related to the timing of HBO treatment performed on the regenerating liver. Our data have shown that HBO treatment can promote functional recovery after PH, however, further studies are necessary to determine the protocol of HBO treatment which can be most beneficial during liver regeneration.

The question of how HBO treatment influences VEGF expression after PH is not resolved by our data. Hypoxia has been reported to be one of the factors that stimulate VEGF production [42], in contrast, hyperoxia also reported to upregulate the expression [43]. Different oxygen tensions seem to activate different signaling pathways to stimulate VEGF expression for angiogenesis. Hyperoxia enhances reactive oxygen species (ROS) production, which potently induces VEGF expression via a hypoxia-inducible factor-1 independent pathway [44,45]. In addition, during wound healing ROS is required for prevention of wound infection, and specific levels of ROS may act as a signaling mediator that regulates a variety of cellular responses [45,46].

In summary, our studies demonstrate that HBO treatment after PH has beneficial effect on regenerating liver, increasing hepatic VEGF, accelerating SEC proliferation, reducing liver injury, and improving biliary function. Because its safety and simplicity in clinical usage have already been confirmed, HBO treatment may become therapeutic modality in situations in which massive hepatectomy is needed. This may happen in surgery for hepatocellular carcinoma or in living donor liver transplantation.

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特集「ウイルス肝炎の肝移植」

生体肝移植後ステロイドフリー免疫抑制法によるC型肝炎再発の制御

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はじめに

非免疫抑制下のC型肝炎ウイルス（HCV）陽性患者では，感染後20年で約20%が肝硬変に進展し，そのうちの3%から6%が毎年非代償性肝硬変へ進行するとされている[1]。従来，HCV陽性レシピエントにおける肝移植後の患者生存率およびグラフト生着率は，その緩やかな経過の予測の下，HCV陰性例と比較して，遜色ないものとされていた[2]。しかし，最近，HCV陽性例では，肝移植後早期に肝炎再発から肝硬変，さらに非代償性肝硬変へと進行し，患者生存率およびグラフト生着率が1990年代に比べ著しく低下していることが報告された[3]。その中で，Berenguerらは近年の過剰な免疫抑制法が，早期の肝硬変への進展と成績の悪化をもたらしている可能性を示唆した。この移植後C型肝炎再発は現在の移植医療従事者が最も頭を悩ませている問題といっても過言ではない。

疫学的に，世界中のHCVキャリアー数は約1億7,500万人で，そのうちわが国におけるHCVキャリアー数は150万から200万人，約4,500人が毎年新規感染していると予想されている。つまり，HCVに起因する肝硬変は元来肝移植の対象として，頻度が高いと考えられる疾患であり，日本肝移植研究会の集計では，2003年までに施行された全成人間生体肝移植数に占めるHCV陽性症例の割合は，22%であった[4]。さらに2004年1月から，ウイルス性肝硬変に対する生体肝移植が保険適応として認められ，HCV陽性レシピエントに対する生体肝移植施行症例数は今後ますます増加するものと思われる。

生体肝移植に用いられるグラフトは部分肝であり，術後の経過でグラフトの再生が起こる。またわが国において，生体ドナーはレシピエントの近親者に限定されているため，ドナーとレシピエントのHLAがより近似している可能性がある。これらのため，生体肝移植術後のC型肝炎再発は，脳死肝移植に比べ，早い時期に起こり予後不良であるとする報告もあるが，脳死肝移植との間に差はないとする報告も多く，いまだ結論は出ていない。

本稿では，HCV陽性例に対する免疫抑制法の最近の報告をレビューするとともに，当施設におけるステロイドフリー免疫抑制法の経験について，概説する。

免疫抑制剤のHCVに与える影響

1. カルシニューリン阻害剤

HCVのレプリコン細胞を用いた実験において，シクロスポリンには濃度依存性に，シクロフィリンBを介するHCV増殖抑制効果があることが報告された[5,6]。臨床例では，シクロスポリン使用によりC型肝炎再発後の線維化が緩徐であるとするものや[7]，肝移植後の再発例で，シクロスポリンはインターフェロンとの併用により抗ウイルス療法に対する相乗効果をもつことが報告された[8]。しかし一方で，移植後5年生存率は，タクロリムス使用群がシクロスポリン使用群より有意に良好であったとする報告がみられ[9]，最近米国でのprospective randomized trialにより，移植後のHCV RNA量やC型肝炎再発率はカルシニューリン阻害剤の種類で差を認めなかったことが報告された[10]。

これら相反する報告から，どちらかのカルシニュー

リン阻害剤が有意に優れているとは，現時点では言えないと考えるのが妥当であろう。

2. ステロイド

Ganeらはメチルプレドニゾロンのbolus投与により，HCV RNA量は4～100倍に増加し，HCV RNA量の高値は，肝移植後C型肝炎の早期再発および再発肝炎の重症度と相関することを報告した[11]。メチルプレドニゾロンのbolus投与に続くHCV量の著増はfibrosing cholestatic hepatitis（FCH）とも関連するとされている[12]。FCHは肝移植後5–10%の症例に起こる，移植約1カ月後の肝機能異常から始まり半年以内に肝不全に進行する特殊なC型肝炎再発パターンである[13]。FCHはインターフェロン治療からの脱落が多く，高い死亡率のため，避けねばならぬ病態である。これらの報告による過剰なステロイドへの拒絶感のためか，rapid steroid taperingのプロトコールを多くの施設が取り入れた。しかし最近，プレドニゾロンを少量長期使用後，移植後6カ月以降に緩徐にtaperするプロトコールで重症再発が予防可能あるいは線維化進展が抑制可能との報告がされた[14,15]。一方，ステロイドフリーの免疫抑制に関する報告もみられ[16,17]，HCVの増殖速度を抑える[16]あるいはグラフト生着率および患者生存率を改善する[17]とされているが，C型肝炎再発自体への影響に関しては，いまだ有意な報告はされていない。

3. Mycophenolate Mofetil（MMF）

*de novo*系の核酸合成阻害剤である本薬は，EBウイルスや黄熱病ウイルスの複製阻害効果，抗ヘルペス薬の効果増強作用，*in vitro*でのHBV複製阻害効果などが知られており，HCVへの抗ウイルス作用の有無が注目されていたが[18]，HCV陽性症例に対する効果に関しては議論が分かれている。HCV陽性症例に対して，タクロリムスとステロイドにMMFを追加すると，C型肝炎の再発には影響を及ぼさず，急性拒絶反応の頻度は低下し，さらに移植後4年生存率およびグラフト生着率は有意に良好であった[19]，術後のALTは有意に低下し，肝炎や線維化の進行を認めなかった[20]というMMFの有用性を示唆する報告がみられる。一方，MMFを用いた免疫抑制導入は移植後2年以内の肝線維化の進行を早める危険因子である[21]，あるいはC型肝炎再発例に対してazathioprineに代えてMMFを使用するとHCV RNA量が増加する[18]といったnegativeな報告もみられ，その効果に関する結論は出ていない。

4. Basiliximab

本薬はIL-2レセプターのα鎖に結合し，Tリンパ球の増殖を抑制するモノクローナル抗体である。術中および術後4日目に使用することで，術後約30日にわたり，CD25陽性活性化T細胞の出現を抑制し，急性拒絶反応の発生率を低下させる。CalmusらはHCV陽性例に使用し肝移植6カ月後の肝炎再発率は48.4%で他の免疫抑制法と差を認めなかったと報告した[22]。その後既出のごとく，Basiliximabとの併用でステロイドフリー免疫抑制法が可能であることが報告された[16,17]。わが国からもMarubashiらが，生体肝移植症例においてもBasiliximabをタクロリムスおよびMMFと併用することで，急性拒絶反応を発生させることなく，ステロイドフリーの免疫抑制が可能であり，術後早期のHCV RNA量を抑制したと報告した[23]。

