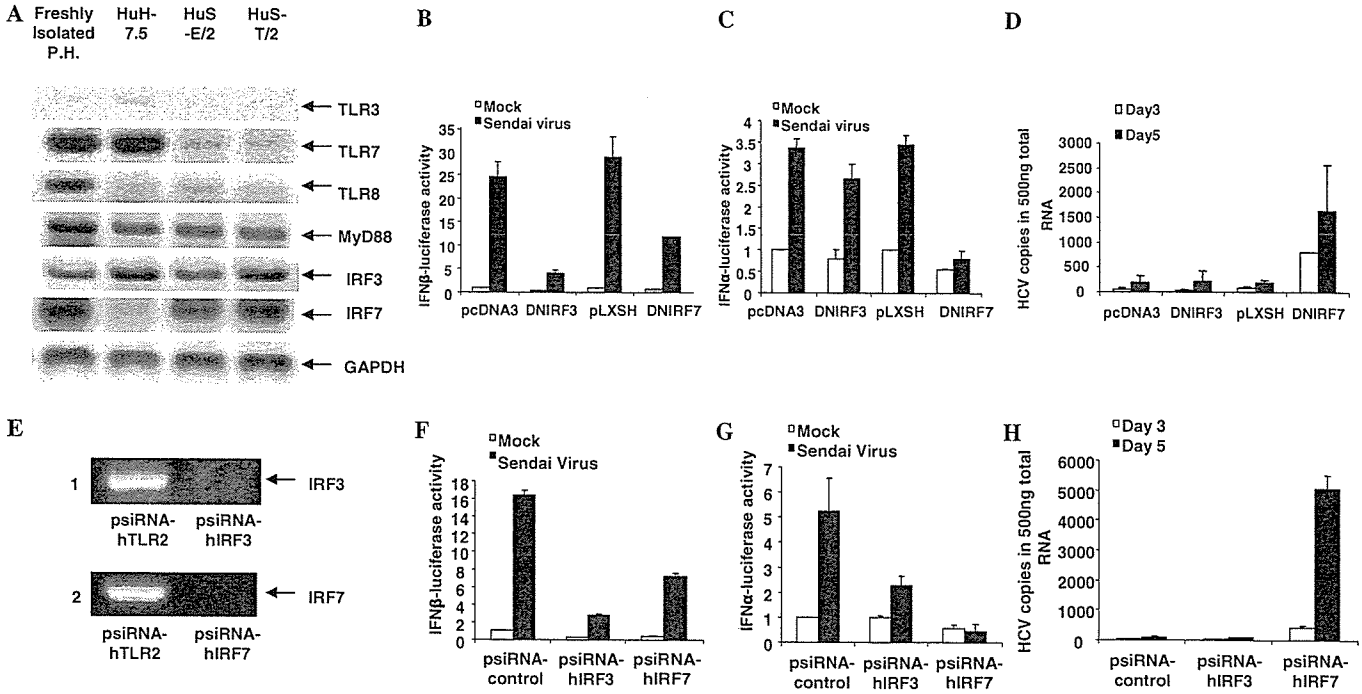

Fig. 5. (A) We examined the expression of TLR3, TLR7, TLR8, MyD88, IRF-3, and IRF-7, as well as GAPDH as an internal control in freshly isolated primary hepatocytes and Huh-7.5, HuS-E/2, and HuS-T/2 cells was investigated by RT-PCR. (B and C) HuS-E/2 cells were cotransfected with pIFNβ-luc (B) or pIFNα-luc (C) with an expression plasmid encoding DNIRF-3, DNIRF-7, or the appropriate empty vector (pcDNA3 and PLXSH, respectively). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (D) IRF-7, but not IRF-3, suppression enhanced HCV infectivity of HuS-E/2 cells. HuS-E/2 cells were transiently transfected with empty pcDNA3, DNIRF-3, empty pLXSH, or DNIRF-7 plasmids. Twenty-four hours later, serum from a patient with HCV was used to infect transfected cells for 24 h. After washing, cells were cultured in fresh medium. The cells were then harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng total RNA was determined by real-time RT-PCR analysis. (E) IRF-3 and IRF-7 levels were suppressed by specific siRNAs. HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, then selected with Zeocin at 250 μg/ml. Two weeks later, cells were harvested and assessed for the expression of IRF-3 and IRF-7 by RT-PCR. (F and G) HuS-E/2 cells were transfected with control psiRNA-hTLR2, psiRNA-hIRF-3, or psiRNA-hIRF-7, followed by selection in Zeocin at 250 μg/ml. Two weeks later, cells were cotransfected with pIFNβ-luc (F) or pIFNα-luc (G). Twenty-four hours later, cells were infected (black bar) with Sendai virus or mock-infected (white bar), then analyzed for luciferase activity after 12 h. (H) Transfected cells were infected with serum from HCV patient; HCV infectivity was assessed as described above.

in HuS-E/2 cells infected with Sendai virus in patterns similar to the effects seen following DNIRF-3 and DNIRF-7 expression, respectively (Figs. 5F and G). Blockade of IRF-7 expression resulted in a significantly higher titer of HCV after infection, while IRF-3 down-regulation did not have any significant effect on HCV titers (Fig. 5H). The enhancement of IRF-7 silencing by siRNA improved the infectivity of HCV (data not shown). These results suggest that IRF-7 plays the major role in the innate immune response to HCV in HuS-E/2 cells.

3.8. Establishment of stable DNIRF-7 expressing clones derived from HuS-E/2 cells

Since DNIRF-7 enhanced HCV infectivity, we transduced the plasmid encoding DNIRF-7 and a hygromycin-B resistance gene, into HuS-E/2 cells. Following selection with hygromycin-B, we obtained the HuS-E7/DN22 and HuS-E7/DN24 clones. As detected by RT-PCR, both clones demonstrated similar expression levels of albumin, apolipoprotein-A1, and HNF4 as the parental HuS-E/2 cells (Fig. 6A). The HuS-E7/DN24 clone exhibited stronger expression of DNIRF-7 than the HuS-E7/DN22 clone by immunoblotting (Fig. 6B). The induction of IFNα in HuS-E7/DN24 in response to infection with an RNA virus (Sendai virus) was low in comparison to the parental HuS-E/2 and HuS-E7/DN22 clones, as detected by IFNα-luciferase reporter assay (Fig. 6C). HuS-E7/DN24 also exhibited a higher HCV infectability in comparison to parental HuS-E/2 cells and the HuS-E7/DN22 clone (Fig. 6D).

