver biopsy 3.8 times on average, and were followed for 8 years on average, and one group of hepatocellular carcinoma (HCC) patients who had undergone hepatectomy. The following items were examined: frequency of OGH, relationship between OGH and the degree of fibrosis and inflammation; amount of mitochondria in resected tissues; activity of mitochondrial enzymes; relationship between the development of HCC and OGH; and relationship between the duration of infection and OGH in the post-transfusion patients.

Results: The incidence of OGH was 35.3% in liver biopsy patients and 46.9% in resected patients. A higher stage of fibrosis was associated with a higher frequency of OGH. Not only OGH but also hepatocyte mitochondria in the peripheral zone increased with the progression of fibrosis. Hepatocytes with or without increased mitochondria were randomly distributed. The mitochondrial enzyme activity was increased in hepatocytes with increased mitochondria. In the post-transfusion patients, a longer duration of infection and a higher stage of fibrosis were associated with a higher frequency of OGH. A high percentage of patients with OGH developed HCC.

Conclusion: Mitochondrial changes are important histological findings related to the progression of liver lesions and the possible development of HCC.

Key words: cytochrome c oxydase, hepatitis C, mitochondria, oxyphilic granular hepatocyte, succinate dehydrogenase

INTRODUCTION

VARIOUS HEPATOCELLULAR CHANGES are observed in viral hepatitis, including oxyphilic granular hepatocyte (OGH). Characteristic acidophilic changes in the cell have been reported to be due to mitochondrial hyperplasia (Fig. 1). OGH was first discovered in hepatitis B by Lefkowitch *et al.*[1] They have extensively examined OGH and clarified the pathological profile of OGH in hepatitis B, although the real pathogenesis remains unknown.[1–3] OGH has also been found in other liver diseases such as primary biliary cirrhosis and alcoholic hepatitis. OGH in hepatitis C has not been analyzed in detail. There is one report that documented OGH in patients with various diseases including hepatitis C. Muller-Hocker found OGH in 20 of 47 cirrhosis livers of various etiology, but the frequency of OGH in patients with hepatitis C was not reported.[4] In this study, we examined the pathological features, pathogenesis and significance of OGH in hepatitis C.

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Received 2 October 2006; revision 3 November 2006; accepted 11 December 2006.

METHODS

Subjects

THE SUBJECTS WERE two groups of patients with hepatitis C: one group comprised patients who had undergone liver biopsy and the other group comprised patients who had undergone liver resection.

Biopsy group

Fifty-one HCV antibody-positive patients with hepatitis C were studied. These patients had been analyzed and reported previously by one of the authors.[5] The patients had been diagnosed with chronic hepatitis C, treated, and followed between 1967 and 1994 in the National