HCV陽性例における生体肝移植と脳死肝移植の相違

慢性的な臓器不足を背景に，欧米においても生体肝移植を施行する施設が増加している。HCV陽性症例に対する生体肝移植の長所として，待機期間が調整可能であるため移植前の抗ウイルス治療が可能であること，短い冷保存時間，比較的若いドナー，重度の脂肪肝のない良好なグラフト機能などが挙げられる。一方，欠点としては親族からの提供の場合にドナーとレシピエント間のHLAの相同性が高いこと，肝細胞の再生のためにHCVの増殖を促進する可能性があること，薬剤代謝の違いから免疫抑制剤が強く作用する可能性があること，高率な胆道系の合併症から持続する胆汁うっ滞を招くこと，などが挙げられる[24]。GaglioらのHCV陽性例における生体肝移植と脳死肝移植を比較した最初の報告では，生体肝移植後のC型肝炎再発は脳死肝移植に比べ早期に起こり，FCHなどのより激しい再発形式をとるとされた[25]。Schianoらは，生体ドナーでは脳死ドナーに比べ，再灌流直後の血中HCV RNAは速やかに低下するが，その後の増殖速度も速く，また移植後2年間のALTが有意に高値であったと報告した[26]。最近の報告では両者間の生存率・グラフト生着率あるいは組織学的再発率に差がないとするものも多いが，いずれも症例数が限られており，さらに各施設間におけるC型肝炎再発の定義の違いか

らか生体ドナーの善悪に関する結論は出ていない[24]。HCV陽性例に対する多数の生体肝移植症例数を持つわが国から，多施設共同研究を行い，積極的に情報を発信していく必要があると思われる。

当施設における生体肝移植後ステロイドフリー免疫抑制法

九州大学第二外科で，2006年3月までに施行した成人間生体肝移植186例中，HCV陽性症例は73例（39.2%）であった。特に2002年以降は全成人症例のうち，約半数をHCV陽性例が占めている。**図1**に当科におけるHCV陽性症例に対する免疫抑制法の変遷を示す。当初はタクロリムスとステロイドを用いていたが，その後MMFおよびBasiliximabの使用，またステロイドの短期中止などを取り入れ，現在では，**図1の3**）に示す如く，BasiliximabとMMFにより導入し，カルシニューリン阻害剤を術後7日目より開始している。カルシニューリン阻害剤のトラフ値はタクロリムスでは10–15 ng/ml，サイクロスポリンでは200–250 ng/mlでコントロールしている。ステロイドは原則的に用いていない。自験例を免疫抑制導入法により3群に分け，3群間における術後HCV RNA量，急性拒絶反応の発生率，肝炎再発率，線維化進展率および生存率を検討した。**表1**に各群の背景因子を示す。ステロイドフリー群でレシピエント年齢が高く，ドナーは女性が多かった。また，術前HCV RNA量が有意に多かった。

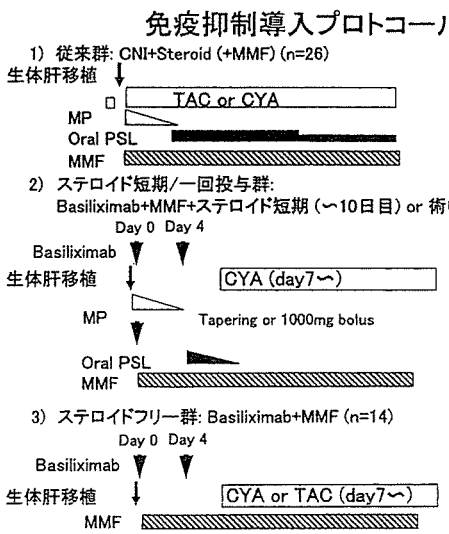
表1

各免疫抑制導入法の背景因子

因子	ステロイドフリー群 (n=14)	ステロイド短期/一回投与群 (n=33)	従来群 (n=26)	P値
Recipient 年齢	56.4	58.5	51.8	<0.05
Recipient 性別 (M/F)	10/4	20/13	18/8	N.S.
Donor 年齢	35.0	35.2	33.4	N.S.
Donor 性別 (M/F)	5/9	26/7	18/8	<0.05
術前HCVRNA (KIU/ml)	554.6	420.3	162.8	<0.005
Virus Grouping 1型/2型	11/0	24/4	13/4	N.S.
Graft種類 (左葉/右葉)	7/7	22/11	17/9	N.S.
GV/SLV (%)	41.0	42.8	42.2	N.S.
術前MELD	11.0	12.3	13.9	N.S.
出血量	6240	5792	12352	0.13
手術時間 (分)	852	788	893	0.10

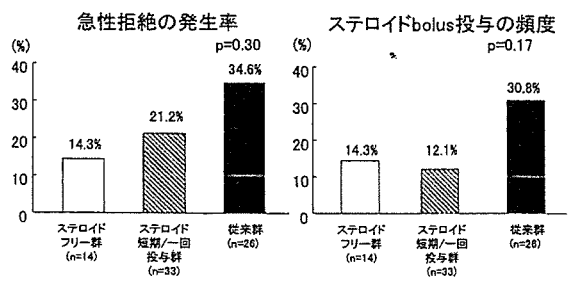
急性拒絶反応の発生率はステロイドフリー群で14.3%と低い傾向にあった。このため，急性拒絶反応の治療として，ステロイドのbolus投与を要した症例も，14.3%に留まった（**図2**）。HCV RNA量の測定法が同一の2004年1月以降の症例で，術後1カ月のHCV RNA量を比較したところ，ステロイドフリー群

図1　九州大学におけるHCV陽性例に対する免疫抑制導入プロトコール



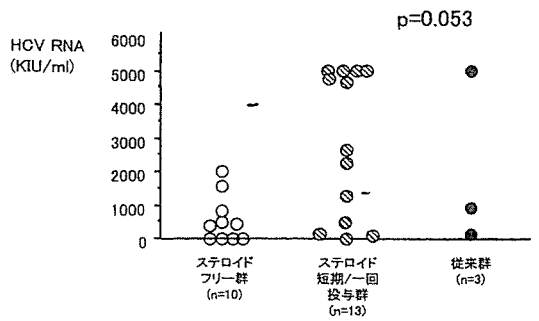
CNI：カルシニューリン阻害剤，TAC：タクロリムス，CYA：サイクロスポリン，MP：メチルプレドニゾロン，PSL：プレドニゾロン，MMF：Mycophenolate Mofetil

図2　各免疫抑制導入法の急性拒絶反応発生率と拒絶反応の治療にステロイドbolus投与を施行した頻度



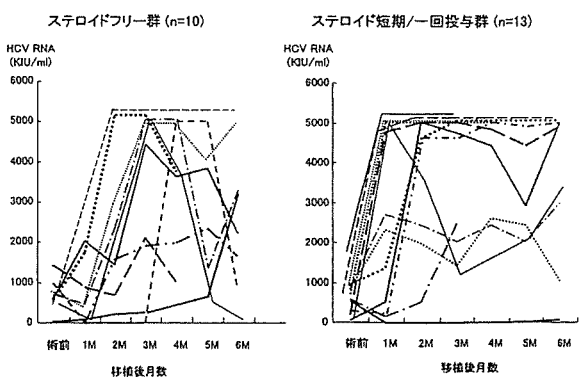
ステロイドフリー群では急性拒絶反応の頻度は14.3%で他群に比べ低い傾向にあった。このため，ステロイドbolus投与を施行したものも14.3%にとどまった。

