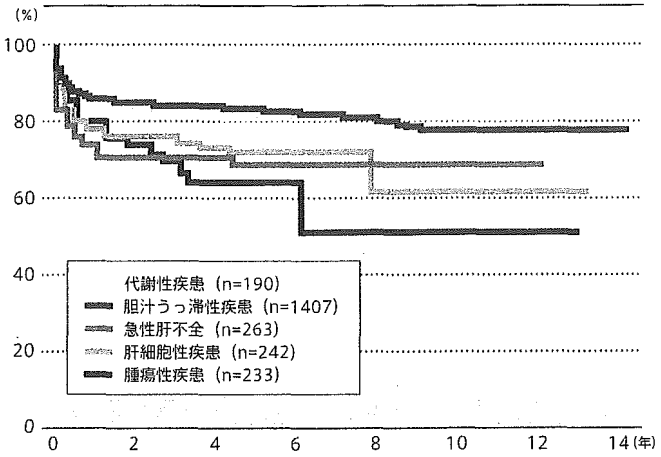
図2 適応疾患別の累積生存率(日本肝移植研究会による)



(非代償期) および劇症肝炎 (ウイルス性、自己免疫性、薬剤性、成因不明を含む) である。また、肝硬変に肝細胞癌を合併している場合には、遠隔転移と血管侵襲を認めないもので、肝内に径5cm以下1個、または3cm以下3個以内が存在する場合に限られている。上記以外の場合は、保険が適応されず、原則的に患者の自費負担となる。

生体移植は、健康人である提供者 (ドナー) の体にメスを入れるという本質的な問題点を有する医療である。ドナーの手術死亡はあってはならないことであるが、2002年4月New England Journal of Medicine誌は、米国で7人の生体肝ドナーの手術死亡があり、他にも2人が肝提供後に肝不全に陥り肝移植を受けたとの論説を掲載し警鐘を鳴らした (後に死亡者数は3人に訂正)。これを受け、日本肝移植研究会は、わが国の全移植施設を対象とし、生体肝ドナーの術後合併症に関する緊急調査を行った。全症例につき回答を得ることができ、手術関連死亡は1人もなかったが、12.4%のドナーに有意な術後合併症が発生していることが分かった。頻度の高い合併症は、胆汁瘻、胃内容停滞 (胃と肝切離面の癒着による)、創感染、腸閉塞など腹部合併症であったが、危険な合併症である肺塞栓が5例あった。ドナーの術後入院日数は15.6±9.6日 (mean±SD) であった。提供肝の種類別で比較すると、肝右葉を提供したドナーは合併症の頻度が高く、術後入院日数が長かった。この調査の後、2003年5月にわが国で初めて生体肝ドナーの手術関連死亡があったが、日本肝移植研究会のドナー安全対策委員会はこの事例につき詳細な検証を行い、誌上報告した。

さらに、日本肝移植研究会は、生体肝ドナー本人を対象として、QOLなどに関するアンケート調査を施行した。61%の方から回答を得ることができ、分析の結果ドナーの健康状態や心理状態などに関して多くの重要な知見を得ることができた。2005年4月に報告書を公開するとともに、日本肝移植研究会のホームページ (http://jlts.umin.ac.jp/) に掲載する予定である。

今後の課題

わが国の脳死肝移植は、症例数が一向に増加しない。生体肝移植はそれを補うべく著明な増加を示しているが、上述したように生体肝ドナーには種々の医学的な合併症が発生しているのみならず、心理的、社会的にも色々な問題があり、決してバラ色の医療ではない。また、海外渡航に道を求める人があるが、C型肝炎の増加に伴い世界的にドナー不足の状態にあり、実際に移植を受けることができる人は限られている。さらに、渡航移植は、海外において、臓器売買に類する行為とのそしりを受ける場合があるとも聞く。以上のような状況を改善するためには、脳死臓器提供の増加が必要であり、いわゆる臓器移植法の改正が望まれる。

繰り返しになるが、脳死肝移植はいまだに保険適応が認められていない。法律で特に規定されている医療が保険適応でないのは異常な状態であり、早急な是正が必要である。

[参考文献]

1) 日本肝移植研究会.肝移植症例登録報告.移植 2004;39:634-642.
2) Surman OS.The ethics of partial-liver donation. N Engl J Med 2002;346:1038.
3) Umeshita K et al.Operative morbidity of living liver donors in Japan. Lancet 2003;362:687-690.
4) 日本肝移植研究会ドナー調査委員会.生体肝移植ドナーに関する調査報告書.2005.
5) 日本肝移植研究会ドナー安全対策委員会.生体肝移植ドナーが肝不全に陥った事例の検証と再発予防への提言.移植 2004;39:47-55.

特集

肝臓の臨床最前線

急性肝不全 劇症肝炎と肝移植

Liver transplantation for fulminant hepatic failure

武田 裕 TAKEDA Yutaka
堂野 恵三 DONO Keizo
梅下 浩司 UMESHITA Koji
永野 浩昭 NAGANO Hiroaki
宮本 敦史 MIYAMOTO Atsushi
丸橋 繁 MARUBASHI Shigeru
門田 守人* MONDEN Morito

Key words 劇症肝炎 肝移植 適応基準

欧米では，1980年代より劇症肝炎の治療として肝移植が施行され，現在では治療法として確立している．一方本邦では，1997年に臓器移植法が施行され，劇症肝炎も脳死肝移植の適応となっているが，日本臓器移植ネットワークのホームページ(http://www.jotnw.or.jp/)によると，2004年12月20日現在で，脳死肝移植はわずかに26例施行されたのみである．したがって，本邦での劇症肝炎に対する肝移植もほとんどが生体肝移植であり，こうした特殊性を含めて，劇症肝炎に対する肝移植について述べたい．

Ⅰ．劇症肝炎とは

急性肝不全(劇症肝炎)はAASLD(American Association for the Study of Liver Disease)のガイドライン[1]では，Acute liver failure(fulminant hepatic failure)として肝移植の適応項目に入っており，先行する肝疾患なく発症から8週間以内に肝性脳症と凝固異常をきたすものと定義され，その原因はアセトアミノフェンとそれ以外の薬剤，A型，B型肝炎，Wilson病と成因不明に分けられている．本邦では，第12回犬山シンポジウムの劇症肝炎の診断基準[2]により，「肝炎のうち初発症状発現後8週間以内に高度の肝機能異常に基づいて昏睡Ⅱ度以上の肝性脳症をきたし，プロトロンビン時間が40％以下を示すものとする．」と定義されている．また，「症状出現後10日以内に脳症が発現する急性型と11日以降に発現する亜急性型がある．」と記載されている(表1)．類縁疾患としては，「プロトロンビン時間が40％以下を示す症例のうち，肝性脳症が認められない，ないしは昏睡Ⅰ度以内の症例は急性肝炎重症型，初発症状出現から8週以降，24週以内に昏睡Ⅱ度以上の脳症を発現する症例は遅発性肝不全に分類する．」とされている．成因に関しては，以前はA型，B型，非A非B型，薬剤性，その他に分類されていたが，現在では，ウイルス性(A型，B型，C型，E型，その他)自己免疫性，薬剤性，成因不明，分類不能の5つに分類されている[3]．

Ⅱ．発症頻度

劇症肝炎の年間推定発生数は1972年の全国集計

大阪大学大学院医学系研究科病態制御外科学教室 *教授

0371-1900/05/¥50/頁/JCLS

表1　劇症肝炎の診断基準(難治性の肝疾患に関する研究班，2003年)

劇症肝炎とは，肝炎のうち初発症状出現後8週間以内に高度の肝機能異常に基づいて昏睡Ⅱ度以上の肝性脳症をきたし，プロトロビン時間が40%以下を示すものとする．そのうちには症状出現後10日以内に脳症が発現する急性型と，11日以内に出現する亜急性型がある．

(注1)　先行する慢性肝疾患が存在する場合は劇症肝炎から除外する．ただし，B型肝炎ウイルスの無症候性キャリアからの急性増悪例は劇症肝炎に含めて扱う．

(注2)　薬物中毒，循環不全，妊娠脂肪肝，Reye症候群など肝臓の炎症を伴わない肝不全は劇症肝炎から除外する．

(注3)　肝性脳症の昏睡度分類は犬山分類(1972年)に基づく(表1)．

(注4)　成因分類は「難治性の肝疾患に関する研究班」の指針(2002年)に基づく(表2)．

(注5)　プロトロビン時間が40%以下を示す症例のうち，肝性脳症が認められない，ないしは昏睡Ⅰ度以内の症例は急性肝炎重症型，初発症状出現から8週以降24週以内に昏睡Ⅱ度以上の脳症を発現する症例は遅発性肝不全に分類する．これらは劇症肝炎の類縁疾患であるが，診断に際しては除外として扱う．

特定疾患の申請に際しての臨床調査個人票には(注3)と(注4)のみが記載されています．

では約3,700例[4]であったが，1989年には約1,000例[5]となり1996年の集計においても約1,000例[6]であった．一方，遅発性肝不全(LOHF：late onset hepatic failure)は年間約100例，急性肝炎重症型は約3,000例と推定されている[7]．

Ⅲ．予　　後

劇症肝炎の予後は，1983年から1997年の救命率は急性型で30～40%，亜急性型は10～20%，またLOHFでは約10%と不良であった．しかしながら，近年では救命率の向上が見られ，2002年の全国集計によると，肝移植非実施症例による救命率は，急性型57%，亜急性型23%，LOHF 0%，肝移植実施症例による救命率は，急性型64%，亜急性型76%，LOHF75%であり，肝移植を含めた全症例での救命率は，急性型58%，亜急性型41%，LOHF 30%と治療成績の向上が見られている[3)8)～10)]．

Ⅳ．肝移植の適応

劇症肝炎の肝移植の適応基準は，欧米では，イギリスのKing's College Hospitalの肝移植適応基準や，フランスのPaul Brousse Hospitalの肝移植適応基準が提唱されている(表2)．King's College Hospitalの適応基準は，アセトアミノフェン中毒とそれ以外の成因を分けて適応を決めている．一方，Paul Brousse Hospitalの適応基準は簡便で，肝性脳症(3または4)および年齢30歳以上で，第Ⅴ因子が30%以下，年齢30歳以下で，第Ⅴ因子が20%以下を適応としている．しかしながらこれらの適応基準は，劇症肝炎の成因や治療法の違いなどから，そのまま国内の症例に適応しても正診率は低いことが分かっている[11]．

そこで本邦での予後予測を正確かつ早期に予測するために1996年に日本急性肝不全研究会により劇症肝炎における肝移植適応ガイドライン(表3)が提示されている．ガイドライン作成当時のPPV(positive predictive value：死亡と予測された症例のうち，実際の死亡例の比率)は1.00，NPV(negative predictive value：生存と予測された症例のうち，実際の生存例の比率)は0.82，sensitivity(死亡例のうち，死亡と予測された症例の比率)は0.86，specificity(生存例のうち，生存と予測された症例の比率)は1.00，PA(predictive accuracy 正診率)は0.91であったが，最近の症例による検討では，PPV 0.83，NPV 0.78，sensitivity 0.93，specificity 0.57，PA 0.82と低下している[12]．Specificityの低下は死亡と予測されたが生存した割合が多いことを示しており，内科的治療による救命率の向上とともに，将来見直すべき可能性があると考えられる．

このガイドラインでは，治療開始(脳症発現)から5日後に予後の再予測を行い，内科的治療に反応する症例の見極めを行うことになっている．当初には多くの施設で5日後の再評価後を待つこと

表2 欧州における急性肝不全の移植適応

a：Criteria Adopted in Paul Brousse Hospital for Liver Transplantation in Fulminant Hepatic Failure.

Factor V＜30％ if age＞30yr
or
FactorV＜20％ if age＜30yr
and
Confusion or coma
(encephalopathy stage 3 or 4)

b：Criteria Adopted in King's College Hospital for Liver Transplantation in Fulminant Hepatic Failure.