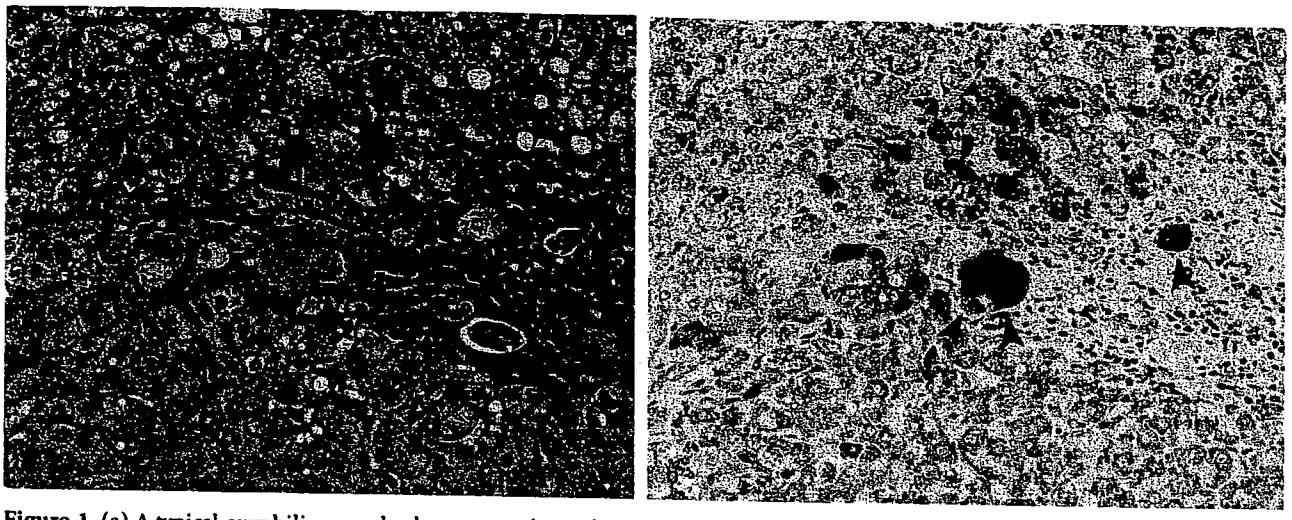

Figure 1 (a) A typical oxyphilic granular hepatocyte (arrowhead) is filled with acidophilic granules (hematoxylin and eosin). (b) A section serial to that in (a) was immunohistochemically stained with antimitochondrial antibody. Mitochondrial hyperplasia was observed in the oxyphilic granular hepatocyte. The arrowhead indicates cells corresponding to those shown in (a). (Original magnification ×400.)

Hospital Organization Nagasaki Medical Center. They usually underwent the first liver biopsy within 6 months of onset, and two or more liver biopsies during follow-up, with a mean of 3.8 biopsies and a follow-up period of 1–21 years (mean 8 years).

Resected group

Sixty-four patients with resected hepatitis C-related hepatocellular carcinoma (HCC) who had undergone hepatectomy from 2001 to 2002 were studied. Eighteen resected patients who had metastatic colorectal cancer during the same period served as controls. Frozen liver tissues from 37 patients with hepatitis C-related HCC and four patients with metastatic colorectal cancer had been stored, and were used as frozen sections for histochemical staining.

Histological examination

Liver biopsy and resected tissues were fixed in 10% formalin and embedded in paraffin. Sections 4-μm thick were stained with hematoxylin and eosin, and examined for OGH and their distribution within the liver lobule. In addition, the degree of liver tissue fibrosis and inflammation was evaluated according to the Ludwig classification of chronic hepatitis.[6]

To examine the relationship between the duration of infection and the appearance of OGH, biopsy patients with post-transfusion hepatitis C were analyzed for the time interval between transfusion and the appearance of OGH, and the degree of fibrosis and inflammation at the time of the appearance of OGH. The frequency of OGH in the liver biopsy patients who developed HCC during follow-up was also determined.

Amount of mitochondria in hepatocytes

To objectively evaluate the amount of mitochondria in resected tissues, immunohistochemical staining with antimitochondrial antibody (Bio Genex, San Ramon, CA, USA) was performed. The amount of mitochondria in the entire liver of each patient was expressed as follows. The amount of mitochondria in each hepatocyte was classified as large, equal, or small, in comparison with that in a control hepatocyte. Five areas per patient were randomly selected from the peripheral zone. The amount of mitochondria in about 500 hepatocytes in each area was classified.

To semiquantify the amount of mitochondria in liver tissue, the percentages of cells with a large, equal, and small amount of mitochondria to the total number of cells observed were calculated to score the amount of mitochondria according to the following formula: Mitochondrial score (MS) = large amount (%) × 3 + equal amount (%) × 2 + small amount (%) × 1.

Hepatocyte mitochondrial function

To evaluate hepatocyte mitochondrial function, frozen sections of resected liver tissues were histochemically stained for the electron transport enzymes succinate dehydrogenase (SDH) and cytochrome c oxydase (COX).

For SDH, the following substrate solution was used: 100 mM disodium succinate, 25 mM phosphate-

buffered saline (PBS; pH 7.4), 1 mM NaN_3, 0.2 mM PMS, and 1.5 mM NitroBT. Unfixed 5-mm thick sections were incubated in the substrate solution at 37°C for 5 min, briefly rinsed in PBS and then water, and sealed with glycerin-gelatin. The substrate disodium succinate was omitted in the processing of controls.[7–9]

For COX, the following substrate solution was used: 50 mM PBS (pH 7.2), 0.15% cytochrome c, and 2.5 mM DAB. Unfixed 10-mm thick sections were incubated in the substrate solution at 37°C for 10 min, rinsed well in PBS, dehydrated in a series of ethanol, cleared in xylene, and mounted with a synthetic mount. The substrate cytochrome c was omitted in the processing of controls.[7,8,10]

Changes in hepatocytes

Resected sections (five OGH positive, five OGH negative, and five control) were immunohistochemically stained with anti-hexokinase (HXK)-II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for the glycolysis enzyme,[11,12] and with anti-Ki-67 antibody (Novo Castra, Newcastle Upon Tyne, UK) for the cell growth cycle. The expression of Ki-67 was evaluated in 10 high-power magnification areas in the peripheral zone and the central zone to calculate the labeling index (LI).