図3　各免疫抑制導入法と移植1カ月後のHCV RNA量



ステロイドフリー群では，他群に比べHCV RNA量が低い傾向にあった。

図4　術後HCV RNA量の推移



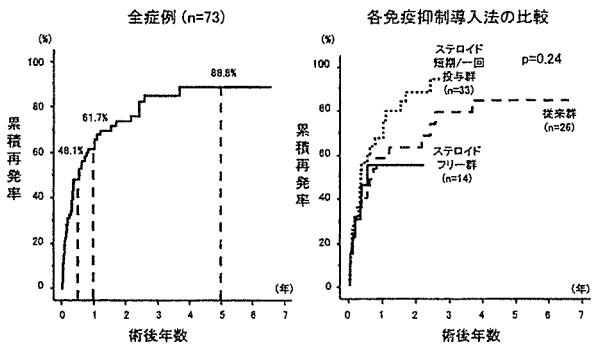
ステロイドフリー群では，ステロイド短期/1回投与群に比べ，術後早期のHCV RNAの増加が緩徐であった。

でHCV RNA量は低い傾向にあった（図3）。

次にステロイドフリー群とステロイド短期/1回投与群の術後6カ月間のHCV RNAの推移を示す（図4）。ステロイドフリー群に対して，短期/1回投与群では，術後早期から急速なHCV RNA量の増加を示し，測定上限である5,000 KIU/mlに達した。これは，rapid steroid taperingがHCVのウイルス動態に悪影響を及ぼすとの報告に矛盾しないと考えられる。ステロイド短期/1回投与群のうち2例では，FCH様の経過を呈し，そのうち1例は急速な肝不全の進行により術後7カ月で失った[13]。McCaughanらが報告しているように術直後の急速なウイルスの増加は，FCHの発生と関連するものと考えられた[12]。ステロイドフリー群においても，多くの症例が術後2～3カ月で測定上限に達していたが，術直後の急速な増殖を避け得たために，FCHを呈した症例は経験しなかった。ウイルスの増殖が上限に達する前に予防的なインターフェロン治療を開始し，ウイルスの陰性化を目指すことが術後管理を行う上で重要と考えられる[27]。

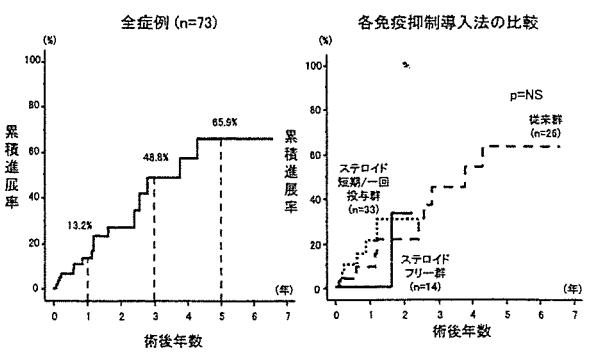
当施設における移植後の組織学的累積再発率は術後6カ月で48.1%，5年では88.8%（図5），F3以上の線維化進展率は，1年で13.2%，5年で65.9%であっ

図5　移植後C型肝炎の組織学的再発率



術後6カ月で48.1%，5年で88.8%に組織学的再発が見られた。ステロイドフリー群では再発率が低い傾向にあった。

図6　移植後F3以上の肝線維化進展率



術後1年で13.2%，5年で65.9%にF3以上の線維化がみられた。各免疫抑制導入法による差は認めなかった。

表2

組織学的線維化(F3, F4)進展の危険因子(多変量解析)

因子	ハザード比 (95% CI)	p 値
Recipient 女性 vs. 男性	3.81 (1.3-11.2)	0.015
Donor 年齢 ≥40才 vs. <40才	2.92 (1.0-8.1)	0.040

た（図6）。予防的治療を開始しない場合の再発はほぼ必発であるため，線維化の進展をいかに防ぐかが，現在の術後治療のポイントと考えられる。組織学的再発率および線維化進展率を各免疫抑制導入法で比較したが，有意な差は認めなかった。多変量解析による再発の危険因子はレシピエントの性別とドナーの年齢40歳以上のみであった。免疫抑制導入法あるいは免疫抑制剤の種類は危険因子とはならなかった（表2）。

まとめ

2004年1月の保険適応改正により，HCV陽性例は，今後しばらくはわが国における生体肝移植の最大の適応であり続けると考えられる。HCV陽性例の移植術後管理の困難さは，急性拒絶反応とC型肝炎再発の鑑別が組織像をもってしても，非常に難しいことにある。わが国において症例が集積される以前は，試行錯誤の中で術後管理が行われていたのが現状であった。自験例での検討から，われわれが現在行っているステロイドフリー免疫抑制法により，組織学的再発あるいは線維化進展の予防は有意には改善しなかった。しかし，ステロイドフリー免疫抑制導入法の最大の長所は，急性拒絶反応の頻度は低いままで，術直後におけるHCV RNA量の急速な増殖を抑制することが可能な点である。これにより，予後不良なFCHを起こすことなく，より早期に安定した状態で，術後の予防的インターフェロン治療を開始することが可能となった。現在のわれわれのプロトコールで術後管理を行う上で，急性拒絶とC型肝炎再発の鑑別に悩むことはほぼ皆無となった。

1980年代まで移植後の成績が不良であったB型肝炎では，HBIG・ラミブジンの使用により，背景に存在する肝炎までも肝移植により同時に根治させることが可能となり，現在では肝移植の最も良い適応とさえ考えられる。現在，HCVが感染した細胞内におけるHCVの翻訳あるいはHCV RNAの複製を制御すると考えられる種々のHCVに対する治療薬が開発されているが[28]，HCVのグラフト肝への再感染は，再潅流時から始まる[12,26]。肝移植の分野から言えば，B型肝炎におけるHBIGの働き，つまり肝細胞へのHCVのbindingそのものを予防しうるモノクローナル抗体あるいは免疫グロブリンなどの開発が，より効率の良い移植後再感染およびC型肝炎再発予防を可能にするであろう[29]。最近，memory T細胞を利用したHCVワクチンのHCV感染に対する有効性が報告された[30]。肝移植術後の症例では，急性拒絶反応とのバランスという問題もあるが，術後のT細胞の特異的な制御が，今後の治療の主役となる可能性もある。いずれにしろ，HCVの分子構造解明に基づく治療法の早急な開発が望まれる。

最後に，HCV陽性例に対する移植後の管理に関しては，いまだコンセンサスが得られていないのが現状である。われわれが積極的に取り組んでいるステロイドフリー免疫抑制法は，そのものがHCVの治療法ではないが，安定した状態で早期に抗ウイルス治療を開始するための周術期管理法としては，大変有効な手段である。現在，厚生労働省班研究による症例の集積が進んでおり，今後さらに新たな知見が得られるものと考えられる。

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REVIEW

Living donor liver transplantation to patients with hepatitis C virus cirrhosis

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Abstract

Living donor liver transplantation (LDLT) is an alternative therapeutic option for patients with end-stage hepatitis C virus (HCV) cirrhosis because of the cadaveric organ shortage. HCV infection is now a leading indication for LDLT among adults worldwide, and there is a worse prognosis with HCV recurrence. The antivirus strategy after transplantation, however, is currently under debate. Recent updates on the clinical and therapeutic aspects of living donor liver transplantation for HCV are discussed in the present review.

Key words: Hepatitis C virus; Living donor liver transplantation; Interferon; Rivabirin

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INTRODUCTION

The use of live donors for liver transplantation was initiated more than a decade ago as a solution to the cadaveric donor shortage for pediatric recipients[1]. After the first successful case in an adult patient in 1994[2], this procedure is now widely applied to adult recipients, especially in countries where the availability of brain-dead donors is severely restricted[3] and also in the United States and European countries, due to a critical shortage of cadaveric organs. Improved surgical techniques and the introduction of new immunosuppressive agents have enhanced the long-term results of living donor liver transplantation (LDLT), leading to an increased demand for liver transplantation that exceeds the number of potential donor organs. In initial experiences with adult LDLT in Japan, the most common indication was cholestatic liver disease, including primary biliary cirrhosis and primary sclerosing cholangitis in Japan. The number of LDLT patients indicated for hepatitis C virus (HCV) has recently increased rapidly.