3.9. Infection of HuS-E7/DN24 cells with different HCV genotypes

Huh7.5 and HuS-E7/DN24 cells were separately infected with serums derived from 3 different HCV-patients or by JFH-1 concentrated medium (HCV-2a). Two serums were infected by HCV-1b, while the third by HCV-2b. Inoculated virus titer was adjusted to be the same in all cases. Except for JFH-1, which efficiently

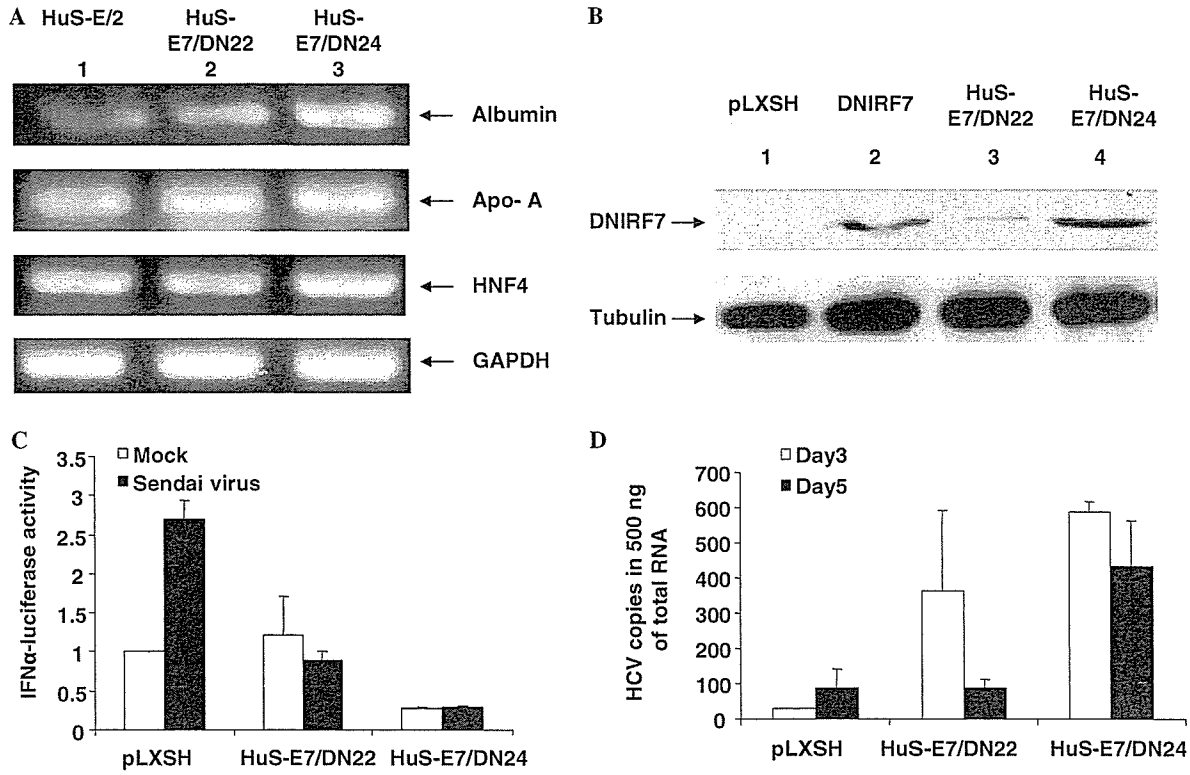

Fig. 6. (A) The pLXSH-HA-DNIRF-7 plasmid was transfected into HuS-E/2 cells, followed by selection in 100 μg/ml Hygromycin B. Two clones, HuS-E7/DN22 (lane 2) and HuS-E7/DN24 (lane 3), were obtained. We investigated the expression of albumin, apo-A, HNF4, and GAPDH as an internal control in parental HuS-E/2, HuS-E7/DN22, and HuS-E7/DN24 hepatocytes cultured for two weeks by RT-PCR. (B) Expression of HA-tagged DNIRF-7 (upper panel) and tubulin (control, lower panel) was detected by immunoblotting analysis. HuS-E/2 cells transiently transfected with either empty pLXSH vector (lane 1) or pLXSH-HA-DNIRF-7 (lane 2) were used as negative and positive controls, respectively, after 48 h. (C) HuS-E/2, HuS-E7/DN24, and HuS-E7/DN22 cells were transfected with IFNα-luc. HuS-E/2 cells were also cotransfected with pLXSH. All of these cells were then infected (black bar) or with Sendai virus or mock-infected, then analyzed for luciferase activity after 12 h. (D) HuS-E7/DN24 cells exhibited high infectivity to HCV samples derived from patient serum. HuS-E/2 cells were transiently transfected with empty pLXSH. Twenty-four hours later, serum from a recurrently transplanted HCV patient was used to infect transfected cells and HuS-E7/DN22 and HuS-E7/DN24 cells for 24 h. After washing three times, cells were cultured in fresh medium. Cells were then harvested and lysed at the indicated time points.

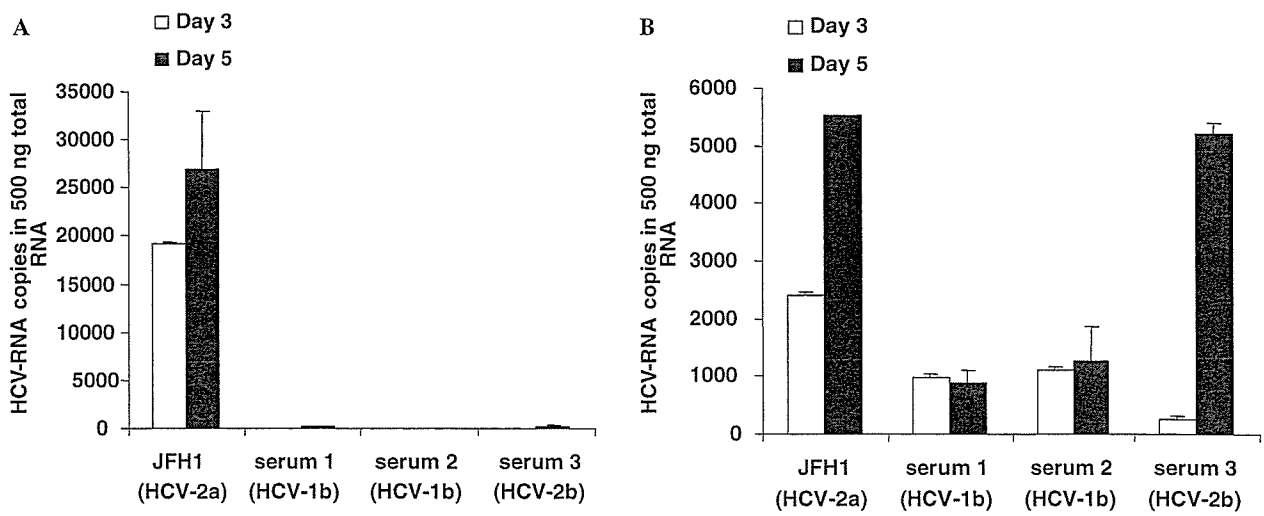

Fig. 7. The infectability of Huh-7.5 and HuS-E7/DN24 cells to different HCV genotypes. Huh-7.5 (A) and HuS-E7/DN24 (B) cells were infected with same titer of JFH1 (HCV-2a), two different HCV-1b serums and one HCV-2b serum. After removing the infected medium, the cells were washed in PBS and recultured in fresh medium. Cells were harvested and lysed at the indicated time points. The quantity of HCV genome RNA per 500 ng RNA was detected by real-time RT-PCR analysis.

replicated in Huh7.5 cells (Fig. 7A), HuS-E7/DN24 cells showed a higher and reproducible infectability for the different HCV strains than Huh7.5 cells (Fig. 7B). Similar higher infectability of HuS-E7/DN24 cells was observed with HCV-4a genotype (unpublished data). These results suggest that the high infectability of Huh-7.5 with JFH-1 is specific among the combinations of HCV strains and cell lines; while HuS-E7/DN24 cells were generally permissive to HCV-infected serum independent of HCV strains.