Acetaminophen
pH＜7.30(irrespective of grade of encephalopathy)
or
Prothrombin time＞100s (INR＞7) and serum
creatinine＞300μmol/l in patients with grade Ⅲ or Ⅳ encephalopathy.
Nonacetaminophen patients
Prothrombin time＞100s (INR＞7) (irrespective of grade of encephalopathy)
or
Any 3 of the following variables (irrespective of grade of encephalopathy)：
Age＜10 or＞40yr
Etiology—non A, non B hepatitis, halothane hepatitis, idiosyncratic drug reactions
Duration of jaundice before onset of encephalopathy＞7days
Prothrombin time(INR＞3.5)＞50s
Serum bilirubin＞300μmol/l

(文献17より)

表3 劇症肝炎における肝移植適応のガイドライン(1996年日本急性肝不全研究会)

Ⅰ．脳症出現時に次の5項目のうち2項目を満たす場合は死亡と予測して肝移植の登録を行う
(1)年齢：≧45歳
(2)初発症状から脳症発現までの日数：≧11日(すなわち亜急性型)
(3)プロトロンビン時間：＜10％
(4)血清ビリルビン濃度：≧18.0mg/dl
(5)直接/総ビリルビン吐：≦0.67
Ⅱ．治療開始(脳症発現)から5日後における予後の再予測
(1)脳症がⅠ度以内に覚醒あるいは昏睡度でⅡ度以上の改善
(2)プロトロンビン時間が50％以上に改善
以上2項目のうち，認められる項目数が
2 ：生存と再予測して肝移植の登録を取り消す
1 or 0 ：死亡と再予測して肝移植の登録を継続する

が多く，感染症や脳症の進行により，移植不能となったり，術前の病状を悪化させる結果となっていた．しかしながら，脳症出現時に5項目のうちの2項目を満たしていれば脳死肝移植の登録は可能であり，登録のために5日後の再予測を待つ必要はない．特に治療開始後5日以内に肝移植を必要とするhyperacute liver failureのような病態では，5日後の再評価を待つべきではないことを強調したい．

またこのガイドラインは，脳死肝移植の際の基準として，グラフトの公平な分配のための客観的な基準となっている．生体肝移植においては，内科的治療によって改善がみられない場合に，適切なドナーが存在し，十分なインフォームドコンセントがなされて，ドナー候補を含む家族が生体肝移植を希望されれば，脳障害や重症感染症を合併する前に肝移植を施行する方が良いと考えられる[13]．脳死肝移植症例数が少ない本邦では，緊急に対応が必要となる，劇症肝炎に脳死肝移植が対応することは困難であり，劇症肝炎における生体

肝移植適応ガイドラインが必要と考えられる．

Ⅴ．内科的治療

内科的治療に関しては詳細を他項に譲るが，原疾患治療としてB型劇症肝炎に対するラミブジン投与やインターフェロンの併用が行われている．また，免疫応答による肝障害を防止する目的にて，サイクロスポリン投与やステロイドパルス療法を，肝再生を目的にPGE1投与やグルカゴンインスリン療法が選択される場合もある．また，劇症肝炎の経過中にはSIRSの状態に陥ることが多く，肝に加えて心肺腎等の多臓器障害をきたし，循環，呼吸，血糖等の全身管理および人工肝補助療法が必要となることから，早期より集中治療の実施が望ましい．人工肝補助療法としては，血漿交換と持続血液濾過透析とを施行する方法の効果が高いと考えられている．劇症肝炎の予後を決定するのみならず，肝移植の禁忌となる感染等の合併症対策も重要である．感染症対策としては，胸部レントゲン，CT等画像検査に加え，各種培養検査を早期に施行し，早期に治療を開始することが重要である．また，選択的消化管内殺菌を施行する施設もある．肝不全に伴う脳浮腫に対しては，脳波，頭部CT，場合によっては頭蓋内圧をモニターする必要があり，アンモニア上昇に対してラルツロース，脳圧降下剤としてマンニトールやグリセオールが投与されている．微小循環障害に対しては，蛋白分解酵素阻害剤が投与される場合もある[14]．

Ⅵ．劇症肝炎に対する肝移植

劇症肝炎に対する肝移植は，先に述べた肝移植の適応にしたがって行うが，もう一つのポイントは移植の禁忌が無いかを検討することである．まずは耐術能があるかで，高齢者(70歳以上)，重篤な心肺合併症，多臓器不全などは適応外となる．脳障害の可逆性に関しては，頭蓋内圧をモニター等から判断されるが，肝性脳症Ⅳ度以上で非可逆性の脳障害をきたしたと考えられる場合も適応外である．また肝移植後は，免疫抑制剤やステロイドが投与されることから感染症の合併は禁忌である．細菌感染症では，明らかな肺炎や敗血症性ショックの状態は移植禁忌である．それ以外の細菌感染症の場合は抗生剤等を投与し適応の検討を続行する．真菌感染症では，アスペルギルス感染症は肝移植の禁忌である[15]．

移植適応のある患者あるいは移植を考慮する患者が現れた場合は，移植施設と綿密な連絡をとり，早期より集中治療を開始し，平行して適応評価と術前検査を行う．移植適応の可能性があれば，早期に患者を移植施設に搬送し，内科的治療を続行するとともに適応判定を行う．画像検査に加えて各種培養検査を早期に施行し，感染症の有無を判定し，感染症が有ればこれに対して，抗生剤投与を行う．予防的抗生剤投与を行う施設もあるが，当施設では予防的抗生剤投与は行っていない．肝性脳症Ⅲ度以上で鎮静剤を使用し，気管内挿管にて人工呼吸管理を行っている場合には，血漿交換後に頭蓋内圧センサーを留置し頭蓋内圧測定を行っている．

また，劇症肝炎の原因検索も平行して行っている．劇症肝炎の成因分類では，ウイルス性，自己免疫性，薬剤性，成因不明，分類不能の5つに分類されるが，例えばB型劇症肝炎であればラミブジン投与やインターフェロンの併用療法の選択や移植後の再感染予防を考慮する．また肝臓の状態や門脈等の血流動態をCT，Echoにて把握している．

これらの情報を踏まえたうえで，当施設では，移植を考慮する患者が現れた場合は，高度救命救急センターのスタッフを中心に，肝臓内科，小児科，消化器外科，小児外科，麻酔科，腎臓内科，神経内科，移植コーディネーターから構成された，劇症肝炎ワーキングを開催し，個別に迅速に検討している．

Ⅶ. 手術方法，術中管理

手術方法に関して，劇症肝移植であるからという理由で特別なことはない．欧米では主に死体肝移植が施行されているが，本邦では他の場合の肝移植と同じく，生体肝移植が選択される場合が多い．通常はレシピエントの肝臓を全摘し同所性にグラフト肝が移植される．しかしながら，劇症肝炎では急性期を乗り切れれば，肝再生の可能性があることから，補助的肝移植が施行される場合がある．補助的肝移植では，レシピエント肝が回復し，グラフト肝による機能補助が不要になった場合に，免疫抑制剤を中止できる可能性がある．APOLT(auxiliary partial orthotopic transplantation)は，レシピエント肝を部分切除または葉切除し，その場所にグラフト肝を移植する術式である．異所性補助的肝移植は，腹腔内スペースの問題と，門脈血流の分配の問題から選択されることは少ない．

また，劇症肝炎では，門脈や静脈の側副血行路が発達していないため，場合によっては，門脈・下大静脈－鎖骨下静脈の体外循環バイパスや，一時的な門脈－下大静脈シャントの作成を行う場合がある．

術中管理に関して通常の生体肝移植または脳死肝移植と大きく変わることはない．ただし，脳浮腫が疑われる場合には，血圧等循環動態の安定に努めるように努力している．

Ⅷ. 術後管理

術後管理に関しても通常の生体肝移植または脳死肝移植と大きく変わることはない．ただし感染症に関しては，重篤なものは禁忌であるが，不顕性に感染が潜んでいる場合が多く，注意を要する．また，術前に肝腎症候群から腎不全を合併している場合も多い．当施設では，無尿の場合は免疫抑制剤をステロイドとMMF(ミコフェノール酸モフェチル)で開始し，腎不全が改善してきた時点でカルシニュリン阻害剤を追加している．

Ⅸ. 当施設での経験

当施設では2001年4月から2004年3月の3年間に17件の劇症肝炎(急性型4例，亜急性型13例)を経験した．ウイルス性(B型)6例，薬剤性3例，成因不明8例であった．脳死肝移植適応ありは13例で，内9例が脳死登録を行い，1例に脳死下の肝移植を施行した．この1例を除く移植適応ありの12例中6例に生体移植ドナーが現れ，生体肝移植を施行した．脳死肝移植を含む劇症肝炎の肝移植症例7例中5例が周術期を乗り越えて生存している(生存率71.4%)．一方，脳死肝移植適応ありとされたが，生体移植ドナー候補がなかった6例中4例が死亡されている(生存率33.3%)．

Ⅹ. 国内および海外の現状

日本肝移植研究会の肝移植症例登録報告[16]によると，2002年末までにわが国での総肝移植数は2,249例であり，ドナー別では，死体肝移植が23例(脳死肝移植21例，心停止肝移植2例)，生体肝移植が2,226例であった．急性肝不全に対して，生体肝移植は266例施行されているが，死体肝移植はわずかに2例施行されたのみである．劇症肝炎の生存率はB型肝炎では3年生存率78.2%，薬剤性では85.7%と成因により若干の差があるが，劇症肝炎全体での1年生存率は72%，3年生存率は71%，5年生存率は68.5%と周術期を乗り越えて生存できれば以後の成績は良好である．一方，欧米では，2003年の米国のUNOS(United Network for Organ Sharing)(OPTN/SRTR)の統計では1993年から2002年までに計3,364例の劇症肝炎に死体肝移植が施行され，1年生存率83.9%，3年生存率75.7%，5年生存率69.4%と報告されている．またヨーロッパのELTR(European Liver Transplant Registry)の統計では1988年から2000

年までに計2,908例の劇症肝炎に肝移植が施行され，1年生存率70%，3年生存率62%，5年生存率59%であった．これらの成績は日本の生体肝移植の成績とほぼ同じである．内科的治療による救命率が，急性型で57%，亜急性型23%であることから考えると，まだまだ限られた状況のなかながら移植医療は標準治療の一選択肢となってきたと考えられる．

XI. ドナーの問題

患者家族にとってみれば，家族の一人が，劇症肝炎により，突然集中治療室に収容されるという緊急な状況下で，短時間の間に治療法の選択を迫られる状況になる．そこで内科的治療が困難であること，肝移植という治療選択があること，脳死肝移植は登録しても現実には可能性が低いこと，生体肝移植にはドナーが必要であることを，切迫した状況下で知らされることになる．日本肝移植研究会の生体肝提供(ドナー)手術に関する指針では，強要のない，自発的意思に基づく提供が明示されている．このことは，緊急性の高い劇症肝炎に対する生体肝移植であっても同じである．限られた時間の中ではあるが，十分なインフォームドコンセントが必要である．当施設でも複数回のインフォームドコンセントを必ず行い，精神科医師による面談も実施している．

おわりに

2004年1月に生体部分肝移植の保険適用疾患が拡大され，劇症肝炎(ウィルス性，自己免疫性，薬剤性，成因不明を含む)も保険適用となった．これまで多額の医療費を患者に負担させてきた現状が改善され，門戸が広げられるとともに，移植医療が内科的治療の困難な劇症肝炎の標準治療となることが期待される．