A recent study[4] of deceased donor liver transplantation (DDLT) reported that HCV infection was associated with a 23% increase in mortality and a 30% increase in the rate of graft failure. The poor results might be due to the recurrence of HCV disease in the graft[5]. HCV-induced graft hepatitis and fibrosis/cirrhosis occur in 75% to 80% and 10 % to 30% of recipients, respectively, at 5 years[6,7]. Once liver cirrhosis is established, the cumulative probability of developing clinical decompensation is close to 50% after 1 year and survival after decompensation is extremely short[8]. Cholestatic hepatitis occurs in approximately 10% of patients infected with HCV and leads to accelerated graft failure and death[9]. One of the hottest debates is the possibility of increased severity of recurrent HCV in LDLT patients. The benefit of LDLT might be offset if the outcome of LDLT for HCV patients is worse than that of DDLT. In this review, we describe current trends and controversies in LDLT for patients with HCV. Our results for LDLT and HCV are also reported.

CURRENT STATUS OF LDLT

According to the Japan Liver Transplantation Society[10], the number of adult patients (≥18 years old) is increasing annually, and has reached 300 in 2003. The most common indication for adults has been hepatocellular carcinoma (n = 311), followed by primary biliary cirrhosis (n = 255), and HCV-related cirrhosis without carcinoma (n = 113). The 1, 3, and 5 year survival rates of all the adult patients were 76%, 72%, and 69%, respectively. Those of HCV-positive patients were 76%, 73%, and 65%, respectively.

In the United States in 2000, there was a high level of enthusiasm for adult LDLT, with 49 centers performing at least one LDLT. Overall, in experienced centers, about a third of adults on the waiting list had a potential living donor and half of them had undergone LDLT; thus, LDLT might be applicable for up to 15% of individuals on the list[11]. The enthusiasm was, however, quickly tempered

Table 1 Comparison between LDLT and DDLT for hepatitis C virus cirrhosis

Study Author	Year	Institution	N LDLT	N DDLT	Dif[1]	Protocol biopsy	Findings
Gaglio[23]	2003	Colombia U.	23	45	Yes	No	Cholestatic hepatitis in 17% of LDLT and 0% of DDLT (P = 0.001). No significant difference in incidence of Rec.
Shiffman[28]	2003	Virginia Commonwealth U.	22	53	No	Yes	79% patient survival in LDLT and 91% in DDLT during 3 year (NS). No significant difference in inflammation score in liver specimen after 3 years
Russo[29]	2004	UNOS data	279	3955	No	No	87% 1-year patient survival in both.
Thuluvath[30]	2004	UNOS data	207	408	No	No	No significant difference in patient survival (P = 0.6).
Van Vlierberghe[32]	2004	Ghent U.	17	26	No	No	Rec in 35% of LDLT and 38% of DDLT during 1 year (P = 0.1)
Bozorgzadeh[34]	2004	Rochester U.	35	65	No	No	Rec in 77% of LDLT and 72% of DDLT during 1 year (NS), 89% patient survival in LDLT and 75% in DDLT during 39 mo (NS)

[1]Difference in short-term outcomes or severity of virus recurrence between living and deceased donor liver transplantation. Abbreviations: Rec, Virus recurrence; U, University; NS, not significant; UNOS, United Network for Organ Sharing.

by the death of a donor in 2002 in the United States[12]. Since 2001, the number of patients who have undergone LDLT has declined[13]. Currently less than 5% of all adult liver recipients use living donors. By July 2005, 2734 LDLT cases had been performed. There were 1761 adult patients and HCV was the most common indication. HCV is the most common indication for LDLT[14] and the number of HCV-positive patients is stable, approximately 100 per year between 2000 and 2002.

By the end of 2003, 1743 LDLT cases were recorded in the European Liver Transplantation Registry[15]. According to the Transplant Procurement Management[16], the number of LDLT peaked in 2003 and has gradually decreased over recent years. LDLT accounts for approximately 5% of the total liver transplants performed in Europe. Among the 806 LDLT cases from October 1991 to December 2001[17], the overall 5-year graft survival rate was 75%, better for children than for adults (80% *vs* 66% at 3 years). Cirrhosis secondary to HCV infection is a leading indication for LDLT among adults in Europe[18]. The number of LDLT patients is shown in the Table 1.

INDICATIONS

In areas with low deceased donor organ availability, the indications for LDLT are similar to those for DDLT. In contrast, in Western countries, LDLT is conducted in an attempt to alleviate the shortage of donor organs and to decrease the mortality among the patients awaiting transplants. That is, a balance needs to be achieved between the candidate's liver disease severity and the adequacy of a partial graft for transplantation. The candidate's liver disease should be advanced to the extent that transplantation is justified, but the liver disease cannot be so advanced that a partial graft will not provide adequate hepatic mass.

According to Russo's report[19] a substantial proportion of patients were United Network for Organ Sharing (UNOS) status 3 at the time of LDLT (43%). The policy at their centers prior to the implementation of a model for end stage liver disease (MELD)-based allocation was not to proceed with LDLT in patients meeting UNOS status 2A criteria. Their patient survival rate was 57% with an average stay of 23 d in the intensive care unit. In comparison, 1-year patient survival was 82% in DDLT recipients who were UNOS status 2A at the time of transplant[20].

The waiting list mortality increases in patients with advanced liver disease and patients with a MELD score of 25 have a 20% 3-mo mortality[21]. In general, it is uncommon to proceed with LDLT in patients with MELD scores above 25. Thus, depending on the region of the country and the average MELD score at the time of the transplant within the area served by the organ procurement organization, LDLT might offer patients transplantation before they die waiting for a deceased donor liver. The lower MELD score limit with LDLT is more controversial and varies from center to center. Russo[19] commented that they do not proceed with LDLT in candidates with MELD scores under 11.

LDLT AS A RISK FACTOR FOR RECURRENCE OF HCV

One study from Barcelona[22] reported that LDLT patients (n = 22) had younger donors, less graft steatosis, more frequent biliary complications, and earlier and more severe acute hepatitis compared with DDLT (n = 95) patients. A report from Colombia University[23] indicates that cholestatic hepatitis or severe HCV recurrence occurs more frequently in LDLT. These reports indicate that more intensive antiviral therapy might be necessary for recipients of living donor grafts.

The possible causes of HCV recurrence include HLA matching between donor and recipient. Because cellular immune reactions restricted by both HLA class I and II antigens are involved in the recognition of HCV peptides[24], HLA matching between donor and recipient could potentially increase damage to the graft from recurrent viral infections by facilitating host recognition of viral antigens[6]. Recently, a beneficial effect of a complete HLA-DQ mismatch was reported in 14 patients after transplantation for HCV cirrhosis[25]. Another possible cause might be related to liver regeneration[26], although recent data[27] did not support this hypothesis. *In vitro*, HCV internal ribosome entry site activity and replication are higher in actively dividing cells, and it is possible that

viral translation is enhanced by factors that stimulate the regeneration of hepatocytes. Moreover, there are experimental data suggesting that liver regeneration induces low density lipoprotein receptor expression, which might facilitate HCV entrance into the hepatocytes.

In contrast, comparable data between LDLT and DDLT for HCV was recently reported[28]. Russo and colleagues[29] compared patient and graft survival in recipients transplanted for chronic HCV who received a living donor organ (n = 279) and deceased donor organ (n = 3955) using the UNOS liver transplant database. One-year patient survival was 87% in both groups and 2-year patient survival was 83% and 81% in the living donor group and deceased donor group (P = 0.68), respectively. Similar results (DDLT, n = 480 *vs* LDLT, n = 207) were obtained from another analysis using the UNOS data base[30]. Analyses from the Mayo Clinic[31] and Gent University[32] also demonstrated no negative impacts of LDLT on the results of liver transplantation for HCV-related cirrhosis.