4. Discussion

This study demonstrates that ectopic expression of the HPV18/E6E7 genes in combination with hTERT could efficiently immortalize mature human hepatocytes, generating a cell line with stable expression of hepatocyte markers and functions for more than 30 weeks in culture. HuS-E/2 cells continuously exhibited higher expression of both HGF and HGFR than HuS-T/2 cells. This result suggests that HPV18/E6E7-immortalized hepatocytes maintain responsiveness to paracrine signals capable of inducing cell differentiation to a greater extent than SV40 T-immortalized hepatocytes. This conclusion is further supported by the increased expression of HNF4 in HuS-E/2 cells in comparison to HuS-T/2 cells. HNF4 is a major hepatocyte transcription factor, required for hepatocyte differentiation and liver-specific gene expression [26]. HNF4 drives hepatocytes differentiation by acting upstream in a transcription factor cascade that included HNF1α [27]. HuS-E/2 cells continued to express HNF1α throughout prolonged culture, while HuS-T/2 cells lost expression completely. Maintenance of hepatocellular functions was demonstrated by continuous and high expression of albumin, apolipoprotein-A, human transferrin, and E-cadherin by HuS-E/2 in comparison to HuS-T/2 cells. These differences became more pronounced in the late passages. In a similar manner, HuS-E/2 cells continued to express all of the examined CYP genes, with the exception of CYP 3A4, while HuS-T/2 cells lost expression of CYP 3A4, 1B, and 2E1 completely and displayed markedly lower expression of CYP 1B1 than HuS-E/2 cells. Thus, human hepatocytes immortalized by HPV E6/E7 transfection are phenotypically similar to primary hepatocytes, even during extended cultures.

Recently, it was reported that the JFH-1 strain and derived chimeras could only infect and propagate efficiently in Huh7.5.1 and Huh7.5 cells, both of which are subclones of Huh7 cells [7–9]. This limitation, however, may be specific to the JFH-1 **strain**, which may not accurately reflect the course of other HCV strains' infection. Thus, usage of HCV particles isolated from patient serum could be more useful to study authentic HCV infection. Using sera from HCV patients as a source of infective virus, HPV18/E6E7-immortalized cell lines exhibited higher reproducible susceptibility to HCV infection than HuS-T, PH5CH8, and Huh-7.5 cell lines.

IRF3 and IRF7 play an important role in the activation of interferon signaling [28]. We suppressed the functions of IRF-3 or IRF-7 to assess their role in HCV infectivity. In fact, we observed significant increase of HCV replication in HuS-E/2 cells bearing dominant-negative IRF7 that impaired IFN signaling. The suppression of IRF-3, however, did not have any significant effect on HCV infectivity or replication in this cell line. This may result from the blockade of IRF-3 activation by an HCV NS3/4A serine protease [29] through at least two independent pathways that inhibit the TLR3-dependent and RIG-I-dependent signaling pathways [29–33]. Although HCV was shown to inhibit basal expression levels of IRF-7 at both mRNA and protein levels and it was shown that NS5A suppresses IRF-7-induced IFNα promoter activation [34], Stimulation of TLR7 was shown to activate IRF-7 and induce suppression of HCV replicon levels in Huh-7 cells [35]. This suggests that the inhibition of IRF7 by HCV is not complete. Using IRF-7-deficient (IRF-7−/−) mice, Honda [36] demonstrated that the transcription factor IRF-7 is essential for the induction of IFNα/β genes. We established a clone stably expressing DNIRF-7 (HuS-7E/DN24), which demonstrated higher infectivity with different HCV strains than the parental HuS-E/2 clone.

In summary, we have established a human hepatocyte-derived cell line that maintains the characteristic features of primary hepatocytes by transduction with HPV18/E6E7. This cell line is highly infectable by HCV, which suggests that these cells may be useful to characterize the molecular mechanisms involved with HCV infection and to develop novel HCV treatment modalities.

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肝炎等克服緊急対策研究事業

生体肝移植後のC型肝炎再発予防を目指した免疫抑制療法及びウイルス除去療法の試みに関する研究

平成16～18年度分担研究報告書

平成19年3月

分担研究者　木　内　哲　也

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

生体肝移植後のＣ型肝炎再発予防を目指した免疫抑制療法及びウイルス除去療法の試み

分担研究者　木内 哲也　名古屋大学大学院医学系研究科 病態外科学講座教授

研究要旨：当該施設で行われたHCV関連疾患に対する肝移植症例を対象に、周術期血中ウイルス動態と再燃の動態を解析し、免疫抑制剤選択の影響と抗ウイルス治療の効果を検討した。手術中無肝期の二重濾過血漿交換療法によりウイルス減量のパイロット・スタディを行い、その安全性と効果を検討した。

A.　研究目的

本邦肝移植症例の 99%以上が生体肝移植であり、4,000 例を超える生体肝移植の過半数が成人で、Ｃ型肝炎ウイルス(HCV)関連の肝硬変・肝細胞癌症例はその約４分の１を占めている。肝移植後早期のウイルス血症は、移植前にウイルス陽性の場合必発であり、急性肝炎を経て慢性化し、放置すると自己肝よりも速い経過で肝硬変・肝不全へと進行する。

移植肝再感染による肝線維化の進行速度や、胆汁鬱滞型の急性増悪は生体肝移植で脳死肝移植よりも高い可能性が示唆されており、本邦において肝移植後Ｃ型肝炎再発予防策を確立することは急務である。本研究では、共同研究の主課題であるステロイド剤不使用による免疫抑制療法を含め、肝移植周術期の血中HCV-RNA動態の解析とウイルス除去療法の試みを通して、再発予防への鍵を探ることを目的とした。

B.　研究方法

当該施設では、これまでに 17 例の HCV 関連疾患に対する肝移植手術を経験した(うち２例が脳死肝移植)が、当初予定されたステロイド剤不使用による免疫抑制療法の無作為比較対照試験に組み込める症例がなかった。そこで、肝移植術中を含めた周術期の血中 HCV-RNA 動態の詳細な解析を行い、一部の生体肝移植症例では術中無肝期における二重濾過血漿交換(DFPP)を用いた血中ウイルス除去の可能性と効果について検討した。