Statistical analysis

The frequency and distribution of OGH and the relationship between OGH and the degree of fibrosis and inflammation were assessed. The Mann–Whitney *U*-test and chi-squared test were employed. $P < 0.05$ was considered statistically significant.

RESULTS

Biopsy group

AT THE TIME of the first liver biopsy, the frequencies of OGH in patients with stage 3 and 4 fibrosis were 18.2% and 28.6%, respectively, but OGH did not appear in patients with stage 1 or 2 fibrosis. OGH appeared in 16.7% of patients with grade 4 portal tract inflammation and 7.7% of patients with grade 3 portal tract inflammation. OGH first appeared at the age of 48–69 years, with a mean of 56.9 years.

Patients in whom OGH appeared at least once were classified as OGH-positive. OGH appeared in 18 (35.3%) of the 51 liver biopsy patients. At the first appearance of OGH, stage 4 fibrosis was observed in 72.2% of the biopsy patients. A more advanced stage of fibrosis tended to be associated with a higher frequency of OGH. At the first appearance of OGH, inflammation tended to be severe in the portal tract, but mild in the liver lobule. Grade 4 portal inflammation was noted in 94.4% of the 18 OGH-positive patients (Fig. 2).

Of the 51 liver biopsy patients, 21 (41.2%) had received blood transfusions, and OGH appeared in six (28.6%) of them. The time interval between blood

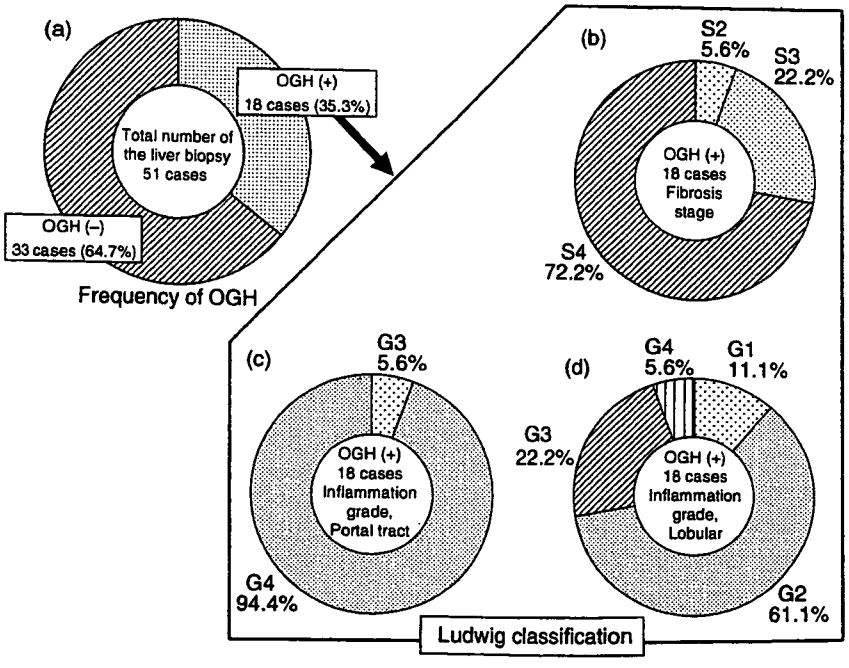

Figure 2 The fibrosis stage and inflammation grade at the first appearance of oxyphilic granular hepatocyte (OGH) on liver biopsy in relation to the frequency of OGH. (a) OGH appeared in 18 (35.3%) of the 51 liver biopsy patients. (b) OGH was observed in 94.4% of patients with stage 3 or 4 fibrosis. (c) OGH appeared in all patients with grade 3 or 4 portal tract inflammation. (d) No relationship was found between the grade of lobular inflammation and the appearance of OGH.