These data should be interpreted with caution, however, because of the important clinical distinction between LDLT and DDLT recipients. At the time of transplantation, the LDLT group recipients are far less sick than their DDLT group counterparts[33]. The LDLT (n = 35) and DDLT (n = 65) data from a single institution, Rochester University, were examined[34]. Patient survival, graft survival, rate of HCV recurrence, severity of HCV recurrence, graft loss from HCV, and interval for HCV recurrence in DDLT and LDLT were similar. It remains unclear, however, whether LDLT is truly disadvantageous compared to DDLT for HCV-positive patients because the number of cases or follow-up duration is not yet sufficient.

According to the data from Russo[29], from 1999 to 2000, the 1-year patient survival in the LDLT group increased from 69% to 90% (P = 0.04), and 1-year graft survival increased from 63% to 79% (P = 0.16). In contrast, in the DDLT group, 1-year patient and graft survival did not substantially change from 1999 to 2000. As a result, 1-year survival rates became similar between the LDLT and DDLT groups in 2000. The results indicated an experience effect and learning curve on outcomes after LDLT for HCV. Therefore, the initial reports indicating poorer results of LDLT might be due to technical problems from a lack of experience. Recent data indicating similar results between LDLT and DDLT might be due to the increased experience with LDLT. The multicenter adult to adult LDLT cohort study (A2ALL) might soon provide some answers to the questions about recurrent HCV after LDLT and DDLT[35].

MANAGEMENT OF HCV

Therapy for reccurrence in DDLT

If HCV recurs earlier and more severely after LDLT, a specific strategy for preventing the detrimental effects of HCV on living donor grafts must be developed. One strategy might be aggressive treatment for HCV. Treatment of recurrent HCV disease with interferon and ribavirin after DDLT is used in some centers[36-38]. One standard regimen includes interferon-alpha2b (3 MU × 3 per week) and ribavirin (1000 mg/d) for 6 mo. In a recent trial, polyethylene glycol-conjugated interferon therapy was used[35,39-44], with a sustained viral response rate ranging from 13% to 47%.

Preemptive therapy for HCV after DDLT

Preemptive therapy in the early post-transplantation period with interferon either alone or in combination with ribavirin has been attempted in DDLT, although its effectiveness is controversial. In one study, HCV-positive recipients were randomized within 2 wk of transplantation to receive either interferon alone (3 MU × 3 per wk, n = 30) or placebo (n = 41) for 1 year[39]. Only 17 patients could complete 1 year of interferon therapy. Eight patients (27%) in the interferon group and 22 (54%) of the untreated patients had recurrent hepatitis (P = 0.02). Patient and graft survival at 2 years did not differ between the groups, however, and the rate of viral persistence was not affected by treatment.

In another controlled trial[45], 24 recipients were randomized at 2 weeks post-transplantation to receive interferon (3 MU × 3 per wk) or placebo for 6 mo. There were no differences in graft or patient survival. There were no differences between groups in the incidence of histological recurrence or its severity differed between groups. Recurrent HCV was delayed 408 d in treated patients versus 193 d in the control cohort.

In a case series by Mazzaferro[46], 36 recipients were treated with interferon-alpha 2b (3 MU × 3 per wk) and ribavirin (10 mg/kg per d). They started treatment at a median of 18 d after the operation and treatment continued for 11 mo. After a median follow-up of 52 mo, the 5-year patient survival was 88%. Serum HCV RNA clearance was obtained in 12 patients (33%). They did not require further antiviral treatment because of negative HCV RNA in serum and normal liver histology for a median of an additional 36 mo. The former two randomized trials on preemptive interferon monotherapy demonstrated minimal benefits of the drug. In contrast, Mazzaferro reported more encouraging results, although their protocol brings into question how long therapy is needed once embarking on a preemptive strategy.

Re-transplantation

The approach to retransplantation for recurrent HCV varies widely among the transplant centers of DDLT[11]. The results after retransplantation for HCV (45% at 5 years) are poorer than that for other causes[47] (56%, P < 0.001). The patients with recurrent HCV in the early timing and graft failure within the first year have poor outcomes after retransplantation. These individuals should be considered contraindicated for retransplantation. The experience of retransplantation for HCV in LDLT has not been well accumulated.

OUR EXPERIENCE

We performed preemptive therapy for LDLT patients with HCV infection[40]. From 1996 to 2004, 67 patients underwent LDLT for HCV cirrhosis at the Tokyo University Hospital. The patients were 51 men and 16

women and their ages ranged from 23 to 63 years (median 55). The HCV genotype was 1b in 53 patients (79%). Forty-one patients (61%) had hepatocellular carcinoma. All the patients received the same immunosuppressive regimens with tacrolimus and methylprednisolone.

All the patients preemptively received antiviral therapy consisting of interferon α-2b and ribavirin, which was started approximately 1 mo after the operation. The therapy was continued for 12 mo after the first negative HCV RNA test. The standard regimen included interferon α-2b (3 MU × 3 per wk) and ribavirin (800 mg/d) for 6 mo. The patients were then observed without the therapy for 6 mo. The therapy was continued for at least 12 mo even if the HCV RNA test remained positive.

Therapy was discontinued when there was significant leukopenia (< 1500 /mL), thrombocytopenia (< 50000 / mL) despite application of granulocyte colony stimulating factor (Gran®, Sankyo, Co. Ltd., Tokyo, Japan), hemolytic anemia (hemoglobin < 8 g/L), renal dysfunction (serum creatinine > 20 mg/L), depressive psychological status, or general fatigue. The subjects were removed from the protocol if they did not continue the therapy for 12 mo due to adverse effects or could not start the therapy due to early death.

Blood counts and liver function tests were checked every 2 wk for the first month, and at 4 wk intervals thereafter. Serum samples were collected once a month for quantitative HCV RNA detection. Protocol liver biopsy was not performed. The log-rank test was used to compare the survival rate of the HCV-positive patients with the HCV-negative patients who underwent transplantation during the same period (n = 168).

A total of 28 patients were excluded from the analysis; 12 patients were removed from the protocol because of early death (n = 9) or because of drug cessation (n = 3). Another 16 patients are currently on the protocol and were therefore excluded from the analysis. Of the remaining 39 patients, 16 (16/39; 41%) obtained a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, comparable with that of patients negative for HCV (n = 168, 86%).

CONCLUSIONS

LDLT will remain an indispensable therapeutic tool for HCV related end stage liver disease and an alternative to DDLT. The association between LDLT and early HCV recurrence remains to be determined, although most of the recent papers suggest that live donor graft has no effect on short-term outcome or severity of virus recurrence. If living donor graft is associated with early HCV recurrence and consequently poorer graft survival, an aggressive antiviral protocol might improve the outcome of LDLT for HCV.

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the graft duodenal mucosa only showed mild infiltration with mononuclear cells. The postendoscopy course was uneventful. The pain subsided spontaneously on the next day. She was discharged home on the 21st postoperative day with both grafts functioning well.

In conclusion, double-balloon enteroscopy is feasible in recipients of pancreatic transplants with enteric drainage. This method makes possible to directly visualize duodenal graft and tissue biopsy. It might be employed to look for and possibly intervene at bleeding from the enteric anastomoses, and in special cases for endoscopic retrograde pancreatography of the graft. Its safety must be, however, carefully evaluated as threading on small bowel might potentially be damaging to anastomosed blood vessels supplying transplanted pancreas.

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Cyclosporin A for Treatment of Hepatitis C Virus After Liver Transplantation

Cirrhosis secondary to hepatitis C virus (HCV) infection is a leading indication for liver transplantation. HCV recurrence, however, is nearly certain and might worsen patient/graft outcome. A recent paper (*1*) reported that HCV infection is associated with a 23% increase in mortality and a 30% increase in the graft failure rate. The goal of HCV management in the transplantation setting is to prevent graft loss due to recurrent HCV infection, raising questions about a possible role for immunosuppression regimens and antiviral therapy.

A previous report (*2*) indicated that cyclosporin A (CyA) and interferon-alpha2b might effectively inhibit HCV replication in vitro. The antiviral effects of CyA for patients with chronic HCV (*3*) and those for HCV recurrence after transplantation (*4*), however, are controversial. We conducted a pilot study of the use of CyA, interferon, and ribavirin for preemptive therapy of HCV after liver transplantation.