症例は男性 11 例女性６例、年齢中央値 58(41-62.5)歳で、12 例に肝細胞癌を合併していた。ドナーは血縁が 11 例で年齢 33(22-60)歳、移植肝は生体肝移植全例で中肝静脈なしの右葉、血液型不適合移植は３例であった。導入免疫抑制はタクロリムス(Tac)(n=10)

またはシクロスポリン(CsA)(n=7)とステロイドとし、ステロイドは術後２ヶ月までに 0.1 mg/kg に減量した後、抗線維化効果を期待してこれを維持した。移植後の追跡期間は 16(0-35)ヶ月であった。HCV ゲノタイプは、生体肝移植の２例で 2a 型、脳死肝移植の１例で 3 型であった以外はすべて 1b 型であった。

生体肝移植のうち５例では、移植術中無肝期(自己肝門脈血流遮断以降)に DFPP を行った。

(倫理面への配慮)

「ステロイド剤不使用による免疫抑制療法の無作為比較対照試験」について施設の治験審査委員会(IRB)に申請して承認を得たが、さらに移植術中の HCV ウイルス除去を目的とした DFPP 療法、血液型不適合移植に使用される薬剤の適応外使用についても、IRB に申請し承認を得、さらに各患者に説明の上で同意を得た。

C. 研究結果

肝移植術前に中央値 484(3-2210) KIU/ml であった血中 HCV-RNA 量は自己肝門脈血流の遮断までに 382(128-589)KIU/ml とやや減少し、門脈血流遮断後には 225(70-895)KIU/ml、自己肝摘出後には 151(40-312)KIU/ml とさらに減少した。移植肝血流再開時のウイルス量は 49(0.5-373)とさらに減少していた。

移植肝血流再開時の HCV-RNA は DFPP 群で中央値 10.5(0.6-22.7)KIU/ml と DFPP 非施行生体肝移植群の 3.9 (0.5-54.3)と有意差を認めなかったが、術前比では DFPP 非施行群 0.0171 (0.0077-0.1852) に対し 0.0090 (0.0006-0.0594)と低下傾向を示し、DFPP 施行前後でも多くの症例で低下が確認された。

移植肝血流再開後は血中 HCV-RNA は減少を続け、移植後再陽性化(>0.5 KIU/ml)は DFPP 群で 9(3-29)日と非 DFPP 群の 2(1-21)日よりも遅かったが、ひとたび陽性化するとウイルス量の差は消失し、術前ウイルス量を凌駕するまでの時間には有意差がなかった。

現在までに、DFPP 群(追跡期間中央値 16 ヶ月)の 40%に中央値３ヶ月で、非 DFPP 群(追跡期間中央値 17 ヶ月)の生存例全例に中央値６ヶ月で組織学的慢性肝炎(>A2F1)を認め、インターフェロン(IFN)+リバビリン治療を開始している。

抗ウイルス療法の経過については現在追跡中であるが、初期の症例に Fibrosing cholestatic hepatitis (FCH)と IFN 治療中の胆管消失性慢性拒絶をそれぞれ１例に認めた。ゲノタイプ 2a ではウイルス学的反応は良好であるが、ゲノタイプ 1b では腎障害や貧血、さらに自己免疫性肝炎の合併などの理由で減量・中止を余儀なくされる例があり、ウイルス学的反応は一般に不良であった。組織学的変化では、IFN/リバビリンが無効であった１例に移植後３年で３度の繊維化を認

めている他は、2 度未満の繊維化に留まっている。

D. 考察

限られた症例数ながら、移植前重症肝不全症例において移植後に強い免疫抑制療法が行われた場合の移植後 HCV 再燃による FCH のリスク、同時に移植後早期の IFN 治療に伴う難治性拒絶発生のリスクが再確認された。

肝移植の術中においては、血中の HCV-RNA の出血などの因子による減少、最大のウイルス源である自己肝の揉み出しによる増加、肝摘出後の自然減衰をを経て、移植肝血流再開に残ったウイルスが移植肝に吸い込まれてさらに減少し、見かけ上短時間の血中消失期を経て、移植肝の中で増殖したウイルスがその後急速に血中に放出され、この次点以降に移植肝の再感染が起こることが示唆された。移植肝への吸収速度や移植肝内でのウイルス増殖速度には、移植肝の機能や大きさも関与していることが推測される。

肝移植後生化学的・組織学的 HCV 肝炎再燃後の IFN/RVB 治療は、脾機能亢進症状の遷延下でも、ウイルス学的には少なくとも一定の効果を示すことが示唆されたが、血中ウイルス陰性化持続状態(sustained viral response)の獲得までに至ることがどの程度可能であるかについては明らかにならなかった。

HCV 除去を目的とした肝移植術中 DFPP の結果は、無肝期の DFPP がそれまでの懸念に反して安全に施行できることを示すものであり、HCV 粒子の除去効率の個人差は、肝外組織へのウイルス分布の個人差をも反映すると思われるが、無肝期における DFPP によって移植肝へ新たに接触する HCV-RNA を減少させることができる可能性が示された。少量でも血中に HCV 粒子が残っている限り移植後の HCV 血症再燃は避けられないが、移植術前術中の他の治療法との組み合わせを図ることで、移植後血中 HCV 陰性期を延長させ、術後の安全な予防的抗ウイルス療法へと繋げることができる可能性が示された。

E. 結論

HCV 関連疾患に対する生体肝移植後において、移植肝の HCV 肝炎再燃は速く、移植前重症例や免疫抑制強度の強い症例はよりハイリスクとなる。一方で、早期 IFN 治療に伴う難治性拒絶合併の危険は、頻度は高くないもののひとたび発生すると重篤化しやすい。

肝移植術中無肝期の残存ウイルス量と、移植直後に血中から消失し肝内で増殖したウイルスが血中に放出されるタイミングとは、互いに密接に関係している可能性がある。肝移植手術中無肝期の DFPP は、フィブリノゲンのモニターと補充によって安全に行うことができ、移植肝血流再開までに血中 HCV-RNA を減少させ、移植後 HCV ウイルス血症再燃までの期間を延長させる可能性がある。

移植後の免疫抑制療法の選択のみならず、こうしたウイルス動態に基づいた周術期の多面的対応を行うことが、移植肝における HCV 再燃制御の鍵となる可能性が示された。

F. 健康危険情報

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H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

別紙４

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IMPACT OF RECIPIENT AGE ON OUTCOME OF ABO-INCOMPATIBLE LIVING-DONOR LIVER TRANSPLANTATION