文　献

1) Robert L Carithers：Liver Transplantation. Liver Transplantation 6：122-135, 2000.
2) 劇症肝炎の診断基準. A型肝炎, 劇症肝炎：第12回犬山シンポジウム. 中外医薬社　110-230, 1982.
3) 藤原研司，持田　智，松井　淳：劇症肝炎，遅発性肝不全(LOHF：late onset hepatic failure)の全国集計(2000年)．厚生労働省疾患対策研究事業「難治性の肝疾患に関する研究」班．平成13年度調査研究報告書，2002.
4) 松下　寛：昭和47年度劇症肝炎全国疫学調査成績補遺．厚生省特定疾患「難治性の肝炎，胆汁うっ滞」調査研究班．昭和50年度調査研究報告書　23-28, 1975.
5) 武藤泰敏：劇症肝炎全国集計：平成元年度．厚生省特定疾患「難治性の肝炎」調査研究班．平成2年度調査研究報告書　85-91, 1991.
6) 森　　満：難治性肝疾患の全国疫学調査成績，厚生省特定疾患に関する疫学調査班．平成8年度研究業績集　23-27, 1997.
7) 持田　智，藤原研司：わが国における劇症肝炎，LOHFの実態．日本消化器病学会雑誌　99：895-904, 2002.
8) 藤原研司，持田　智，松井　淳：劇症肝炎，遅発性肝不全(LOHF：late onset hepatic failure)の全国集計(1998年)．厚生科学研究費特定疾患対策研究事業「難治性の肝疾患に関する研究班」．平成11年度調査研究報告書　59-62, 2000.
9) 藤原研司，持田　智，松井　淳：劇症肝炎，遅発性肝不全(LOHF：late onset hepatic failure)の全国集計(1999年)．厚生科学研究費特定疾患対策研究事業「難治性の肝疾患に関する研究班」．平成11年度調査研究報告書　24-31, 2001.
10) 藤原研司，持田　智，松井　淳：劇症肝炎および遅発性肝不全(LOHF：late onset hepatic failure)の全国集計(2002年)．厚生労働省疾患対策研究事業「難治性の肝疾患に関する研究」班．平成15年度調査研究報告書　85-96, 2004.
11) 武藤泰敏，杉原潤一，内藤智雄：わが国の急性肝不全の肝移植適応基準．モダンフィジシャン19：1227-1230, 1999.
12) 杉原潤一，内藤智雄，石木佳英，ほか：わが国における劇症肝炎の予後予測と肝移植の適応に関する多施設研究．肝臓　42：543-557, 2001.

13) 池上俊彦，川崎誠治：生体肝移植の現況と展開　適応　劇症肝炎患者への肝移植．日本外科学会雑誌　103：401-407　2002.
14) 猪股裕紀洋，阿曽沼克弘：劇症肝不全の治療．今日の移植　16：135-143，2003.
15) 橋倉泰彦，川崎誠治：劇症肝炎・LOHF の現況　肝移植．日本消化器病学会雑誌　99：913-916，2002.
16) 日本肝移植研究会：肝移植症例登録報告．移植　38：401-408，2003.
17) 石崎陽一，川崎誠治：診療の実際と最新のトピックス　急性肝不全に対する肝移植．救急・集中治療　16：947-955，2004.

Low Incidence of Acute Rejection after Living-Donor Liver Transplantation: Immunologic Analyses by Mixed Lymphocyte Reaction using a Carboxyfluorescein Diacetate Succinimidyl Ester Labeling Technique

Yuka Tanaka, Hideki Ohdan, Takashi Onoe, Hiroshi Mitsuta, Hirotaka Tashiro, Toshiyuki Itamoto, and Toshimasa Asahara

Background. To monitor antidonor alloreactivity for accurate diagnosis of acute rejection after living-donor liver transplantation (LDLT), we used a mixed lymphocyte reaction (MLR) assay using an intracellular fluorescent dye carboxyfluorescein diacetate succimidyl ester (CFSE)-labeling technique (CFSE-MLR) in 29 consecutive patients who underwent adult-to-adult LDLT.

Methods. For patients who developed moderate or severe disorders in liver function, CFSE-MLR was performed together with needle biopsy of the liver allografts immediately after liver dysfunction had occurred. CFSE-labeled peripheral blood mononuclear cells (PBMC) from recipients and irradiated autologous, donor, or third-party PBMC were cultured, and then proliferation and CD25 expression in each of the $CD4^+$ and $CD8^+$ T cell subsets were analyzed by flow cytometry.

Results. Twelve (41.4%) of the 29 patients developed moderate or severe disorders in liver function within 6 months after LDLT. Eight of the 12 patients (overall incidence of 27.6%) suffering from liver function disorder were diagnosed on the basis of liver biopsy results as having mild or moderate acute rejection. However, only 4 of the 12 patients (overall incidence of 13.8%) showed remarkable proliferation of $CD8^+$ T cells in association with CD25 expression on antidonor CFSE-MLR. The other eight patients were eventually diagnosed as having recurrence of original hepatitis, drug-induced hepatotoxicity, or congestion of the anterior segment of the liver allograft by further extensive examinations or in retrospect.

Conclusions. The results of CFSE-MLR assays, which could be used for rigorously monitoring rejection, provided evidence of low incidence of acute rejection after LDLT.

Keywords: Living-donor liver transplantation, Flow cytometry, Mixed lymphocyte reaction, Acute rejection, Immune monitoring.

(*Transplantation* 2005;79: 1262–1267)

The incidence of acute cellular rejection (ACR) after liver transplantation has been reported to be approximately 30% to 60% (*1–3*). Such a wide range might be caused by the difficulty in differential diagnosis from rejection, recurrence of original disease (such as viral hepatitis), and drug-induced hepatotoxicity even by pathologic examinations (*4*). More accurate diagnosis of ACR would reduce risks of morbidity and mortality caused by inappropriate immunosuppressive therapy. To practice a necessary and sufficient immunosuppressive therapy in clinical transplantation, the development of a reliable assay for monitoring immune response is needed. The mixed lymphocyte reaction (MLR) is a widely used method for evaluating immune response to alloantigens in both experimental and clinical transplantation. The MLR assay was initially used to determine the proliferation of host (responder) T cells in response to antigens expressed on leukocytes obtained from the donor (*5, 6*). Later, it was shown that host cytotoxic T cells against antigens of the donor could be generated in MLR (*7*). In addition to constituting the majority of the proliferating cells in allogeneic MLR, the $CD4^+$ T-helper cells secreted cytokines that enabled the killer T cells to undergo functional maturation to possess killer activity (i.e., collaboration between $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells). However, traditional MLR using tritiated thymidine incorporation for quantifying cell division does not enable phenotypic or functional analysis of proliferating cells in such heterogeneous MLR. Flow cytometric (FCM) analysis of lymphocyte division by serial halving of the fluorescence intensity of the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) has recently been used instead of the tritiated thymidine method in the MLR (*8–10*). CFSE stably stains intracellular proteins

Selected as one of the top abstracts for presentation at the XX International Congress of The Transplantation Society (ICTS) held in Vienna, September 5–10, 2004; the invited manuscript was peer-reviewed and accepted for publication in the special issue dedicated to the XX Congress.

This work was in part supported by the COE21 Program and Grant-in-Aid for Scientific Research (B)(16390364) from the Japan Society for the Promotion of Science.

Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Hiroshima, Japan.

Address correspondence to: Hideki Ohdan, Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan.

E-mail: hohdan@hiroshima-u.ac.jp.

Received 5 October 2004. Accepted 31 January 2005.

Copyright © 2005 by Lippincott Williams & Wilkins

ISSN 0041-1337/05/7909-1262

DOI: 10.1097/01.TP.0000161667.99145.20

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

without toxicity, and the fluorescence of each stained cell segregates equally to daughter cells upon cell division, resulting in sequential halving of cellular fluorescence intensity with each successive generation (*11*). When analyzed by FCM, this sequential halving of fluorescence is visualized as distinct peaks or populations of cells and can be used to track cell division in populations of proliferating cells. This, then, allows phenotypic analysis of proliferating cells in addition to determining the number of cells produced in each generation by multicolor FCM analysis. To monitor antidonor alloreactivity for more accurate diagnosis, we have used an MLR assay using intracellular a CFSE-labeling technique (hereafter referred to as CFSE-MLR assay) in patients who underwent adult-to-adult living-donor liver transplantation (LDLT).

PATIENTS AND METHODS

Patient Population

Twenty-nine consecutive patients who underwent adult-to-adult LDLT at Hiroshima University Hospital were enrolled in this study. The 29 patients included 17 males and 12 females, ranging in age from 28 to 68 (mean±SD 52.3±7.2) years. Original diseases of the patients are shown in Table 1. The graft donors were 18 children, 4 siblings, 4 spouses, 2 parents, and 1 other relative, with ages ranging from 18 to 61 (mean 36.3±6.0) years.

Immunosuppressive Protocol

The basic immunosuppressive regimen after LDLT consisted of tacrolimus/cyclosporine and methylprednisolone, with doses gradually being tapered off. In patients with hepatitis B or C virus (HCV), the dose of methylprednisolone was rapidly tapered off, and administration was stopped within 1 month after LDLT, which would be beneficial for preventing enhanced viral replication. Instead, basiliximab was usually administered (20 mg on days 0 and 4 after LDLT) to those patients. When patients developed significant disorders in liver function as determined by laboratory tests after LDLT, CFSE-MLR was performed together with needle biopsy of the liver allografts immediately after liver dysfunction had occurred. Unless graft liver dysfunction was progressive, immunosuppressive treatment usually was not intensified. However, some patients in whom liver function

TABLE 1. Patient characteristics and compatibility

Patient no.	Age at LTx (years)	Sex	Original diagnosis	Viral hepatitis	Donor	HLA mismatches, A-B-C-DR	Liver allograft dysfunction
1	63	M	Liver cirrhosis with HCC	HCV	Offspring	0-1-0-1	−
2	50	M	Liver cirrhosis with HCC	HCV	Spouse	1-1-1-1	−
3	66	F	Liver cirrhosis with HCC	HCV	Offspring	1-1-0-2	−
4	62	M	Liver cirrhosis with HCC	HBV	Offspring	1-1-0-1	−
5	40	M	Fluminant hepatitis	—	Sibling	0-1-0-0	−
6	48	F	Autoimmune hepatitis	—	Sibling	2-2-1-1	+
7	52	M	Liver cirrhosis	HCV	Offspring	0-1-0-1	−
8	57	M	Liver cirrhosis with HCC	HBV	Offspring	1-1-0-1	+
9	58	M	Liver cirrhosis (Alcoholic)	—	Offspring	1-1-1-0	+
10	46	F	Liver cirrhosis (Alcoholic)	—	Spouse	2-1-0-2	−
11	66	F	Liver cirrhosis with HCC	HCV	Offspring	1-0-0-1	−
12	56	M	Fluminant hepatitis	HBV	Offspring	1-1-0-1	−
13	59	F	Liver cirrhosis	HCV	Offspring	1-1-0-1	+
14	56	F	Liver cirrhosis with HCC	HCV	Offspring	1-1-1-1	−
15	49	M	Liver cirrhosis with HCC	HCV	Offspring	0-1-1-1	+
16	60	M	Liver cirrhosis with HCC	HCV	Offspring	1-1-0-ND	−
17	54	M	Liver cirrhosis with HCC	HBV	Offspring	1-1-1-ND	−
18	55	F	Liver cirrhosis with HCC	HCV	Sibling	0-0-0-0	−
19	49	M	Liver cirrhosis with HCC	HBV	Offspring	0-1-0-2	−
20	28	M	Liver cirrhosis	HCV	Parent	1-0-0-ND	−
21	47	M	Liver cirrhosis with HCC	HCV	Offspring	1-1-1-1	+
22	51	F	Secondary biliary chirosis	—	Other relative	0-0-0-0	+
23	43	M	Liver cirrhosis with HCC	HBV	Spouse	1-1-1-2	+
24	28	M	Insulinoma (Liver metastasis)	—	Parent	0-0-0-1	+
25	57	F	Liver cirrhosis	HCV	Spouse	2-1-1-1	+
26	58	M	Liver cirrhosis	HBV	Offspring	1-1-1-1	−
27	44	F	Autoimmune hepatitis	—	Sibling	0-0-0-1	+
28	46	F	Liver cirrhosis with HCC	HBV	Offspring	1-1-0-1	−
29	68	F	Liver cirrhosis with HCC	HCV	Offspring	1-1-0-1	+

LTx, liver transplantation; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HBV, hepatitis B virus; ND, not done; HLA, human leukocyte antigen.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

progressively worsened, received low-dose steroid-pulse therapy (125–250 mg/day of methylprednisolone for 2–3 days). Patients who were eventually diagnosed as having acute rejection by liver-allograft biopsy and CFSE-MLR assay received additional steroid-pulse or OKT 3 therapy if necessary. However, when antidonor MLR revealed a hypo-response, the patient did not receive further antirejection therapy.