Until October 2003, 41 HCV-positive patients underwent liver transplantation from living donors at the University of Tokyo Hospital. The immunosuppression regimens consisted of steroids and tacrolimus (*5*). The targeted whole-blood tacrolimus level was 15 to 20 ng/ml during the seven days after living donor liver transplantation (LDLT), which was gradually tapered to 5 ng/ml six months after LDLT. Predonisolone was tapered to 0.05 mg/day/kg six months after LDLT but was not stopped. All of the HCV-positive patients preemptively received interferon-alpha2b and ribavirin therapy (*6*), which was started approximately one month after transplantation. HCV RNA level was measured by real-time-polymerase chain reaction (*7*) and Amplicor HCV (Roche Molecular Systems, Pleasanton, USA). Of the 41 patients, six died within two years; 14 obtained a viral response (<50 IU/ml by Amplicor HCV) at the end of the treatment period (one year) and the response was sustained for another six months without the antiviral therapy; 21 did not respond to the antiviral therapy. Of the 21 nonresponders, eight patients continued with the protocol, which called for a change from tacrolimus to CyA without changing the antiviral therapy. The targeted CyA trough level was 100 ng/ml.

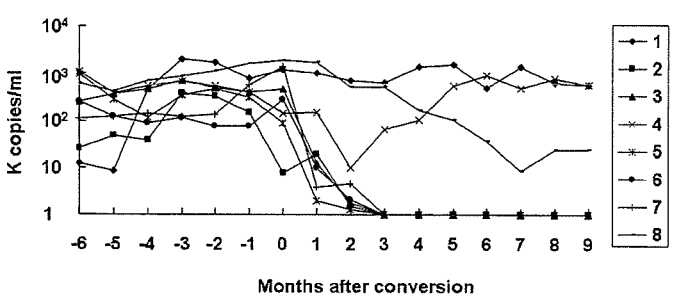

FIGURE 1. Change of the viral titer in the eight patients.

All of the patients were genotype 1b. The serum HCV titer was measured once a month by reverse transcriptase–polymerase chain reaction. The Institution Review Boards at University of Tokyo Hospital approved the protocol.

In five of the eight subjects (63%), the HCV titer was negative by Amplicor HCV within three months after the conversion and remained negative for another six months on CyA, interferon, and ribavirin (Figure 1). Liver and renal functions remained stable in all of the patients, and none of them had complications of acute cellular rejection after the conversion.

Our findings support the use of CyA in combination with interfron and rivabirin for the eradication of HCV in previous nonresponders. These findings suggest that a controlled study to confirm the benefit of CyA for preemptive treatment of HCV after liver transplantation is warranted.

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Does Using HTK Solution for Cold Perfusion of Cadaveric Kidneys Save Money?

Since 1987, University of Wisconsin (UW) solution has been the standard solution for cold perfusion and preservation of kidneys for transplant. In 2001, the U.S. Food and Drug Administration approved histidine-tryotophan-ketoglutarate (HTK) for cold perfusion and storage of renal allografts. Data has shown comparable outcomes between UW and HTK for preservation of renal, liver, and pancreatic allografts (*1–3*). Theoretical advantages for HTK compared to UW solution include: lower viscosity (which might lead to better microperfusion of the allograft), lower potassium concentrations, and easier handling properties, although the actual effects on clinical outcomes are not fully understood. On the other hand, HTK is significantly less expensive than UW solution on a per liter basis. For this reason, HTK has become an attractive alternative to UW solution for some Organ Procurement Organizations. In reported clinical experience with HTK, larger volumes of solution have been used to achieve exsanguination of the recovered organs. The argument is frequently made that these larger volumes negated any significant cost savings associated with the cheaper HTK solution.

Gift of Life Michigan switched from UW to HTK solution in December 2003. This decision was based on an impression of clinical equipoise between UW and HTK solutions, with a projected materials cost savings of 47% per donor.

We compared the material costs of 77 consecutive kidney-pancreas donor procurements, in which the organs were used for transplant. The perfusion volumes include the aortic flush, the back-table flush of the renal allografts, and the storage volume of the renal grafts. As predicted, significantly larger volumes of fluid were used in the perfusion of the organs with HTK (Table 1). The cost of a liter of UW (UW solution $282, filter $10, and additives $30) was $322/L compared to $148/L for HTK (no additives or filters needed). Costs of UW perfusion were significantly higher than HTK perfusion. The transition from UW to HTK saved $548 (USD) per renal donor. This represents a 43% actual cost reduction, which is similar to the 47% projected reduction. In conclusion, the transition from UW to HTK has resulted in a significant cost savings for Gift of Life Michigan.

TABLE 1. UW vs. HTK flush solution for cold perfusion of kidneys for transplant

	UW	HTK	Difference
N	41	36	
Mean flush volume per donor (mL)	3516±795	5575±1506	2059[a]
Cost per liter solution (US $)	$322	$148	$174
Cost per renal donor (US $)	$1288	$740	$548[a]

[a] $P < 0.05$.

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Identification of an Oligopeptide Binding to Hepatocellular Carcinoma

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Biopanning · Hepatocellular carcinoma · Peptide · Phage display · Tumor targeting

Abstract
Objectives: We carried out identification of a small peptide binding to human hepatocellular carcinoma (HCC) cells with the aim of applying the peptide for future HCC-targeted therapy or imaging. ***Methods:*** The biopanning technique using phage peptide display libraries was performed on HCC cells in vitro, and a phage clone expressing the HCC-binding peptide motif was selected. The binding activity of the selected phage was evaluated by plaque infection assay and immunofluorescence on cell lines. In addition, the binding activity of the peptide-expressing phage was investigated using HCC specimens derived from patients who had undergone hepatectomy for HCC. ***Results:*** A heptapetide, Thr-Thr-Pro-Arg-Asp-Ala-Tyr (TTPRDAY), was identified as a motif binding to HCC. TTPRDAY bound specifically to HCC cells in comparison with other cancer cells, and the binding to HCC cells was also confirmed by immunofluorescence. In addition, the synthesized TTPRDAY peptide showed binding activity and a non-mitogenic effect on HCC cells in vitro. TTPRDAY-presenting phage showed more significant binding to HCC cells derived from specimens obtained from actual patients than to non-cancerous liver tissue. ***Conclusion:*** The motif TTPRDAY, identified by the biopanning technique, shows significant binding to HCC cells.

Introduction

Hepatocellular carcinoma (HCC) is one of the commonest malignancies worldwide [1], and the prognosis of affected patients remains poor [2–4]. In many patients with HCC, obtaining curativity by surgery or radiofrequency ablation of the cancer is difficult due to the high frequency of intrahepatic metastases or multicentric occurrence. Other therapeutic options for intrahepatic localized HCC are percutaneous ethanol injection, microwave therapy, cryotherapy and transarterial chemoembolization. However, the therapeutic efficacy of these treatments is limited. For example, the 5-year survival rate after transarterial chemoembolization for HCC is only 12.5% [5]. Patients with distant metastatic HCC are treated with systemic chemotherapy, but its reported

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therapeutic efficacy is dismal; Chung et al. [6] reported that intra-arterial cisplatin infusion and systemic interferon-α yielded a response rate of 33% and a median survival time of 19 weeks for HCC patients with major portal vein thrombosis or distant metastases.

The chief obstacles to establishing effective therapies for HCC are the multiplicity of the lesions (multicentric occurrence of the tumor and intrahepatic or distant metastases) and severe liver dysfunction due to underlying liver cirrhosis. Therefore, establishment of a methodology that could target HCC efficiently would bring about a dramatic improvement in the efficacy of treatments for HCC. For example, HCC-selective ligands would enable targeted delivery of a variety of therapeutic agents, such as genes or cytotoxic drugs, and would also facilitate better imaging of multiple HCC lesions. Such agents are clearly appropriate for HCC patients with liver cirrhosis, because they could minimize the degree of drug entry into non-HCC cells and the resulting damage to liver function.