HIROTO EGAWA,[1,5] FUMITAKA OIKE,[1] LEO BUHLER,[2] A. M. JAMES SHAPIRO,[3] SACHIKO MINAMIGUCHI,[4] HIRONORI HAGA,[4] KENJI URYUHARA,[1] TETSUYA KIUCHI,[1] SATOSHI KAIHARA,[1] AND KOICHI TANAKA[1]

Background. **Transplantation of hepatic grafts from ABO-incompatible donors is controversial because of the risk of hyperacute rejection mediated by preformed anti-ABO antibodies. The aim of the present study was to evaluate the outcome of liver transplants performed with ABO-incompatible living-donor livers and to detect risk factors for development of complications.**

Methods. **From June 1990 to February 2000, 66 patients, 10 months to 55 years old (median, 2 years old), received 68 ABO-incompatible living-donor liver grafts. The antibody titer and clinical course were followed prospectively during a period ranging from 3 to 11 years.**

Results. **The 5-year patient survival was 59%, 76%, and 80% for ABO-incompatible, ABO-compatible, and ABO-identical grafts, respectively ($P<0.01$). In patients <1 year old, ≥1 to <8, ≥8 to <16, and and ≥16 years old, 5-year survival was 76%, 68%, 53%, and 22%, respectively. The incidence of intrahepatic biliary complications and hepatic necrosis in ABO-incompatible living-related grafts (18% and 8%, respectively) was significantly ($P<0.0001$) greater than in ABO-compatible and ABO-identical grafts (both 0.6% and 0%, respectively). Predictive risk factors for increased mortality and morbidity were age greater than 1 year and elevated anti-ABO titers before transplantation.**

Conclusions. **ABO-incompatible liver transplantation was carried out with relative safety in infants <1 year old but was not satisfactory in children >1 year in long-term follow-up. Patients aged >8 years remain at considerable risk of early fatal outcome because of hepatic necrosis, and new strategies to prevent antibody-mediated rejection are required.**

The indication for liver transplantation (LTx) across an ABO-incompatible (ABO-I) barrier remains controversial. The additional risks of ABO-I LTx include antibody (Ab)-mediated hyperacute rejection and a higher incidence of severe acute rejection, hepatic artery thrombosis, and biliary complications (*1–3*). The impact of preformed antidonor ABO Ab and strategies to reduce their titers have been reported previously (*4–8*). Prophylactic antilymphocyte globulin, plasmapheresis, splenectomy, soluble antigen, and ABO immunoabsorbents have been applied, with varying degrees of success (*4–10*). It has been suggested that acceptable results of ABO-I LTx may be achieved in young children (*9*, *10*). Yandza et al. (*7*) demonstrated that children less than 2 years old had lower anti-ABO Ab titers and lower morbidity compared with adults. Gugenheim et al. (*1*) suggested that ABO-I LTx is only justifiable in adult recipients as an emergency. On the contrary, Hanto et al. (*11*) recently reported encouraging results in adults.

Living-donor LTx (LDLT) provides the only access to donor organs in countries where cadaveric donors are not available. In Japan, approximately 2,000 patients have undergone LDLT. LDLT offers advantages in terms of minimal cold preservation time in healthy and hemodynamically stable donors. The technique, however, is generally used only for immediate family relatives of the recipient. Therefore, because of a limited donor supply and because the need of patients with end-stage liver failure is critical, use of grafts from ABO-I donors may be the only available option.

The current study reports our single-center results in 68 ABO-I LDLT carried out in 66 patients with a minimum follow-up period of 3 years for surviving patients. We report here our current protocol and focus on the risk factors for development of major complications after ABO-I LDLT.

PATIENTS AND METHODS

Patients

From June 1990 to February 2000, 523 patients underwent 540 LDLT (including 19 second LTx; 201 male patients and 339 female patients) at Kyoto University Hospital. The age range was 3 months to 68 years, with a median age of 4 years. The weight range was 3 to 77 kg, with a median body weight of 23 kg. Three hundred sixty-two patients underwent 370 ABO-identical LTx (69%), 99 patients underwent 102 ABO-compatible LTx (18%), and 66 patients underwent 68 ABO-I LTx (13%). The age range was 1 month to 66 years (median, 5 years) in ABO-identical LTx, 3 months to 68 years (median, 5 years) in ABO-compatible LTx, and 3 months to 55 years (median, 2 years) in ABO-I LTx. Patient status was intensive care unit bound in 16 patients, hospitalized in 28 patients, and at home in 21 patients. The principal indications for ABO-I LTx were biliary atresia in 42 cases, metabolic liver diseases in 8 cases, fulminant hepatic failure in 5 cases, cryptogenic cirrhosis in 2 cases, Alagille syndrome in 1 case, primary biliary cirrhosis in 2 cases, primary sclerosing cholangitis in 1 case, autoimmune hepatitis in 1 case, and graft failure in 6 cases. The proportion of biliary atresia among indications was 60%, 49%, and 62% in ABO-identical LTx, ABO-compatible LTx, and ABO-I LTx, respectively.

Blood Groups and Antibody Status

The ABO blood group barrier between donor and recipient was A to O in 22, B to O in 17, B to A in 9, AB to A in 9, A to B in 6, and AB

[1] Department of Transplantation Immunology, Faculty of Medicine, Kyoto University, Kyoto, Japan.
[2] Department of Surgery, University Hospital of Geneva, Geneva, Switzerland.
[3] Department of Surgery, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada.
[4] Department of Clinical Pathology, Faculty of Medicine, Kyoto University, Kyoto, Japan.
[5] Address correspondence to: Hiroto Egawa, M.D., Department of Transplantation Immunology, Transplant Surgery, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606, Japan. E-mail: egawa@kuhp.kyoto-u.ac.jp.
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to B in 5 patients. In the Japanese population, 98.8% with blood type A are type A1. The reasons for selection of incompatible donors were as follows: ABO incompatibility in both parents in 42 pediatric cases, liver dysfunction or hepatitis of one of the parents in 9 pediatric cases, pregnancy of a potential ABO-compatible donor in 1 pediatric case, size mismatch in 1 pediatric case, and only one available potential donor in another 15 cases including 6 retransplant procedures. In the six patients receiving second grafts from ABO-I donors, two received the first liver from ABO-I donors and the other four received the first liver from ABO-identical donors. The titer was measured on a regular basis after transplantation: every day during the first 2 weeks, two times during the next 2 weeks, and occasionally thereafter.

Antidonor blood type titers were measured in a microhemagglutination assay serially before and after transplantation (Tx). In ABO-I combinations, exchange transfusion was carried out in children weighing less than 10 kg, and plasmapheresis or hemofiltration was performed preoperatively in larger children or adults to decrease the antidonor ABO titer below 1:8 before Tx. For emergency cases, target levels of titers could not be obtained before Tx. These procedures were considered when the titers increased over 1:64 after Tx. Titers were followed weekly during 1 month after Tx.

Transfusion Policy

For transfusion and blood exchange, red blood cell products with the same type as a recipient were used. Plasma and platelets of blood group AB were used in all cases, because platelet products could contain plasma.