CFSE Labeling

Peripheral blood mononuclear cells (PBMC, 1×10^7 cells/mL) were resuspended in phosphate-buffered saline (PBS). 5-(and 6)-CFSE (Molecular Probes, Inc., Eugene, OR) was added to make a final concentration of 5 μM, and the cells were gently mixed and incubated for 15 minutes at 37 °C in a CO_2 incubator protected from light. Labeling of cells was stopped by adding cold PBS with 2% fetal bovine serum (Sanko, Tokyo, Japan), and the cells were then washed and resuspended in MLR medium, that is, RPMI culture medium containing 15% controlled process serum replacement-type 3 (Sigma, St. Louis, MO), 50 μM 2-mercaptoethanol (Katayama, Osaka, Japan), 1% HEPES buffer (Gibco, NY, NY), and 100 IU/mL penicillin-100 μg/mL streptomycin (Gibco, NY, NY).

MLR Assay

The PBMC prepared from recipients (autologous control), donors, and healthy volunteers (third-party control) as stimulator cells were irradiated with 30 Gy, and those as responder cells from recipients were labeled with CFSE, as described above. Both the stimulator and responder cells in the MLR medium were adjusted to 2×10^6 cells/mL of medium and cocultured in a total volume of 2 mL of medium in 24-well flat-bottom plates (BD Labware, Franklin Lakes, NJ) at 37°C in a 5% CO_2 incubator in the dark for 5 days. After MLR culture, nonadherent cells were harvested and stained with either phycoerythrin-conjugated CD4 or CD8 monoclonal antibodies (mAbs; BD Farmingen, San Diego, CA) together with allophycocyanin-conjugated CD25 mAb (BD Farmingen). Four-color FCM was performed on a FACSCalibur dual-laser cytometer (Becton Dickinson, Mountain View, CA) using standard Cell Quest acquisition/analysis, and fluorescence compensation was achieved using an appropriate single fluorochrome-labeled sample. Dead cells were excluded from the analysis by light-scatter or propidium iodide.

Quantifying Proliferation of $CD4^+$ and $CD8^+$ T Cells

Precursor frequency (PF), proliferation index (PI), and stimulation index (SI) were quantitatively estimated using a method described previously (*10, 12*). The CFSE fluorescence intensity of the peak of cell division, which was divided once, shows a half value of CFSE-fluorescence intensity of the peak of nonreactive cell division. Divisions of reactive cells, which were identified and determined by their CFSE intensities, were labeled from 0 to n as dividing time. A single cell dividing n times will generate 2^n daughter cells. With use of this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division and from proliferation events and PF in $CD4^+$ and $CD8^+$ T-cell subsets. With use of these values, proliferation events and PI were calculated. SI was calculated by dividing PI of allogeneic combinations by those of self-control.

RESULTS

Clinical Characteristics

The target blood levels of calcineurin inhibitors were achieved in all 29 recipients in this series (i.e., trough whole-blood levels of tacrolimus were maintained between 8 and 15 ng/mL in the first few postoperative weeks and thereafter between 5 and 10 ng/mL, and those of cyclosporine were maintained between 100 and 200 ng/mL in the first few postoperative weeks and thereafter between 100 and 150 ng/mL). Twelve (41.4%) of the 29 patients developed significant disorders in liver function (levels of serum bilirubin, aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were routinely measured as indexes of liver function) within 6 months after LDLT (Table 2). In 6 of those 12 patients, immunosuppressive treatment was not intensified because liver dysfunction was not progressive. However, the other six patients (including 4 patients who were eventually diagnosed by CFSE-MLR as having ACR) in whom liver function progressively worsened received low-dose steroid-pulse therapy (125–250 mg/day of methylprednisolone for 2–3 days) after CFSE-MLR, and needle biopsy had been performed but before their results had been obtained. Eight of the 12 patients suffering from liver function disorder were diagnosed as having mild or moderate ACR by liver-allograft biopsy (overall incidence of 27.6%). The other four patients in whom ACR was ruled out by results of liver biopsy were retrospectively diagnosed as having either drug-induced hepatotoxicity (in 2 patients) or congestion of the anterior segment of the liver allograft (in 2 patients). In contrast, only 4 of the 12 patients suffering from liver-function disorder showed remarkable proliferation of $CD8^+$ T cells in association with CD25 expression on anti-donor CFSE-MLR (overall incidence of 13.8%). In those four patients, antirejection therapy consisting of steroid pulse (250 mg/day for 3–5 days) with OKT 3 therapy (in 2 patients) or without OKT3 therapy (in 2 patients) resulted in remarkable improvement. Four patients who were diagnosed as having ACR but did not show significant antidonor responses of CD8 T cells in the CFSE-MLR were eventually diagnosed as having recurrence of original hepatitis (i.e., HCV in 2 patients and autoimmune hepatitis [AIH] in 1 patient) or drug-induced hepatotoxicity (in 1 patient) by results of further extensive examinations (i.e., detection of elevation of HCV RNA levels or autoantibodies/globulins in peripheral blood). In the patients diagnosed as having HCV recurrence, immunosuppressive treatment was not intensified. In contrast, in the patients diagnosed as having AIH recurrence, mycophenolate mofetil was administered (750–1,500 mg/day) together with tacrolimus and methylprednisolone.

Histology

Discrepancy between liver-biopsy and MLR results is likely to be caused by the difficulty of differential diagnosis between ACR and recurrence of original hepatitis (HCV and AIH) (*4*). In general, histologic characteristics of ACR are portal inflammatory infiltrates and nonsuppurative cholangitis with or without endotheliitis (Fig. 1, A and B). In con-

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

TABLE 2. Results of liver allograft biopsy and CFSE-MLR

Patient no.	Timing of biopsy and MLR (postLTx days)	Histopathologic diagnosis	CFSE-MLR stimulation index				$CD25^+$ cells among proliferating $CD8^+$ cells (%)	
			CD4		CD8			
			Donor	Third	Donor	Third	Donor	Third
6	12	Acute rejection (mild)	1.4	2.0	1.6	2.4	41.7	69.1
8	170	Acute rejection (mild)	0.4	0.4	1.1	0.9	19.9	17.1
9	68	Focal necrosis and bile stasis	0.6	2.6	0.3	0.4	ND	ND
13	59	Acute rejection (mild)	1.2	2.4	3.5[a]	1.4	69.7	30.1
15	29	Acute rejection (mild)	0.7	1.5	1.2	1.3	27.5	20.2
21	58	Acute rejection (mild)	0.4	1.1	0.9	1.3	9.0	43.6
22	26	Bile stasis	1.8	1.8	1.0	3.8	33.3	85.5
23	15	Acute rejection (mild)	14.5	5.2	68.3[a]	14.7	76.6	82.8
24	21	Centilobular hepatocellular degeneration	4.4	4.8	1.5	4.0	27.0	64.6
25	14	Acute rejection (mild)	3.3	1.5	3.6[a]	1.2	67.0	7.6
27	21	Acute rejection (moderate)	5.1	12.8	16.3[a]	9.3	80.8	82.3
29	30	Focal necrosis and bile stasis	4.5	2.5	2.4	2.1	1.4	5.7

[a] Data showing significant proliferation of CD8+ T cells in anti-donor MLR.
ND, not done; MLR, mixed lymphocyte reaction; CFSE, carboxyfluorescein diacetate succinimidyl ester; LTx, liver transplantation.

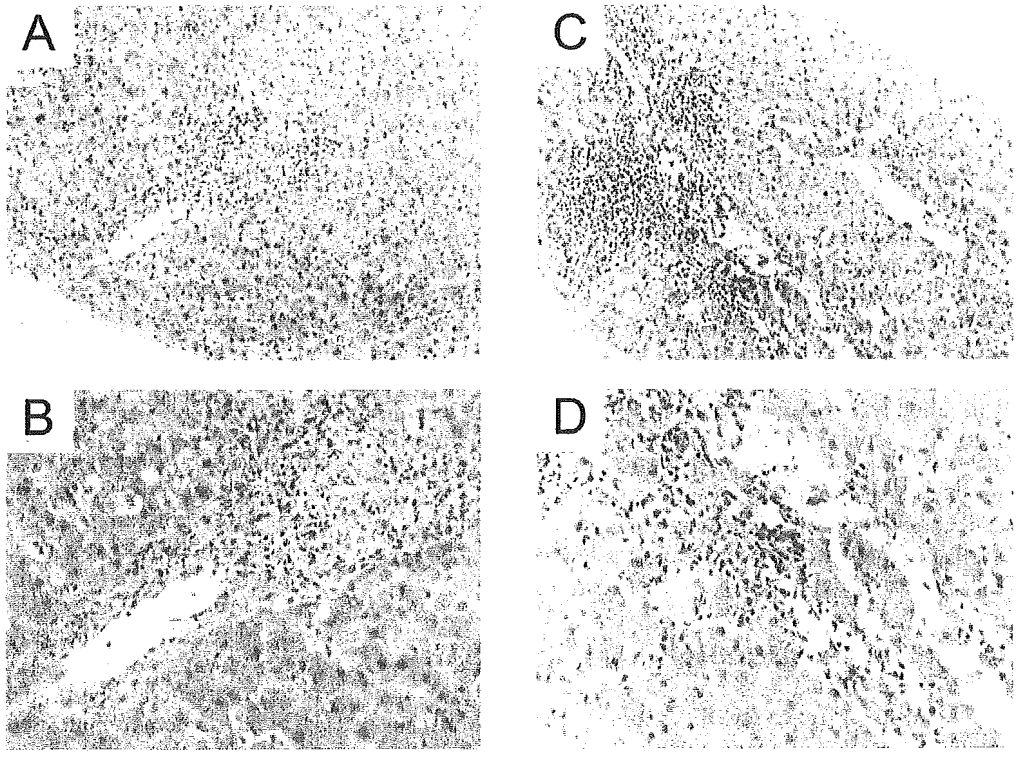


FIGURE 1. Representative histopathologic findings of liver allograft biopsies. (A) (magnification, ×100) and (B) (×200): portal inflammatory infiltrates and nonsuppurative cholangitis with endotheliitis were observed, leading to the histologic diagnosis of acute rejection (patient 23). (C) (×100) and (D) (×200): mild periportal hepatitis with lymphoid aggregates, the most common biopsy presentation of recurrent hepatitis C virus (HCV), was observed (patient 21). Because lymphoid cholangitis and endotheliitis were also found, recurrent HCV was difficult to distinguish from acute rejection. This patient was eventually diagnosed as having HCV recurrence.

trast, mild periportal hepatitis with lymphoid aggregates, with or without fatty change, is the most common biopsy presentation of recurrent HCV. It has been well noticed that lymphoid cholangitis and endotheliitis also are found, and in these instances, recurrent HCV is difficult to distinguish from acute rejection (Fig. 1, C and D). This was true of the two patients in this series who were diagnosed as having ACR by liver biopsy but were eventually diagnosed as having HCV recurrence. Recurrence of AIH is usually defined by the presence of autoantibodies and elevated globulins in association with periportal hepatitis in the absence of viral infection or rejection. The presence of periportal hepatitis with lymphoplasmacytic infiltrates, plasma cells, piecemeal necrosis, and bridging fibrosis in liver-biopsy samples is consistent with recurrent disease of AIH. However, the features of histopathology at the early phase of AIH recurrence were similar to

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

those for ACR in this series, making it difficult to distinguish between them.