Candidate targeting agents have been studied by several groups in attempts to achieve tumor tropism. The ligands that have been evaluated include some antibodies, as well as fragments and single-chain Fv molecules and growth factors. Recently, however, this empirical approach to the identification of targeting ligands has been largely superseded by the use of library-based screening systems, which have been designed to allow iterative selection of high-affinity ligands by repeated screening and enrichment of living libraries.

Barry et al. [7] have introduced peptide-presenting phage libraries to enable selection of peptides binding to several different cell types. Since then, the biopanning technique using phage peptide display libraries has been shown to be a powerful tool for identifying specific ligands on target organs and tumors [8–10]. The peptide-presenting phage library used is based on a combinatorial library of random peptide heptamers fused to a minor coat protein (pIII) of phage M13 and contains about 2.8×10^9 different sequences. With successive rounds of biopanning, different peptides selective for the target cells can be identified.

In the present study, we used the phage panning technique to identify a peptide that would have the specific ability to bind to human HCC cells. In addition, in order to confirm the feasibility of applying the identified peptide to clinical practice, its HCC-binding activity was assessed on HCC samples derived from patients who had undergone hepatectomy for operable HCC.

Materials and Methods

Cell Lines

The cell line Huh-7 (human HCC) was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan), and the cell lines Hep3B (human HCC), HepG2 (human hepatoblastoma), DLD-1 (human colorectal carcinoma), AZ521 (human gastric cancer) and Saos2 (human osteosarcoma) were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Sigma Chemical, Poole, UK) or RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. The cells were passaged and expanded by trypsinization of the cell monolayers followed by replating every 4 days.

Selection of Cell-Targeting Peptides

The Ph.C7C M13 heptapeptide phage display library (New England Biolabs, Beverly, Mass., USA) was used for biopanning. Huh-7 cells were grown to confluence in a 6-well plate. They were acclimated to 4°C for 30 min and washed twice in phosphate-buffered saline (PBS, Dainippon, Osaka, Japan) containing 1% (w/v) bovine serum albumin (BSA, Sigma). The phage library of 2×10^{11} plaque-forming units (pfu)/well diluted in 1 ml DMEM containing 1% BSA was added to each well at 4°C. After 1 h of incubation with gentle agitation, medium containing unbound phage was removed, and the cells were washed 4 times in PBS containing 1% BSA. This was followed by addition of 1 ml/well 0.2 M glycine-HCl (pH 2.2) as a general buffer for nonspecific disruption of binding interactions for 5 min, and fluids were neutralized with 150 μl 1 M Tris-HCl (pH 9.1).

The cells were scraped from the 6-well plate. Numbers of eluted phages were established by titering on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Wako, Osaka, Japan) and IPTG (isopropyl-β-D-thiogalactopyranoside; Wako) agar plates containing *Escherichia coli* strain ER2738. Remaining phages were amplified by addition to a 20-ml early log phase culture of ER2738 for 5 h at 37°C with vigorous shaking (150 rpm). Amplified phages were isolated from the resulting culture using the manufacturer's recommended protocol, concentrated, titered and used for subsequent rounds of biopanning. In total, four rounds of biopanning were performed in triplicate.

Isolation and Sequencing of Phage DNA

After each round of biopanning, individual phage clones were isolated from each replicate and their total DNA was isolated according to the recommended protocol of the sequencing kit manufacturer (Biosystems, Perklin Elmer, Foster City, Calif., USA). The resulting DNA was used for sequencing analysis using the -96 primer together with a BigDye terminator cycle sequencing kit (Amersham Biosciences, Amersham, UK). The DNA sequences were determined using an automated ABI PRISM sequencer 3100 Genetic Analyzer (Applied Biosystems). Next, searches for human proteins mimicked by the selected peptide motifs were carried out using online databases through the National Center for Biotechnology Information (http:www.ncbi.nlm.nih.gov/BLAST/).

Evaluation of Selected Phage Clones for Binding to Human Cancer Cells

The cells were grown to confluency in 6-well plates, acclimated to 4°C for 30 min and washed briefly in PBS containing 1% BSA two times. Then, each selected phage clone diluted in 1 ml of DMEM containing 1% BSA at a concentration of 5×10^7–10^9 pfu was added to each well at 4°C. After 1 h of incubation with gentle agitation, medium containing unbound phages was discarded, and the cells were washed four times in PBS containing 1% BSA. Then, the phage binding to cells was evaluated as described in 'Selection of Cell-Targeting Peptides'. In addition, the ability of selected phage clones to bind to other human cancer cell lines (Hep3B, HepG2, AZ521, DLD-1 and Saos2) was determined in 6-well plates as described above.

Immunofluorescence Analysis of Binding of Selected Phage Clones to Huh-7 Cells in vitro

Huh-7 cells were grown to confluence on an 8-well chamber slide in DMEM containing 10% FBS. The cells were washed with PBS containing 1% BSA, and incubated for 1 h at 4°C in DMEM (containing 1% BSA) containing 2×10^{11} pfu of either a selected phage clone or a control phage. A phage clone displaying no oligopeptide insert (insertless) was used as a negative control. The medium was discarded and the cells were washed four times with PBS containing 1% BSA, fixed with methanol/acetone (–20°C), and blocked with 10% goat serum for 20 min at room temperature. An anti-M13 monoclonal antibody diluted 1:600 in 2% goat serum was added and incubated for 1 h at room temperature. The cells were washed in PBS three times, and then incubated with FITC-conjugated goat anti-mouse immunoglobulin for 1 h at room temperature. The cells were washed in PBS three times, 4′-6-diamino-2-phenylindole was used for nuclear counterstain, and visualized using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan).

The motif Thr-Thr-Pro-Arg-Asp-Ala-Thr (TTPRDAY) was selected as the most promising consensus sequence binding to HCC and studied in more detail.

Competitive Inhibition of the Synthesized Peptide on Phage Accumulation in vitro

To confirm the capacity of the synthesized peptide (TTPRDAY) to bind to HCC, its inhibitory effects on phage accumulation were examined. Huh-7 cells were pre-incubated with the TTPRDAY peptide (synthesized by Sigma Genosys Japan, Ishikari, Japan) or control peptide at 1 or 10 μM for 30 min at 4°C, and then 5×10^8 pfu of the selected phage diluted in 1 ml DMEM containing 1% BSA were added. The phages were allowed to bind to the cells for 1 h at 4°C with gentle agitation. Medium containing unbound phages was discarded, and the cells were then washed four times for 5 min each time in PBS containing 1% BSA, before the cell-associated phages were recovered by lysing the cells in 1 ml/well of 30 mM Tris-HCl (pH 8.0) containing 10 mM EDTA on ice for 1 h. The number of phages recovered was determined by titering multiple dilutions of the eluted phages as described above. The same experiment was repeated using insertless phages and TTPRDAY peptide.

Effect of the TTPRDAY Peptide on Cell Viability

Huh-7 cells were plated in 96-well plates at 5×10^3 cells/well and incubated at 37°C in DMEM containing 10% FCS in either the presence or absence of the TTPRDAY peptide at 0.1, 1 or 10 μM. After 24, 48, 72 and 96 h, the viability of the Huh-7 cells was assessed using the MTS assay, as described previously [9]. Media were replaced with 120 μl of FCS-free DMEM containing 20 μl of CellTiter®96 AQueous One solution reagent (Promega, Madison, Wisc., USA), and the culture plates were incubated at 37°C for 2 h. Next, 100 μl of the medium were transferred to a new 96-well plate and the quantity of the formazan product present was determined by measuring the absorbance at 490 nm using a microplate autoreader (Molecular Devices, Sunnyvale, Calif., USA).