Intraoperative Procedures

Details pertaining to donor evaluation and surgical techniques used in Kyoto have been reported elsewhere (*12*). Intraoperative cholangiography was performed routinely to exclude aberrant anatomy. The liver graft was flushed with cold University of Wisconsin solution or histidine-tryptophan-ketoglutarate (*13*). Anastomosis of the hepatic artery was carried out using a microvascular technique.

Immunosuppressive Therapy

The basic immunosuppressive regimen consisted of tacrolimus and steroids in all groups (*14*). Target trough levels of tacrolimus in whole blood were 10 to 15 ng/mL in the first week, and then 5 to 10 ng/mL during the first post-Tx month. Methylprednisolone (20 mg/kg) was administered intravenously (IV) during the anhepatic phase of surgery, followed by 2 mg/kg administered IV for the first 3 days, then tapered to 1 mg/kg for 3 days, and converted to 1 mg/kg/day of prednisone, which was weaned gradually and discontinued routinely after 6 months. In ABO-I cases, prophylactic steroid pulse (methylprednisolone; 10 mg/kg, 5 mg/kg, and 2.5 mg/kg IV for 3 days) was administered every week for the first month (weekly pulse). Prostaglandin E_1 was infused (0.01 mg/kg/min IV) for 7 to 14 days after Tx. Cyclophosphamide therapy (2 mg/kg/day) was initiated 1 week pretransplant and given daily for 1 month after Tx, and was then converted to azathioprine (1 mg/kg/day). Splenectomy was performed basically in patients aged 6 years and older, unless patients had risks for infection such as surgical injury of the intestine because of massive adhesions, and malnutrition. OKT3 was administered in initial cases (*10*) but discontinued because of frequent severe infection.

ABO-I–Related Complications

Hepatic necrosis was diagnosed by enhanced computed tomographic scan and confirmed by liver biopsy. Initial radiologic findings were spotty or patchy low-density lesions that progressively developed diffusely throughout the graft. Intrahepatic biliary complications (IHBC) were diagnosed by cholangiography, showing multiple stenoses of intrahepatic bile ducts similar to sclerosing cholangitis, multiple bile lakes, or both.

Statistical Analysis

Data of titers are expressed as mean SE and were analyzed using StatView (version 5.0 for Macintosh; StatView, Corp., Berkeley, CA), with P 0.05 being considered significant. Analysis of variance was used for comparison between groups. The chi-square test and Fisher's exact test were used to examine the association between age of recipients and incidence of complications. The difference in survival between subgroups was assessed by the Cox-Mantel test.

RESULTS

Morbidity and Mortality

Patient survival curves are shown in Figure 1. Five-year actuarial survival was 59%, 76%, and 81% in ABO-I, ABO-compatible, and ABO-identical cases, respectively (P 0.01, Cox-Mantel test). ABO-I recipients developed a significantly higher incidence of liver necrosis (8% vs. 0% and 0%, P 0.0001) and IHBC (15% vs. 0% and 0.6%, P 0.0001) compared with ABO-compatible and ABO-identical cases. ABO-I recipients developed a significantly higher incidence of cytomegalovirus disease compared with ABO-compatible and ABO-identical cases (45% vs. 17% and 27%, respectively; P 0.05). Among these three groups, there was no significant difference in incidence of biopsy-proven acute cellular rejection (46%, 41%, and 43%, respectively) or ductopenic rejection (3%, 5%, and 5%, respectively) (*16*). The incidence of hepatic artery thrombosis suspected by loss of Doppler ultrasound signal was significantly higher in ABO-I cases compared with ABO-identical and ABO-compatible cases (10%, 1%, and 3.5%, respectively; P 0.05). In the early period of this study, we used OKT3 for induction (*10*). A total of 14 patients received it, and 6 patients survived. Three of the six patients (50%) who survived were under 1 year old, and the other three were younger than 3 years old. Four patients died as a result of sepsis.

For ABO-I cases, the patient and graft survival rates were related to the age of the recipient and were decreased in older patients, as shown in Figure 2. Patients were divided into four groups: younger than 1 year old (1; n 21), 1 year old or older and younger than 8 (1– 8; n 25), 8 years old or older and younger than 16 (8– 16; n 11), and 16 years old or older (16; n 9). In patients 1 year old, 1 to 8, 8 to 16, and 16 years old, 5-year patient survival was 76%, 68%, 53%, and 22%, respectively. Five-year graft survival was 76%, 55%, 49%, and 22%, respectively. Both patient and

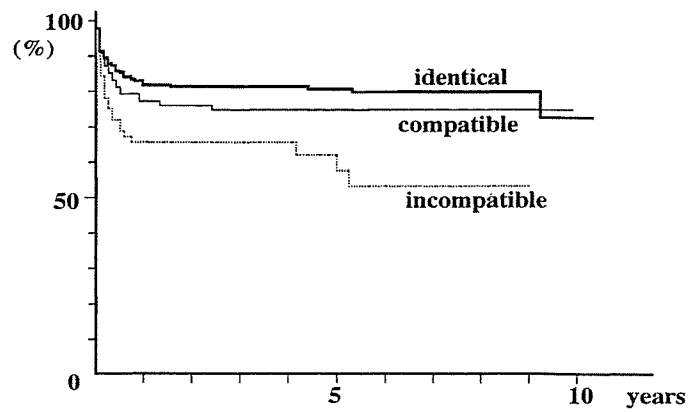

FIGURE 1. Actuarial survival of patients with ABO-I, ABO-compatible, and ABO-identical grafts.

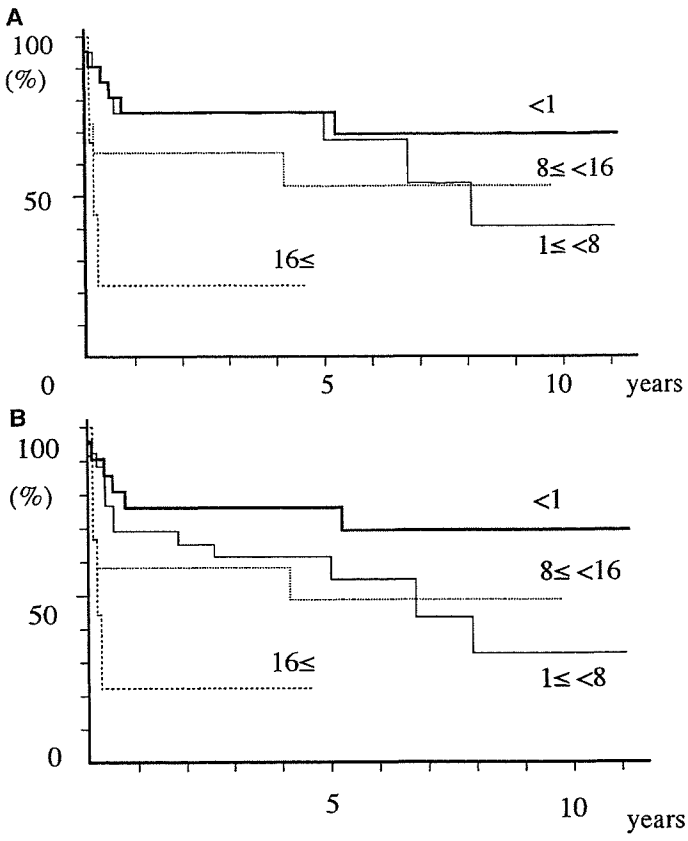

FIGURE 2. Actuarial patient (A) and graft (B) survival of patients with ABO-I living-related liver grafts in four age groups (<1 year old, ≥1–>8 years old, ≥8–<16 years old, and ≥16 years old).