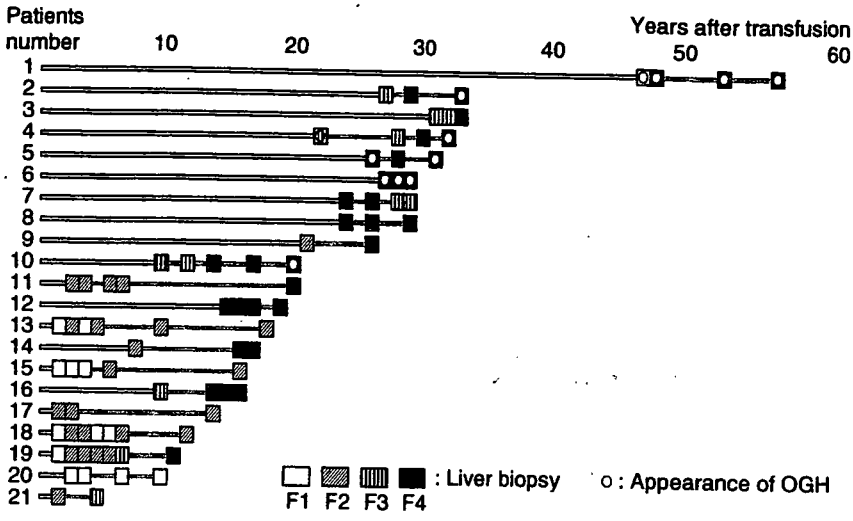

Figure 3 Post-transfusion course of patients. The mean time interval between blood transfusion and the appearance of oxyphilic granular hepatocyte (OGH) was 28 years and 4 months (range 20–40 years). OGH tended to appear in patients with severe liver fibrosis at more than 20 years after blood transfusion. In contrast, OGH was not observed in patients with severe liver fibrosis at less than 20 years after blood transfusion.

transfusion and the appearance of OGH ranged from 20 to 40 years, with a mean of 28 years and 4 months (Fig. 3).

Hepatocellular carcinoma developed in 17 (33.3%) of the 51 liver biopsy patients, and OGH was observed in eight (47.1%) of them. HCC was not observed in 34 patients, in 10 (29.4%) of whom OGH appeared (Fig. 4). At the last liver biopsy, 21 patients with stage 3–4 fibrosis and two patients with stage 1–2 fibrosis had developed HCC. Therefore, 20 patients with stage 3–4 fibrosis and eight patients with stage 1–2 fibrosis did not develop HCC. No significant difference was noted in stage of fibrosis between the patients who did or did not develop HCC ($P = 0.0914$)

Resected group

Oxyphilic granular hepatocyte appeared in 30 (46.9%) of the 64 resected patients (Fig. 5), but in none of the control patients. Inflammatory cell infiltration was prominent, especially in periportal areas with piecemeal necrosis (Fig. 1). The frequency of OGH tended to become higher with the progression of fibrosis (Fig. 5), and was significantly higher in the group with severe fibrosis than in the group with mild fibrosis (Table 1). A higher grade of portal tract inflammation tended to be associated with a higher frequency of OGH: the frequency was significantly higher in the group with severe portal tract inflammation than in the group with mild portal tract inflammation (Table 1), but did not significantly differ with respect to the degree of hepatic lobular inflammation (Table 1). Thus, the histological findings in the resected patients were similar to those of the biopsy patients.

Amount of mitochondria in hepatocytes

The amount of hepatocyte mitochondria was evaluated in the resected patients. Immunohistochemical staining with antimitochondrial antibody was strongly positive in OGH (Fig. 1). Mitochondria were also increased in hepatocytes other than OGH. However, in patients with stage 3–4 fibrosis, the amount of mitochondria was decreased markedly in some hepatocytes in the central

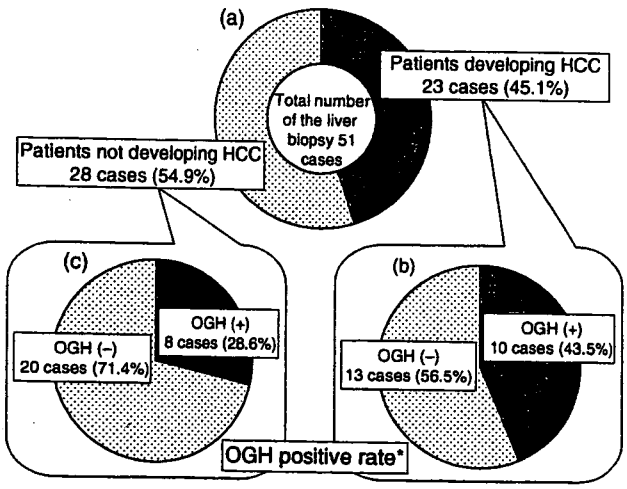

Figure 4 The frequency of oxyphilic granular hepatocyte (OGH) in patients who developed hepatocellular carcinoma (HCC). (a) HCC developed in 23 (45.1%) of the 51 liver biopsy patients. (b) OGH appeared in 10 (43.5%) of the 17 patients who developed HCC. (c) OGH appeared in 8 (28.6%) of the 34 patients who did not develop HCC. No significant difference was noted in the frequency of OGH between the patients who did or did not develop HCC (*$P = 0.4156$, chi-squared test).