Mutagenicity Study

The Ames test [11, 12] was carried out using histidine-deficient (*his*$^-$) *Salmonella typhimurium* tester strains, TA100 and TA98. The tester strains 100 are responsive to base-pair substitutions, whereas the 98 detect deletions or additions of base pairs (frameshifts). Strains TA100 and TA98 were cultured at 37°C in the presence of different concentrations (0.1, 1, 10, 100 and 1,000 μM) of the selected peptide and revertant *his*$^+$ colonies were counted after a 48-hour incubation period. The presence of revertant colonies after plating on histidine-poor growth media indicates the presence of a mutagen. Each experimental condition was run with duplicate samples. Additionally, the Ames test was performed in the absence or presence of a S9 fraction (mix of metabolizing enzymes from rat liver) to allow not only detection of a direct mutagenic effect, but also of an indirect mutagenic effect brought about by possible metabolites of the applied compound.

Binding of the Selected Phage Clone to Surgically Resected HCC Specimens

Samples of tumor and non-cancerous liver tissue were obtained at the time of hepatectomy from patients with operable HCC. Samples were weighed and homogenized using a motor-driven Teflon-glass homogenizer. The homogenized samples of both the tumor and non-cancerous liver tissue were each divided into two portions, one being used to assess the binding activity of the selected phage clone and the other to assess that of the control phage. Either the selected or the control phage was added to the homogenates of tumor and non-cancerous liver tissue at 5×10^{10} pfu per 100 mg tissue, and allowed to bind to the samples for 20 min at 37°C with agitation (150 rpm). The samples were washed with PBS twice, and then 2 ml of 0.2 M glycine-HCl were added as a general buffer for nonspecific disruption of binding interactions for 3 min, and fluids were neutralized with 300 μl of 1 M Tris-HCl. After washing with PBS, 5 ml of PBS containing 0.5% Tween 20 were added to the cells. Recovery was quantified by titering multiple dilutions of the homogenates, as described above. The experiment was performed on 6 patients with HCC, and in each case, the results were presented as a ratio relative to the recovery of the insertless phage from the non-cancerous liver tissue. In addition, the binding activity of the selected phage clone on surgically resected specimens was evaluated by immunofluorescence analysis. Sample from the tumor tissue was mounted in OCT compound and frozen, and sectioned at 6 μm in a cryostat at –20°C. After washing with PBS twice, the sectioned sample was incubated with 5×10^{10} pfu of the selected phage clone or control phage (insertless) for 30 min. The sample was then washed four times in PBS. This was followed by addition of 100 μl 0.2 M glycine-HCl (pH 2.2) as a general buffer for nonspecific disruption of binding interac-

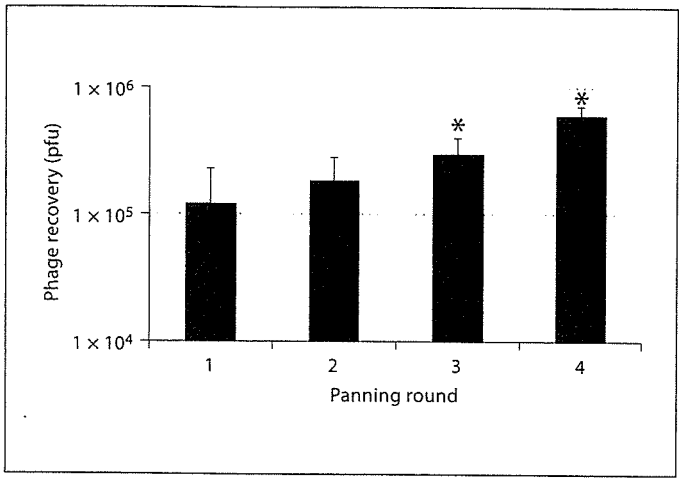

Fig. 1. Phage recoveries from Huh-7 cells in each round of biopanning. Four consecutive rounds of biopanning were performed on the human HCC cell line Huh-7. The phage recovery from each round increased with the number of biopanning passages, being approximately 6-fold higher in the final round than in the first. * $p < 0.05$ compared to the recovery in round 1.

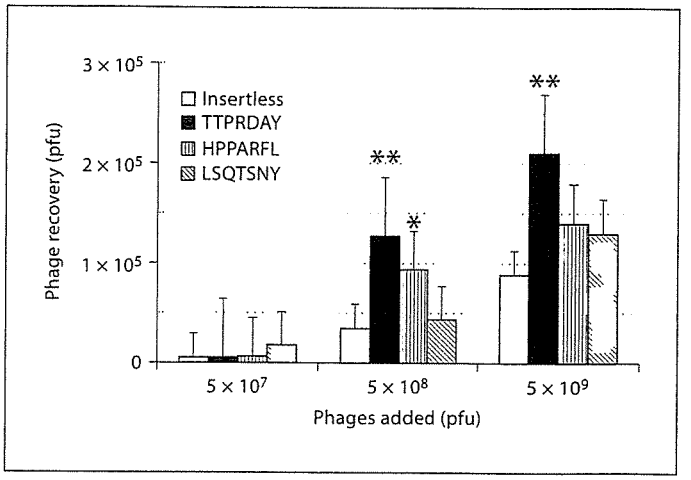

Fig. 2. Assessment of binding activities of selected phage clones expressing TTPRDAY, HPPARFL and LSQTSNY on Huh-7 cells in vitro. Huh-7 cells were cultured in 6-well plates and incubated with phage clones expressing TTPRDAY, HPPARFL or LSQTSNY or control phage (insertless) at a concentration of 5×10^7–10^9 pfu. Cells were then scraped off and phage recoveries were titered by plaque infection assay. The phage clone expressing TTPRDAY showed the highest binding activity at plural concentrations of added phage. ** $p < 0.01$ and * $p < 0.05$ compared to the recovery of insertless phage.

tions for 5 min, and fluids were neutralized with 15 μl 1 M Tris-HCl (pH 9.1). Then, the sample was washed with PBS and immunostained as described in 'Immunofluorescence Analysis of Binding of Selected Phage Clones to Huh-7 Cells in vitro'.

Statistics

Results are presented as the mean and standard deviation of data from three independent experiments, with significance of differences evaluated using Student's t test. In the experiment, to examine the binding of phages to surgically resected human tissue specimens, the results are presented as the mean and standard deviation of the data from six patients.

Approval from the Shinshu University Ethics Committee was obtained before the study, and written informed consent was obtained from all patients for the use of their tissue specimens.

Results

Isolation of the Specific Peptide Binding to Huh-7 Cells

Four consecutive rounds of in vitro biopanning were performed on human HCC. The phage recovery from each round increased with the number of biopanning passages, indicating selection of phage binding to HCC cells (fig. 1).

After each round of biopanning, individual phage plaques were picked up. Their DNA was isolated and sequenced, and the corresponding amino acid sequences of the inserts were deduced. After the first and second rounds of biopanning, the HCC-derived sequences displayed no distinguishable homology (data not shown). However, the HCC-derived sequences from the third and fourth rounds displayed some consensus motifs, and these were selected as candidate peptides that could bind to HCC. After the third and fourth rounds of biopanning, 24 phage plaques were picked up from each replicate, and their DNA was sequenced. We then compared the relative frequencies of every tripeptide motif in each replicate. The motif frequencies were calculated as the prevalence of each motif-containing peptide divided by the total number of isolated peptides. The Thr-Thr-Pro-Arg-Asp-Ala-Tyr (TTPRDAY) motif was the most frequently encountered (5.6%), followed by His-Pro-Pro-Ala-Arg-Phe-Leu (HPPARFL; 4.2%) and Leu-Ser-Gln-Thr-Ser-Asn-Thr (LSQTSNY; 2.8%).

To determine which motif was the best binding peptide, the binding activities of selected phage clones expressing the candidate oligopeptides were assessed in vitro as described above. The phage clone expressing the TTPRDAY motif showed the highest binding activity at plural concentrations of added phage clones (fig. 2), and therefore this motif was selected as the most promising for binding to HCC.