Immune Monitoring by CFSE-MLR Assay

A representative FCM profile in a patient showing hyper-response in antidonor MLR is shown in Figure 2A. When compared with anti–third-party MLR, higher levels of CD4 and CD8 T-cell proliferation were observed. A comparable or even higher level of CD25 expression on the proliferating CD8 T cells in antidonor MLR suggested cytotoxicity activity against donor cells in this patient. A representative FCM profile in a patient showing hypo-response in antidonor MLR is shown in Figure 2B. When compared with anti–third-party MLR, limited levels of CD4 and CD8 T-cell proliferation were observed. Absence of CD25 expression on the proliferating CD8 T cells in antidonor MLR but the presence of that in anti–third-party MLR suggested a lack of cytotoxicity against donor cells in this patient (we have confirmed that only $CD25^+$ proliferating $CD8^+$ T cells have cytotoxic activity against donor cells in our preliminary studies). The SIs in each alloreactive $CD4^+$ and $CD8^+$ T cells in response to antidonor and anti–third-party MLR are shown in Table 2. CFSE-MLR could be a useful tool for precise diagnosis even when differential diagnosis between rejection and recurrence of viral hepatitis is difficult by pathologic examinations. The higher SI in both CD4 and CD8 T cells in antidonor MLR than those in anti–third-party MLR reflects strong antidonor reactivity, confirming the accuracy of diagnosis of ACR.

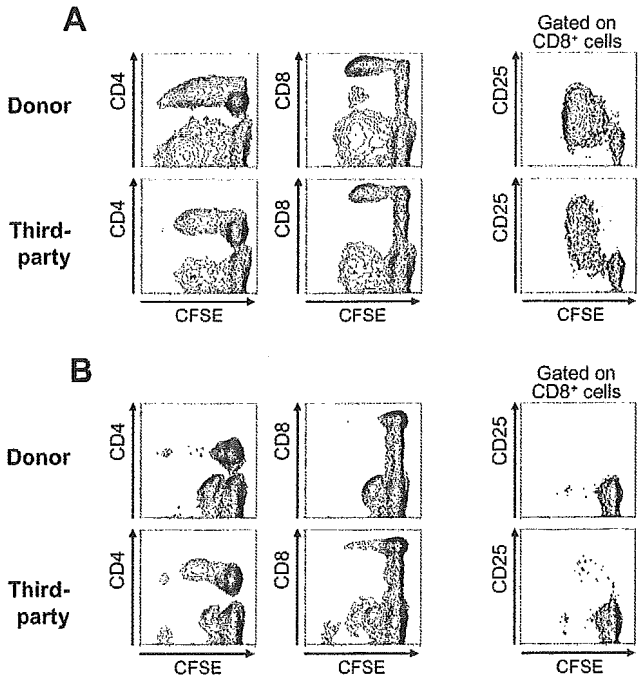


FIGURE 2. (A) Flow cytometry (FCM) profiles in the patient whose histologic appearance of liver allograft biopsy is shown in Figure 1, A and B (patient 23). When compared with anti–third-party mixed lymphocyte reaction (MLR), high levels of CD4 and CD8 T-cell proliferation were observed. A comparable or even higher level of CD25 expression on early proliferating CD8 T cells in both antidonor MLRs was observed. (B) FCM profiles in the patient whose histologic appearance of liver allograft biopsy is shown in Figure 1, C and D (patient 21). When compared with anti–third-party MLR, limited levels of CD4 and CD8 T-cell proliferation were observed. CD25 expression on proliferating CD8 T cells was undetectable in the anti-donor MLR. CFSE, carboxyfluorescein diacetate succinimidyl ester.

DISCUSSION

Antidonor alloreactivity, defined as the number and phenotype of alloreactive precursors in the recipient, can be used to monitor rejection or reduction/withdrawal of immunosuppression. Monitoring such alloreactivity using PBMCs in recipients of transplants does not necessarily mirror what will occur in the allograft tissue because allografts are regulated by infiltrating lymphocytes. However, it has been reported that the frequencies of donor-reactive T cells in PBMCs are closely linked with those in lymphocytes infiltrating allografts (*13*), indicating the validity of evaluation of T-cell responses to allogeneic stimulation using PBMCs for monitoring alloreactivity in transplant recipients. MLR using PBMCs is a widely used method for evaluating T-cell responses to allogeneic stimulation in both experimental and clinical transplantation. However, in conventional forms using tritiated thymidine incorporation, proliferative MLR bulk cultures have very little predictive value because of its low level of reproducibility in the context of transplantation (*14*). The low level of reproducibility of conventional MLR might be caused at least in part by the presence of nonviable cells (which might include unexpectedly surviving stimulator cells) that still have the ability to incorporate tritiated thymidine. By applying a CFSE-based method, the proliferation of viable $CD4^+$ and $CD8^+$ responder T cells in response to allostimulation could be separately quantified using multiparameter FCM. The lack of proliferation of both $CD4^+$ and $CD8^+$ T cells in antidonor MLR would reflect suppression of antidonor response. When remarkable proliferation was observed in $CD4^+$ T cells but not in $CD8^+$ T cells, we did not observe cytotoxic activity against donor cells in the subsequent CML assay in our preliminary studies (data not shown). In contrast, remarkable proliferation of $CD8^+$ T cells would reflect strong antidonor response. We further examined CD25 expression on the proliferating $CD8^+$ T cells by multicolor FCM. The remarkable elevation of CD25 expression on proliferating $CD8^+$ T cells might reflect their cytotoxic activity toward donor cells. In our preliminary studies, the proliferative activity levels of $CD4^+$ and $CD8^+$ T cells were generally higher in MLRs using PBMCs from the spouse than in MLRs using PBMCs from offspring or parents, even before transplantation. This may reflect the greater susceptibility to ACR in recipients of liver allografts from unrelated donors than in recipients of liver allografts from related donors. Consistent with this speculations, MLR-proven ACR occurred in 2 (50%) of the 4 patients receiving LDLT from their spouse but in only 2 (4%) of the 25 patients receiving LDLT from their offspring or parents in the present study. Although the usefulness of CFSE-MLR for the prediction of ACR remains to be elucidated, the results obtained by using this method provided evidence of low incidence of ACR after LDLT compared with previously reported results.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Possible alternative methodologies to diagnose ACR using laboratory-based immunologic modalities might include limiting dilution assay (LDA) and enzyme-linked immunospot (ELISPOT). LDAs provide precise quantification of immunity to a given stimulus and allow the estimation of frequencies of antigen-specific cells participating in an immune response (*15*). Although LDAs have been shown to be specific and reproducible as a measurement of alloreactivity (*16*), conflicting data regarding the usefulness of measurement of cytotoxic T-cell precursors for diagnosis/prediction of rejection in the context of solid-organ transplantation have been reported (*17, 18*). The ELISPOT assay is based on the detection of a cytokine produced by single cells after stimulation with mitogens or antigens (*19*). It has been used to identify the presence of donor-specific T cells in patients before surgery (*20*). However, data indicating the usefulness of this method for diagnosing ACR are less abundant at present.

One of the most difficult challenges in the care of HCV-positive liver-transplant recipients is the differentiation between ACR and HCV recurrence, which can have considerable histologic overlap (*4*). Although polymerase chain reaction allows identification of HCV RNA in biopsy tissue in such difficult cases (*21*), this method could not distinguish between HCV infection alone versus HCV infection complicated by ACR. Attempts to distinguish between these at the intrahepatic gene response level have been made in two studies. The first study analyzed ACR and HCV infection versus HCV infection alone by using gene array analysis (*22*). It has been found that ACR and recurrence of HCV are associated with distinct mRNA expression pattern (i.e., ACR is most notably associated with the relative over-expression of immune activation genes such as major histocompatibility complex classes I and II, tumor necrosis factor [TNF]-α, granzyme B, and complement components). Zekry et al. (*23*) concentrated on Th1 versus Th2-like gene expression and found that ACR in the setting of HCV infection was more like ACR in non–HCV-infected patients and was associated with increase in interleukin (IL)-10 and IL-4 gene expression rather than the IL-2/interferon-γ/TNF-α response seen more in chronic HCV alone. Higher average daily steroid dose and use of OKT 3 have both been associated with more severe recurrence of HCV, presumably through enhancing viral replication or attenuating viral clearance (*24–26*). Minimizing exposure to immunosuppressants for HCV-infected liver-transplant recipients thus requires accurate distinction of recurrence of HCV from ACR. Diagnosis of ACR is based on the detection of biochemical evidence of graft dysfunction and the presence of suggestive allograft histology, including distinct lymphocytic infiltrate patterns. The presence of a modest cellular infiltrate and biochemical abnormalities, however, are not specific to ACR. In addition to the above-described methods, CFSE-MLR also has potential as a tool for diagnosing ACR in HCV-reinfected patients. Additional studies with larger sample sizes are required to confirm this possibility.