graft survival rates for patients 16 years old were significantly lower than those for children 1 year old and children 1 to 8 years old (P 0.01 and P 0.01, respectively). Patients 1 year old did not develop hepatic necrosis or IHBC (Table 1).

Hepatic necrosis was observed in five recipients of ABO-I grafts (7%) 8 to 40 years old. The demographic data of these patients are shown in Table 2. Two of these patients developed hepatic artery thrombus detected by Doppler ultrasound. The onset of hepatic necrosis ranged between 2 and 21 days after Tx. All five of these patients developed jaundice and fever accompanied by a marked increase in anti-ABO immunoglobulin (Ig) M and IgG in four cases (80%). Liver biopsy and radiologic investigations demonstrated hepatic necrosis. All five patients with hepatic necrosis died 20 days to 59 days after Tx, despite institution of plasma exchange and aggressive immunosuppression. One patient received a second ABO-I graft and died because of intracranial hemorrhage immediately after liver retransplantation.

IHBC was observed in 12 patients (17%), 1 to 26 years old. The demographic data and outcomes of these patients are shown in Table 3. There was no hepatic artery thrombus detectable by Doppler ultrasound. The antidonor ABO IgM titer before LTx was high (1:128) in all patients but one. The patient with a low IgM titer before LTx (patient 485) showed increased IgM titers (1:1,024) after Tx. Two types of IHBC were observed radiologically: (1) multiple intrahepatic cysts in three cases and (2) a sclerosing cholangitis complication with stenosis throughout the biliary tree in nine cases. In both types, liver biopsy showed acute and chronic cholangitis. There was no significant difference in Ab titers between these two types. IHBC usually developed within 3 months after Tx, except in two cases in which it occurred at 8 months and 5 years. Eight of 12 patients developed graft failure (66%). Four patients underwent retransplantation (two with ABO-identical cadaveric grafts 2 years and 8 years after the first Tx, one with an ABO-identical LDLT 31 months after the first Tx, and one with an ABO-I LDLT 2 months after the first Tx). Two patients were placed on the waiting list for cadaveric LTx but died because of pulmonary hypertension and hemorrhagic shock secondary to rupture of esophageal varices 4 and 7 years after LTx, respectively. Another two patients died because of sepsis secondary to cholangitis 3 months after Tx. Four patients are alive 2, 4, 6, and 8 years after LTx, and two older children attend school every day, even with sporadic cholangitis.

Arteriopathy consisting of intimal thickness and obstruction of the arterial lumen was observed in the explant livers obtained from two patients with hepatic necrosis and from one with IHBC. These two patients had normal hepatic artery flow according to Doppler ultrasound.

Other causes of death in 17 patients were surgical complications (n 3) and fungal or bacterial infection (n 9), including three patients with intracranial bleeding caused by *Aspergillus* infections, recurrent fulminant hepatic failure (n 2), de novo hepatitis C cirrhosis (n 2), and posttransplant lymphoproliferative disorder (n 1).

Serum Antibody Titers before and after Tx

Anti-ABO Ab titers were decreased in all recipients before Tx by transfusion or plasma exchange. There were significant differences of pre- and post-Tx Ab titers among age groups. Figure 3(A) shows pre- and post-Tx anti-ABO IgM peak titers in four age groups (1, 1– 8, 8– 16, and 16 years). Pre-Tx anti-ABO IgM peak titers in age groups 1 to 8, and 8 to 16 years were significantly higher than

TABLE 1. Age and complications[a]

Age (yr)	Patient groups				
	Necrosis	IHBC	Normal	Other death	Total
1	0	0	15	6	21
1– 8	0	8	11	6	25
8– 16	3	2	5	1	11
16	2	2	1	4	9
Total	5	12	32	17	66

[a] P 0.01, [2] test. Two patients surviving 1 wk were not included in this analysis.

TABLE 2. Data of patients with hepatic necrosis

Patient	Age (yr) at LT/gender	Blood type (D/R)	Disease	Peak titer of donor blood type			HAT onset		Outcome
				IgM/IgG before LTx	IgM/IgG after LTx	(POD)			
266	40/M	AB/A	FHF HB	32/4	4,096/1,024	(4)	–	2	Died (POD 20)
276	19/M	A/O	Biliary atresia	64/512	128/512	(2, 36)	–	9	Died (POD 59)
286	8/M	A/O	Biliary atresia	256/128	512/512	(5)		Not clear	Died (POD 54)
294	8/F	A/O	Biliary atresia	512/16,380	2,048/2,048	(18)		21	Dead (POD 33)[a]
311	14/F	AB/A	Biliary atresia	256/32	64/16	(3)	–	18	Died (POD 32)

D, Donor; R, recipient; FHF HB, Fulminant hepatic failure caused by hepatitis B; HAT, hepatic artery thrombus; POD, postoperative day; M, male; F, female.

[a] She underwent retransplantation with a graft from another parent of the ABO-I blood type and died.

in the other two age groups (1– 8 vs. 1, P 0.0040; 1– 8 vs. 16, P 0.0280; 8– 16 vs. 1, P 0.0040; and 8– 16 vs. 16, P 0.0120). At Tx, IgM titers in the 1 year age group were significantly lower than in the 1 to 8 age group (P 0.0241) and the 8 to 16 age group (P 0.0070). Post-Tx IgM titers in the 16 age group were significantly higher than those of the two younger groups 1 (P 0.0006) and 1 to 8 (P 0.0019), and those in the age group 8 to 16 years were significantly higher than those of the 1 (P 0.0207) age group. All long-term survivors showed low levels of IgM titers. Patients who had ABO-I–related complications showed an increase of titers before onset of complications, followed by a decrease of titers while complications persisted.

Figure 3(B) shows pre-and post-Tx anti-ABO IgG peak titers in the four age groups. Pre-Tx, anti-ABO IgG titers in the 8 to 16 age group were significantly higher than in the 1 (P 0.0290) and 1 to 8 (P 0.0019) age groups. At Tx, IgG titers in the 8 to 16 age group were higher than in other age groups, but there was no significant difference. Post-Tx IgG titers in the 16 age group were significantly higher than in the 1 (P 0.0014) and 1 to 8 (P 0.0089) age groups. All long-term survivors showed low levels of IgG titers. Patients who had ABO-I–related complication also showed a decrease of the IgG titers during their clinical courses similar to IgM titers.