REFERENCES

1. Minamiguchi S, Sakurai T, Fujita S, et al. Living related liver transplantation: histopathologic analysis of graft dysfunction in 304 patients. *Hum Pathol* 1999; 30(12): 1479.
2. Russo FP, Bassanello M, Senzolo M, et al. Functional and morphological graft monitoring after liver transplantation. *Clin Chim Acta* 2001; 310(1): 17.
3. Sugawara Y, Makuuchi M, Kaneko J, et al. Risk factors for acute rejection in living donor liver transplantation. *Clin Transplant* 2003; 17(4): 347.
4. Demetris AJ, Eghtesad B, Marcos A, et al. Recurrent hepatitis C in liver allografts: prospective assessment of diagnostic accuracy, identification of pitfalls, and observations about pathogenesis. *Am J Surg Pathol* 2004; 28(5): 658.
5. Bain B, Vas MR, Lowenstein L. The Development of Large Immature Mononuclear Cells in Mixed Leukocyte Cultures. *Blood* 1964; 23: 108.
6. Bach F, Hirschhorn K. Lymphocyte interaction: a potential histocompatibility test in vitro. *Science* 1964; 143: 813.
7. Solliday S, Bach FH. Cytotoxicity: specificity after in vitro sensitization. *Science* 1970; 170(965): 1406.
8. Popma SH, Krasinskas AM, McLean AD, et al. Immune monitoring in xenotransplantation: the multiparameter flow cytometric mixed lymphocyte culture assay. *Cytometry* 2000; 42(5): 277.
9. Nitta Y, Nelson K, Andrews RG, et al. CFSE dye dilution mixed lymphocyte reactions quantify donor-specific alloreactive precursors in non-human primate cardiac graft rejection. *Transplant Proc* 2001; 33(1–2): 326.
10. Tanaka Y, Ohdan H, Onoe T, et al. Multiparameter flow cytometric approach for simultaneous evaluation of proliferation and cytokine-secreting activity in T cells responding to allo-stimulation. *Immunol Invest* 2004; 33(3): 309.
11. Paramore CG, Turner DA, Madison RD. Fluorescent labeling of dissociated fetal cells for tissue culture. *J Neurosci Methods* 1992; 44(1): 7.
12. Wells AD, Gudmundsdottir H, Turka LA. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J Clin Invest* 1997; 100(12): 3173.
13. Orosz CG, Zinn NE, Sirinek LP, et al. Size of the donor-reactive CTL pool determined by limiting dilution analysis in sponge matrix allografts. *Transplantation* 1986; 41(3): 400.
14. Segall M, Noreen H, Edwins L, et al. Lack of correlation of MLC reactivity with acute graft-versus-host disease and mortality in unrelated donor bone marrow transplantation. *Hum Immunol* 1996; 49(1): 49.
15. Lefkovits I. Induction of antibody-forming cell clones in microcultures. *Eur J Immunol* 1972; 2(4): 360.
16. Sharrock CE, Kaminski E, Man S. Limiting dilution analysis of human T cells: a useful clinical tool. *Immunol Today* 1990; 11(8): 281.
17. Hu H, Robertus M, de Jonge N, et al. Reduction of donor-specific cytotoxic T lymphocyte precursors in peripheral blood of allografted heart recipients. *Transplantation* 1994; 58(11): 1263.
18. Mestre M, Massip E, Bas J, et al. Longitudinal study of the frequency of cytotoxic T cell precursors in kidney allograft recipients. *Clin Exp Immunol* 1996; 104(1): 108.
19. Miyahira Y, Murata K, Rodriguez D, et al. Quantification of antigen specific CD8+ T cells using an ELISPOT assay. *J Immunol Methods* 1995; 181(1): 45.
20. Heeger PS, Greenspan NS, Kuhlenschmidt S, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. *J Immunol* 1999; 163(4): 2267.
21. Gottschlich MJ, Aardema KL, Burd EM, et al. The use of hepatitis C viral RNA levels in liver tissue to distinguish rejection from recurrent hepatitis C. *Liver Transpl* 2001; 7(5): 436.
22. Sreekumar R, Rasmussen DL, Wiesner RH, et al. Differential allograft gene expression in acute cellular rejection and recurrence of hepatitis C after liver transplantation. *Liver Transpl* 2002; 8(9): 814.
23. Zekry A, Bishop GA, Bowen DG, et al. Intrahepatic cytokine profiles associated with posttransplantation hepatitis C virus-related liver injury. *Liver Transpl* 2002; 8(3): 292.
24. Charlton M, Seaberg E. Impact of immunosuppression and acute rejection on recurrence of hepatitis C: results of the National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. *Liver Transpl Surg* 1999; 5(4 Suppl 1): S107.
25. Rosen HR, Shackleton CR, Higa L, et al. Use of OKT3 is associated with early and severe recurrence of hepatitis C after liver transplantation. *Am J Gastroenterol* 1997; 92(9): 1453.
26. Sheiner PA, Schwartz ME, Mor E, et al. Severe or multiple rejection episodes are associated with early recurrence of hepatitis C after orthotopic liver transplantation. *Hepatology* 1995; 21(1): 30.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Difference in Cytotoxicity Against Hepatocellular Carcinoma Between Liver and Periphery Natural Killer Cells in Humans

Kohei Ishiyama,[1] Hideki Ohdan,[1] Masahiro Ohira,[1] Hiroshi Mitsuta,[1] Koji Arihiro,[2] and Toshimasa Asahara[1]

In rodents, liver natural killer (NK) cells have been shown to mediate higher cytotoxic activity against tumor cells than do peripheral blood (PB) NK cells. However, such differences between liver and PB NK cells have not been extensively investigated in humans. The phenotypical and functional properties of NK cells extracted from liver perfusates at the time of living donor liver transplantation were investigated. The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a critical molecule for NK cell–mediated anti-tumor cell killing, was not expressed by freshly isolated PB NK cells or by liver NK cells. Stimulation with interleukin (IL)-2, significantly up-regulated the expression of TRAIL on liver NK cells, but this effect was barely observed on PB NK cells. Donor liver NK cells showed the most vigorous cytotoxicity against HepG2, a hepatocellular carcinoma (HCC) cell line, after IL-2 stimulation (90.5% ± 2.2% at E: T = 10:1), compared with donor and recipient PB NK cells and recipient liver NK cells (64.8% ± 8.2%, 56.1% ± 8.9%, and 34.6% ± 7.5%, respectively). IL-2 stimulation resulted in an increased expression of killing inhibitory receptors on liver NK cells in parallel with TRAIL expression. Consistently, the cytotoxicities of IL-2–stimulated donor liver NK cells against self and recipient lymphoblasts were negligible. In conclusion, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver graft perfusate could mount an anti-tumor response without causing toxicity against 1-haplotype identical recipient intact tissues. These findings present a concept to prevent recurrence of HCC after liver transplantation. (HEPATOLOGY 2006;43:362-372.)

Natural killer (NK) cells are thought to provide a first line of defense against invading infectious microbes and neoplastic cells by exerting an effector function without the necessity for priming.[1,2] Given the efficacy of NK cells in selectively killing abnormal cells, a variety of approaches have been taken to try and selectively augment NK cell response to tumors.[3,4] Several therapeutic cytokines primarily act via NK cells [such as interleukin (IL)-2, IL-12, IL-15, and interferons (IFNs)] and many studies have shown that activation of NK cell differentiation and function leads to a more efficient elimination of tumor growth.[5-9] Despite these promising advances, the systemic administration of cytokines such as IL-2, that nonspecifically activate a broad range of different immune cell types, is associated with significant toxicity.[5,10] The adoptive transfer of NK cells further demonstrates the ability of NK cells to mount a therapeutic anti-tumor response and suggests that NK cells can be used in controlling human malignancy.[11,12] In these studies, autologous or even haploidentical lymphokine-activated killer cells obtained from peripheral blood mononuclear cells (PBMCs) have been administered to patients, although their comprehensive role in the treatment of selected malignancies remains to be elucidated.

NK cells are quite abundant in the liver of mice, in contrast to a relatively small percentage in the periph-

Abbreviations: NK; natural killer; IL, interleukin; IFN, interferon; PBMC, peripheral blood mononuclear cell; PB, peripheral blood; LDLT; living donor liver transplantation; HCC; hepatocellular carcinoma; TNF, tumor necrosis factor; TRAIL; TNF-related apoptosis-inducing ligand; DR, death receptor; LMNC; liver mononuclear cell; FCM, flow cytometric; MAb, monoclonal antibody; HLA, human leukocyte antigen; E:T, effector:target; MC, mononuclear cell; KIR, killer cell immunoglobulin-like receptors; MHC, major histocompatibility complex.

From the [1]Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences; and the [2]Department of Anatomical Pathology, Hiroshima University, Hiroshima, Japan.

Received August 12, 2005; accepted November 18, 2005.

Supported by a Grant-in-Aid for Scientific Research (B) (2) (16390364), Japan Society for the Promotion of Science, Grant-in-Aid for Health Labour Sciences Research Grant, and Grant-in-Aid for the Creation of Innovations through Business-Academic-Public Sector Cooperation, The Ministry of Education, Culture, Sports, Science and Technology.

Address reprint requests to: Hideki Ohdan, M.D., Ph.D., Department of Surgery, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: hohdan@hiroshima-u.ac.jp; fax: (81) 82-257-5224.

Copyright © 2006 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21035

Potential conflict of interest: Nothing to report.

Table 1 Clinical Characteristics of Living-Related Liver Transplant Donors and Corresponding Recipients With Cirrhosis

	Donor								Recipient									
Case No.	Age(y)/ Sex	Graft Liver	Graft Wt(g)	LMNC (×10⁵/ g)	HLA A	HLA B	HLA C	Relation	Age(y)/ Sex	Child Pugh	MELD	Original Disease	HCC	Liver Wt(g)	LMNC (×10⁵/ g)	HLA A	HLA B	HLA C
1	27/F	Left	262	3.7	2,26	51,54	1,–	Offspring	59/F	B	13.4	HCV	-	800	4.4	11,26	54,–	1,–
2	29/F	Left	460	7.8	2,26	56,61	1,8	Offspring	56/F	C	13.6	HCV	-	776	2.7	26,–	61,–	8,–
3	21/M	Right	550	6.4	2,24	7,35	3,7	Offspring	49/M	C	18.9	HCV	+	718	0.4	2,24	13,35	3,–
4	24/M	Right	564	1.2	24,26	62,-	3,4	Offspring	60/M	B	10.4	HCV	+	846	0.6	24,–	60,62	3,4
5	20/M	Right	896	1.9	2,33	37,46	1,3	Offspring	54/M	B	13.3	HBV	+	665	0.2	31,33	37,61	3,4
6	20/M	Right	632	3.2	24,26	52,62	3,12	Offspring	47/M	A	10.6	HCV	+	1150	3.1	24,–	52,–	12,–
7	57/F	Right	678	9.2	2,24	52,54	1,11	Spouse	43/M	C	35.2	HBV	+	810	7.0	24,–	7,52	7,12
8	59/M	Left	398	4.2	11,31	39,61	7,8	Spouse	57/F	C	18.9	HCV	-	684	9.5	24,26	35,39	3,7
9	30/M	Right	550	3.7	26,33	44,62	3,14	Offspring	58/M	C	29.4	HBV	-	820	0.4	2,26	51,62	3,15
10	49/F	Right	660	7.4	2,24	7,61	7,8	Sibling	44/F	C	18.1	AIH	-	753	1.2	2,24	7,61	7,8
11	44/F	Right	576	4.0	26,-	35,62	3,–	Spouse	48/M	C	37.5	HCV	-	410	13.4	24,–	52,–	12,–
12	29/M	Right	900	4.4	2,24	51,54	1,14	Offspring	58/M	C	16.9	HCV	+	714	4.2	24,–	54,–	1,8
13	43/M	Left	350	10.6	24,–	7,46	1,7	Sibling	46/F	C	17.6	HBV	-	425	1.4	24,–	7,46	1,7
14	18/M	Right	630	3.2	2,31	54,61	1,3	Offspring	57/F	A	3.1	HBV	+	932	8.2	24,31	54,61	1,3

Abbreviations: F; female, M; male, Graft wt; graft weight, LMNC; liver mononuclear cell, HLA; human leukocyte antigens, MELD; Model for End-Stage Liver Disease, HCV; hepatitis C virus, HBV; hepatitis B virus, AIH; autoimmune hepatitis, HCC; hepatocellular carcinoma.

eral lymphatics.[13-15] The underlying reason for this anatomically biased distribution has not been fully elucidated. In addition, liver NK cells have been shown to mediate higher cytotoxic activity against tumor cells than spleen or peripheral blood (PB) NK cells in rodents.[13-16] However, such differences between liver and PB NK cells have not been extensively investigated in human because of the limited availability of appropriate human samples.

In the current study, we have determined phenotypical and functional properties of liver NK cells extracted from donor and recipient liver perfusates in clinical living donor liver transplantation (LDLT). Donor liver NK cells showed the most vigorous cytotoxicity against a hepatocellular carcinoma (HCC) cell line after *in vitro* IL-2 stimulation, compared with donor and recipient PB NK cells and recipient liver NK cells. IL-2 stimulation led to an increased expression of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) on liver NK cells, which has been shown to be critical for NK cell–mediated anti-tumor cell killing without affecting normal cells.[17-19] In addition, we have confirmed that HCC expressed the death-inducing TRAIL receptors (TRAIL-Rs), TRAIL-R1/death receptor (DR) 4, and TRAIL-R2/DR5 that contain cytoplasmic death domains and signal apoptosis.[20,21] These findings raise a novel concept to prevent recurrence of HCC after liver transplantation, in other words, adoptive transfer of IL-2–stimulated NK cells extracted from donor liver graft into 1-haplotype identical recipients.

Patients and Methods

Patients. Fourteen patients who underwent adult-to-adult LDLT at The Hiroshima University Hospital (Hiroshima, Japan) were involved in this study. The 14 patients with hepatic cirrhosis included 8 men and 6 women, ranging in age from 43 to 60 years [mean age (in years) ± SD, 52.6 ± 5.5]. Original diseases of the patients are shown in Table 1. Nine of the graft donors were offspring, two were siblings, and three were spouses, with ages ranging from 18 to 59 years (33.6 ± 12.0).

Isolation of Liver and Peripheral Blood Lymphocytes. Donor hepatectomy and the recipient transplantation procedure were performed as described previously.[22] In brief, the right or left lobe was harvested from the donor. After hepatectomy, *ex vivo* perfusion of the liver graft was performed through the portal vein. The initial perfusate consisted of saline solution (500 mL) followed by University of Wisconsin solution (1,000 mL). For the recipient, the implantation was performed after total hepatectomy. *Ex vivo* perfusion of the removed recipient liver was also performed through the portal vein by using the same perfusates. Liver mononuclear cells (LMNCs) were obtained from those perfusate effluents from healthy donor liver grafts and recipient livers with cirrhosis as follows. The effluents were condensed by centrifuging and LMNCs were isolated by gradient centrifugation with Separate-L (Muto Pure Chemicals Co., Ltd, Tokyo, Japan). PBMCs were also isolated by gradient centrifugation with Separate-L from 40 mL heparinized peripheral

blood from donors and recipients. LMNCs and PBMCs were suspended in RPMI 1640 medium that was supplemented with 10% heat-inactivated fetal calf serum (Sanko Chemical Co., Ltd., Tokyo, Japan), 25 mmol/L HEPES Buffer (Gibco, Grand Island, NY), 50 μmol/L 2 mercaptoethanol (Katayama Chemical Co., Osaka, Japan), 50 U/mL penicillin, and 50 μg/mL streptomycin (Gibco) (from hereon we refer to this medium as 10% RPMI). Ethical approval for this study was obtained from the Ethics Committee at The Hiroshima University Hospital.

Flow Cytometric Analyses. All flow cytometric (FCM) analyses were performed on a FACS Calibur dual-laser cytometer (BD Biosciences, Mountain View, CA). For phenotyping of NK cells, LMNCs and PBMCs were stained with fluorescein isothiocyanate–conjugated anti-CD3 (BD Pharmingen, San Diego, CA), phycoerythrin-conjugated anti-CD56 (B159) (BD Pharmingen), and biotin-conjugated anti-TRAIL monoclonal antibodies (MAbs) (RIK-2) (e Bioscience, Oxford, U.K.). For analyzing inhibitory receptors on NK cells, LMNCs and PBMCs were stained with peridin chlorophyll protein–conjugated anti-CD3 (SP34-2) (BD Pharmingen), phycoerythrin-conjugated anti-CD56, biotin-conjugated anti-TRAIL and fluorescein isothiocyanate–conjugated anti-CD-158a (HP-3E4) (BD Pharmingen), anti-CD-158b (CH-L) (BD Pharmingen), or anti-CD94 MAbs (HP-3D9) (BD Pharmingen). For analyzing TRAIL receptors on the HCC cell line, HepG2 cells were stained with biotin-conjugated anti-TRAIL-R1/DR4 (DJR1), anti-TRAIL-R2/DR5 (DJR2-4), anti-TRAIL-R3/decoy receptor (DcR) 1 (DJR3), or anti-TRAIL-R4/DcR2 (DJR4-1) MAbs (all MAbs from eBioscience). All the biotinylated MAbs were visualized with allophycocyanin-streptavidin (BD Pharmingen). Dead cells were excluded from the analysis by light-scatter and propidium iodide staining.

Cell Culture. LMNCs and PBMCs were cultured with or without human recombinant IL-2 (100 U/mL) (Takeda, Tokyo, Japan) in 10% RPMI at 37°C in a 5 % CO_2 incubator. After 4 days in culture, cells were harvested for further analyses.

Cytotoxicity Assay. HepG2 cells established from HCC tissue from a hepatitis B virus– and hepatitis C virus–negative HCC patient (HLA; human leukocyte antigens: −A02,24, −B35,51 −CO,4) were purchased from The Japanese Cancer Research Resources Bank and were maintained in 10% RPMI.[23] HepG2 were labeled with 100 μCi Na2 (^{51}Cr) O4 for 60 minutes at 37°C in 5% CO_2 in 10% RPMI, washed 3 times with medium, and then subjected to the cytotoxicity assay. The labeled HepG2 cells were adjusted to 1×10^6 cells in 10 mL volumes (1×10^4/well) and were incubated in a total volume of 200 μL with effector cells in 10% RPMI in round-bottomed 96-well microtiter plates (Nunclon; Inter Med, Denmark). LMNCs or PBMCs from healthy donors or recipients with cirrhosis were used as effectors at effector-target (E:T) ratios of 2.5:1 to 40:1. When indicated, LMNCs and PBMCs were cultured *in vitro* with IL-2 for 4 days before using as effectors. As a control, the target cells were incubated either in culture medium alone to determine spontaneous release, or in a mixture of 2% Nonidet P-40 (Nacalai Tesque, Inc., Kyoto, Japan) to define the maximum ^{51}Cr release. The plates were centrifuged at 1,000 rpm for 3 minutes to pack the cell layer at the end of the reaction, after which the cell-free supernatants were carefully harvested, and its radioactivity was measured with a gamma counter. The percentage of specific ^{51}Cr release was calculated by the following formula: % cytotoxicity = [(cpm of experimental release − cpm of spontaneous release)]/[(cpm of maximum release − cpm of spontaneous release)] × 100. The spontaneous release was less than 20% of the maximum release. All assays were performed in triplicate.

Isolation of NK Cells. LMNCs and PBMCs were separated into a $CD3^-CD56^+$ NK cell fraction and a non-NK cell fraction (T cells, NKT cells, B cells, and monocytes/macrophages) by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) using the human NK cell isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. The purity of isolated fractions was assessed by FCM, and only preparations whose purities were greater than 95% were used for functional studies. Using a similar method as described, NK and non-NK cells isolated from IL-2–stimulated donor LMNCs and PBMCs were subjected to the cytotoxicity assay against HepG2 cells. When indicated, the isolated NK cells were pre-incubated for 30 minutes at 4°C with neutralizing Abs against TRAIL (purified anti-TRAIL MAbs; eBioscience) (final concentration 20 μg/mL) or with isotype-matched non-reactive Abs before the cytotoxicity assay. In addition, a similar cytotoxicity assay with those donor NK cells was performed against autologous and allogenic lymphoblasts, which had been prepared from PBMCs of donors and corresponding recipients by cultivating with 5 μg/mL PHA (Sigma, Poole, UK) for 4 days.

Immunohistochemistry. Surgically resected liver specimens were obtained from patient with HCC who had undergone potentially curative tumor resection at The Hiroshima University Hospital. All HCC tissues were pathologically confirmed. Normal liver samples obtained from patients with metastatic liver tumors were used as control. Informed consent was obtained from all

patients. To localize TRAIL receptors *in situ* in the liver, immunohistochemistry was performed on frozen tissue sections of normal liver tissues and carcinoma cases as described previously.[24] For protein detection by immunohistochemistry, 4-μm frozen sections from representative tumor tissue of at least 1 cm^2 were fixed in acetone for 15 minutes. Sections were incubated for 60 minutes with the following MAbs: biotin-conjugated anti-TRAIL-DR4, anti-TRAIL-DR5, anti-TRAIL-DcR1, and anti-TRAIL-DcR2 at appropriate concentrations in phosphate-buffered saline. Binding sites of primary antibodies were visualized using the Dako EnVision kit (Dako, Copenhagen, Denmark) according to the manufacturer's instructions. Finally, sections were faintly counterstained with Harris' hematoxylin and mounted with glycerol gelatin. Negative controls were performed by omission of the primary antibody.

Statistical Analyses. Data are presented as mean ± SEM. The statistical differences of the results were analyzed by ANOVA analysis with the Scheffe F test using the Stat View program. A P value of .05 or less was accepted as statistically significant.

Results

LMNCs Contained a Large Population of NK Cells. The liver contains significant numbers of resident mononuclear cells (MCs) in human. These cells include a large number of T cells, B cells, NK cells, and NKT cells, many of which differ phenotypically and functionally from circulating lymphocytes.[25,26] Characterization of liver NK cells requires the isolation of viable LMNCs that can be analyzed by FCM and in functional assays. The techniques used to isolate LMNCs usually involve mechanical and enzymatic dissociation of liver tissue.[25-27] However, the difficulties in getting liver samples and processing liver biopsy have been obstacles to such studies. In the current study, instead of dispersal of liver tissue, *ex vivo* perfusion of the liver through the portal vein, which was inevitably done to flush blood from the liver graft before implantation in LDLT, provided LMNCs by extraction from liver perfusates. In our preliminary experiments, the proportions of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ NKT cells in LMNCs extracted from liver perfusates (38.1% ± 4.5 % and 14.0% ± 3.0 %, respectively) were almost identical to those in LMNCs collected by method using enzymatic dissociation (33.7% ± 1.3 % and 14.8% ± 0.7 %, respectively, n = 3). These data were consistent with data in previous reports that used the enzymatic dissociation method.[25,26] Although this nondestructive method might allow contamination with circulating MCs to some extent, the possibility of enzyme-induced alteration/disruption of specific epitopes could be disregarded.

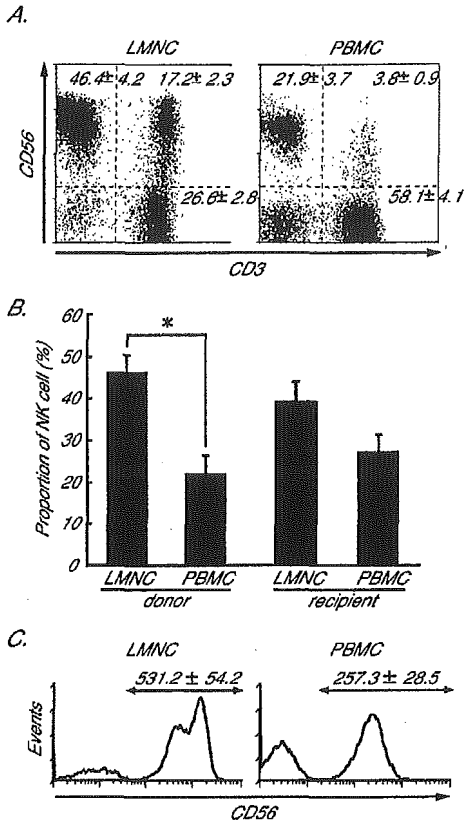


Fig. 1. Human liver mononuclear cell extracted from liver perfusates contain a large population of NK cells. (A) Flow cytometric (FCM) analysis of freshly isolated LMNCs obtained from liver perfusates and PBMCs from same donor after staining with MAbs against CD3 and CD56 were analyzed. Lymphocytes were gated by forward scatter and side scatter. FCM profiles shown are representative of 14 independent experiments. Percentages of $CD3^+CD56^-$ (T), $CD3^-CD56^+$ (NK) and $CD3^+CD56^+$ (NKT) cells are indicated at each quadrant (mean ± SEM, n = 14 each). (B) The numbers represent the means ± SEM of the $CD3^-CD56^+$ NK cell populations in total LMNCs or PBMCs obtained from 14 adult healthy donors and 14 corresponding recipients with liver cirrhosis. Statistical analyses were performed using ANOVA (*$P < .05$). (C) Expression of CD56 on electronically gated $CD3^-$ cells were analyzed by FCM. Histogram profiles shown are representative of 14 independent experiments. The data shown were obtained from donor LMNCs and PBMCs (similar results were obtained from recipient LMNCs and PBMCs). The numbers indicate the mean ± SEM of the mean fluorescence intensity (MFI) of CD56 expression on $CD3^-$ LMNCs and PBMCs cells (n = 14 each). NK, natural killer; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody; FCM, flow cytometric.

The number of LMNCs extracted from donor normal livers and recipient livers with cirrhosis were 0.5 ± 0.1 and 0.4 ± 0.1 × 10^6 cells/g, respectively, and were not statistically different (Table 1). FCM profiles of LMNCs and PBMCs from donor are shown in Fig. 1A. Proportions of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ NKT cells in LMNCs were significantly higher than in PBMCs from same donors (Fig. 1B). Such a difference in NK cell pro-

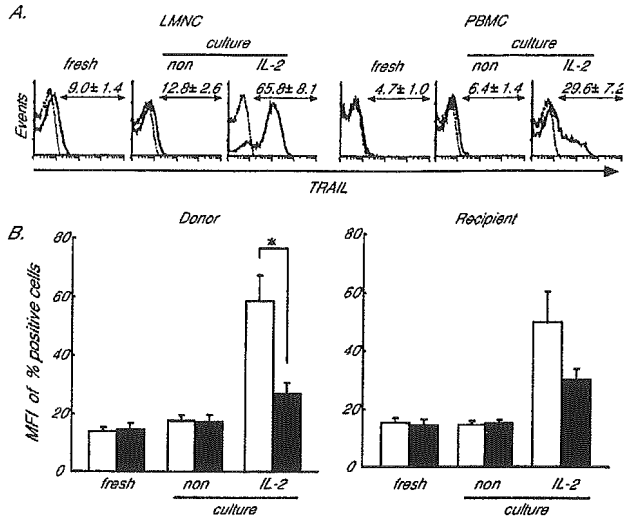


Fig. 2. Liver NK cells inductively express remarkable levels of TRAIL, but PB NK cells do not. Freshly isolated or cultivated with or without IL-2 LMNCs and PBMCs obtained from healthy donors and corresponding recipients were stained with CD3 and CD56 MAbs together with TRAIL MAb. (A) Histograms represent the log fluorescence intensities obtained on staining for TRAIL after gating on the $CD3^-CD56^+$ NK cells subsets obtained from healthy donors. Dotted lines represent negative control staining with isotype-matched MAbs. The numbers (mean ± SEM) indicate the percentages of cells in each group that were positive for TRAIL expression (n = 7 each). Histogram profiles shown are representative of 7 independent experiments. (B) The numbers indicate the mean fluorescence intensity (MFI) of cells in each group that were staining positively for TRAIL on freshly isolated, or cultivated with/without IL-2 NK cells (LMNC; open column, PBMC; closed column). The data represent mean ± SEM (n = 7). Statistical analyses were performed using ANOVA (*$P < 0.05$). NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody.

portion between LMNCs and PBMCs was not conspicuous in recipients with cirrhosis, because of a relatively reduced proportion of NK cells in LMNCs from livers with cirrhosis. In LMNCs from donors and recipients, the $CD3^-CD56^{high}$ NK cell subpopulation, which is known to produce preferentially large amounts of cytokines,[28,29] was easily detectable, whereas it was undetectable in PBMCs (Fig. 1C).

Liver NK Cells Inductively Expressed TRAIL. TRAIL is a type II transmembrane protein that belongs to the TNF family, which preferentially induces apoptotic cell death in a wide variety of tumor cells but not in most normal cells.[17-19] We and others have previously reported that a subpopulation of NK cells in adult mouse liver, unlike other tissues, constitutively express TRAIL, and these liver NK cells were partially responsible for the natural anti-tumor function against TRAIL-sensitive tumor cells.[9,16,17] As shown in Fig. 2, freshly isolated liver NK cells from normal liver and liver with cirrhosis barely expressed TRAIL, whereas freshly isolated PB NK cells completely lacked TRAIL-expression. *In vitro* stimulation with IL-2 significantly up-regulated the expression of TRAIL on liver NK cells (the expression of TRAIL on liver NK cells from donor normal livers was somewhat higher than that on liver NK cells from recipient livers with cirrhosis). Conversely, even after IL-2 stimulation, PB NK cells expressed little TRAIL. Thus, liver NK cells inductively expressed remarkable levels of TRAIL, but PB NK cells did not.

We have recently demonstrated that most murine TRAIL-expressing liver NK cells lack expression of Ly-49 inhibitory receptors, which recognize self-MHC class I.[16] To address whether the same is true in humans, we have analyzed inhibitory receptors on human liver NK cells. Inhibitory receptors on human NK cells can be subdivided into 2 groups: killer cell immunoglobulin-like receptors (KIRs) (belonging to the immunoglobulin superfamily) including CD158a and CD158b; and C-type lectin-like receptors, CD94/NKG2. KIRs are major histocompatibility class (MHC) class I–restricted molecules that recognize HLA-A, -B, -C, and -G molecules, whereas CD94 recognizes the nonclassical MHC class Ib molecule HLA-E. CD94 is expressed essentially on all NK cells, and uses HLA-E expression as a sensor for the overall MHC class I level of a cells.[30,31] In contrast, individual KIR family members express on certain NK cell subsets, exhibit finer specificity for HLA class I allotypes, and can distinguish between groups of HLA-A, -B, and -C allotypes. Ligation of such KIRs/CD94 to HLA class I molecules on self cells results in inhibition of NK cytotoxic activity, as originally predicted by the "missing-self" hypothesis.[32,33] This regulation ensures that cells expressing none, altered, or reduced MHC-I molecules, such as malignant or virus-infected cells, can be killed by NK cells. All freshly isolated liver NK cells expressed CD94 and subpopulations of those cells expressed CD158a/CD158b (Fig. 3A). Cultivation of liver NK cells with no stimulants resulted in reduced expressions of CD158a, CD158b and CD94 (Fig. 3B). However, IL-2 stimulation led to the maintenance of those expressions even on TRAIL expressing liver NK cells (Fig. 3A-B). Thus, as opposed to TRAIL-expressing NK cells in mice, those cells in humans equip a compensatory mechanism to protect the self-MHC class I–expressing cells from NK cell-mediated cell killing.

HCCs Express the Death-Inducing TRAIL-DR4 and -DR5. The susceptibility to TRAIL-induced apoptosis may be related to the expression levels of multiple receptors on target cells. Recent molecular cloning of the TRAIL-receptors elucidated that TRAIL binds to at least four receptors, two of these death-inducing receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) contain cyto-

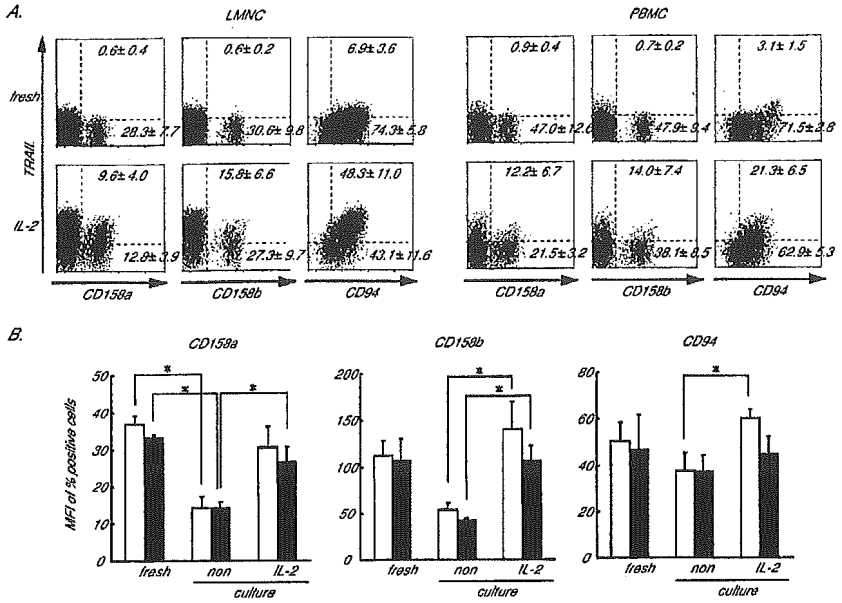


Fig. 3. IL-2 stimulation leads to maintenance of CD158a, CD158b, and CD94 expressions even on TRAIL expressing liver NK cells. Expression of various inhibitory receptors on the NK cells subsets among LMNCs or PBMCs freshly isolated or cultivated with or without IL-2 was analyzed. The LMNCs and PBMCs obtained from healthy donors were stained with CD3, CD56, and TRAIL MAbs together with CD-158a, CD-158b, or CD94 MAbs. (A) Expression of CD158a, CD158b, CD94, and TRAIL on electronically gated $CD3^{-}CD56^{+}$ NK cells were analyzed by FCM. Representative dot plots from freshly isolated NK cells (top panel) or IL-2–stimulated NK cells (second panel) obtained by FCM analysis. Percentages of $TRAIL^{+}$ NK and $TRAIL^{-}$ NK cells expressing killing inhibitory receptors are shown in the right upper and lower quadrants, respectively (mean ± SEM, n = 4 each). The results shown are representative of 4 independent experiments. (B) The percentages of cells staining positively for CD158a, CD158b, and CD94 on $CD3^{-}CD56^{+}$ NK cells are shown (LMNC; open column, PBMC; closed column). Data represent mean ± SEM of 4 cases per group. Statistical analyses were performed using ANOVA (*$P < .05$). IL-2, interleukin-2; NK, natural killer; TRAIL, TNF-related apoptosis-inducing ligand; LMNC, liver mononuclear cell; PBMC, peripheral blood mononuclear cell; MAb, monoclonal antibody; FCM, flow cytometric.

plasmic death domains and signal apoptosis, whereas two other death-inhibitory receptors (TRAIL-R3/DcR1 and TRAIL-R4/DcR2) lack a functional death domain and do not mediate apoptosis, all have similar affinities, and the latter may act as decoys.[20,21] Many cancer cell lines preferentially express TRAIL-DR4 and -DR5, suggesting differential regulation of the death and decoy receptors.[24] The preferential expression of these decoy receptors in normal tissue suggests that TRAIL may be useful as an anti-cancer agent that induces apoptosis in cancer cells while sparing normal cells. Recent studies have demonstrated that NK cells can destroy many solid tissue-derived malignant cells, such as melanoma, breast cancer, lung cancer, gastric cancer, colon cancer, renal cancer, and ovarian cancer cell lines, and that this process is mediated primarily by death receptor/ligand interactions.[34] We investigated the expression patterns of TRAIL-DR and -DcR on or in both normal liver tissues and HCC samples. As shown in Fig. 4A, the endothelial cells in normal liver tissues expressed TRAIL-DR4 and -DR5 together with TRAIL-DcR1 and -DcR2, but hepatocytes did not. Well-differentiated HCCs weakly expressed TRAIL-DR4, -DR5, -DcR1, and -DcR2. Moderately differentiated HCCs showed a higher expression of TRAIL-DR4 and -DR5 than well-differentiated HCCs but little TRAIL-DcR1 and DcR2. In particularly, poorly differentiated HCCs expressed remarkable levels of TRAIL-DR4 and -DR5 but did not express TRAIL-DcR1 and -DcR2, suggesting a susceptibility to TRAIL-expressing NK cell–mediated cell killing. To address this possibility, HepG2, an HCC cell line, could be used as target cells for natural killing activity of LMNCs, because HepG2 expressed high TRAIL-DR4 and -DR5 but no TRAIL-DcR1 and -DcR2, most resembling poorly differentiated HCCs (Fig. 4B).

IL-2–Stimulated Donor Liver NK Cells Showed the Vigorous Cytotoxicity Against HepG2. NK cell cytotoxicity assays using LMNCs and PBMCs isolated from same donors and recipients with liver cirrhosis as effectors and HepG2 as targets were performed. As shown in Fig. 5A, the freshly isolated donor LMNCs were able to mediate potent cytotoxicity against HepG2, whereas the recipient LMNCs and both donor and recipient PBMCs were not able to mediate cytotoxicity without stimulation. After IL-2 stimulation, the donor LMNCs showed the most vigorous natural cytotoxicity against HepG2 (90.5% ± 2.2% at E:T = 10:1), when compared with the donor and recipient PBMCs and the recipient LMNCs