The pre- and post-Tx anti-ABO IgM and IgG peak titers were correlated to the four age groups (1, 1– 8, 8– 16, and 16 years) and to four clinical outcomes: (1) hepatic necrosis; (2) IHBC; (3) the group with an uneventful postoperative course; and (4) the group with death from causes other than hepatic necrosis or IHBC. Figure 4(A) shows pre-Tx and Figure 4(B) shows post-Tx anti-ABO IgM peak titers. Pre- and post-Tx IgM titers in the 1 year age group were low. Post-Tx IgM titer in the 1 to 8 age group were significantly higher in patients with IHBC than in patients with uneventful courses (P 0.0004) and other death (P 0.0476). In the 8 to 16 age group, pre- and post-Tx IgM titers were significantly higher in patients with IHBC than hepatic necrosis (P 0.0059), uneventful death (P 0.0021), and other death (P 0.0087). In the 16 age group, pre-Tx IgM titers were low, but post-Tx titers were high in patients with hepatic necrosis and death as a result of other causes, although there was no significant difference.

Figure 5(A) shows pre-Tx and Figure 5(B) shows post-Tx anti-ABO IgG peak titers in correlation with age and clinical outcome. Pre- and post-Tx IgG titers in the 1-year age group were low. In the 8 to 16 age group, pre-Tx IgG titers in patients with hepatic necrosis and post-Tx IgG titers in patients with hepatic necrosis and IHBC were high compared with other groups. For the 16 age group, although there was no statistical significance, only patients with normal outcome had relatively low pre- and post-Tx IgG titers.

DISCUSSION

ABO-I LTx may be considered a lifesaving procedure for patients with life-threatening fulminant liver failure (*4,17*). Application of LDLT has recently reduced the number of ABO-I cadaveric LTx in children in the United States (*18*). In countries with limited access to cadaveric donors, however, the use of grafts from ABO-I donors occurs more frequently, particularly when the donor source is restricted to immediate relatives. An evaluation of ABO-I LDLT, which takes into account the additional risks of morbidity and mortality, is now required.

Our report is the first study of ABO-I LDLT and analyzes the outcome of 68 ABO-I LDLT. The overall 5-year patient survival was 58%, compared with 80% in ABO-identical allograft recipients. However, analysis of the different age groups showed that 5-year patient survivals for patients 1 year old and 16 years old were 77% and 22%, respectively. The incidence of intrahepatic biliary complications and hepatic necrosis in ABO-I LDLT increased significantly with the age of the recipients. Predictive risk factors for increased morbidity and mortality were age 1 year and elevated anti-ABO Ab titers pre-Tx.

Anti-ABO Ab are present at birth as a result of diaplacental transport of maternal Ab, but not as a result of self-production. Maternal Ab disappear from the neonate after 2 weeks, and at approximately 8 to 12 weeks the newborn starts producing IgM and IgG of its own (*19*). Adult levels are reached by age 5 to 10 years (*20*). Although the stimulus for the production of Ab against A, B, or both determinants remains uncertain, one commonly held hypothesis is that it is a response to the presence of A, B, or both carbohydrates on bacteria or other micro-organisms that colonize the infant gastrointestinal tract. Anti-ABO Ab baseline production is thought to be T-cell independent, as is the production of other anticarbohydrate Ab, such as anti-Gal 1,3 Gal Ab (*21*). Therefore, anti–T-cell–directed immunosuppression is not successful in inhibiting baseline anticarbohydrate Ab production (*22*), and nonspecific immunosuppression, such as cyclophosphamide, has shown limited effects on anticarbohydrate Ab production (*22*). Arteriopathy was observed in our cases with ABO-I–related complications, and the mechanisms of arteriopathy relating endothelial injury by humoral rejection might be a key for the strategy for ABO-I Tx (*15*).

TABLE 3. Data of patients with intrahepatic biliary complications

Patient	Age at LT/gender	Blood type (D/R)	Disease	Peak titer of donor blood type antibody IgM/IgG before LTx	IgM/IgG after LTx (POD)	Type	Onset	Outcome	Follow-up period
54	3 yr/F	AB/B	Biliary atresia	512/16	512/64 (1)	Sclerosing cholangitis	8 mo	PTCD	8 yr
66	7 yr/F	B/O	Biliary atresia	256/64	128/256 (7)	Sclerosing cholangitis	5 yr	Died after re-CLT	3 yr
111	6 yr/F	A/B	Biliary atresia	512/32	512/128 (5)	Sclerosing cholangitis	1 mo	Died[a]	6 yr
175	12 yr/M	B/O	Alagille syndrome	2,048/1,024	2,048/1,024 (5)	Sclerosing cholangitis	3 mo	Cholangitis	6 yr
189	3 yr/M	A/O	Glycogen storage	256/256	512/256 (18)	Multiple cysts	2 mo	Died after re-LRLT	7 mo
203	2 yr/F	A/O	Biliary atresia	1,024/32	1,024/2,048 (6)	Sclerosing cholangitis	3 mo	Died[d]	3 mo
210	15 yr/F	A/B	Biliary atresia	1,024/1	2,048/128 (7)	Multiple cysts	2 mo	Died[a]	5 yr
231	1 yr 1 mo/M	A/O	Biliary atresia	512/64	16/4 (2)	Sclerosing cholangitis	1 mo	Re-LRLT[b]	4 yr
282	1 yr 9 mo/F	B/O	Biliary atresia	128/64	32/32 (6)	Sclerosing cholangitis	1 mo	Improved	4 yr
362	4 yr 6 mo/F	A/O	Biliary atresia	512/512	2,048/1,024 (19)	Sclerosing cholangitis	1 mo	Re-CLT[c]	4 yr
450	50 yr/M	A/O	FHF	128/2,048	128/512 (3)	Sclerosing cholangitis	1 mo	Improved	2 yr
485	26 yr 8 mo/F	B/A	PSC	16/0	1,024/1,024 (12)	Multiple cysts	1 mo	Died[d]	3 mo

D, Donor; R, recipient; PSC, primary sclerosing cholangitis; FHF, fulminant hepatic failure; CLT, cadaveric LTx; LRLT, living-related LTx; POD, postoperative day; PTCD, percutaneous transhepatic cholangiography and drainage; M, male; F, female.

[a] On the waiting list for cadaveric LTx from compatible blood type donor.

[b] Retransplantation from his grandmother of the identical blood type.

[c] She underwent auxiliary partial orthotopic LTx and survived the cholangitis with the remnant native liver and underwent successful CLT from a compatible blood type donor.

[d] Sepsis resulting from cholangitis.

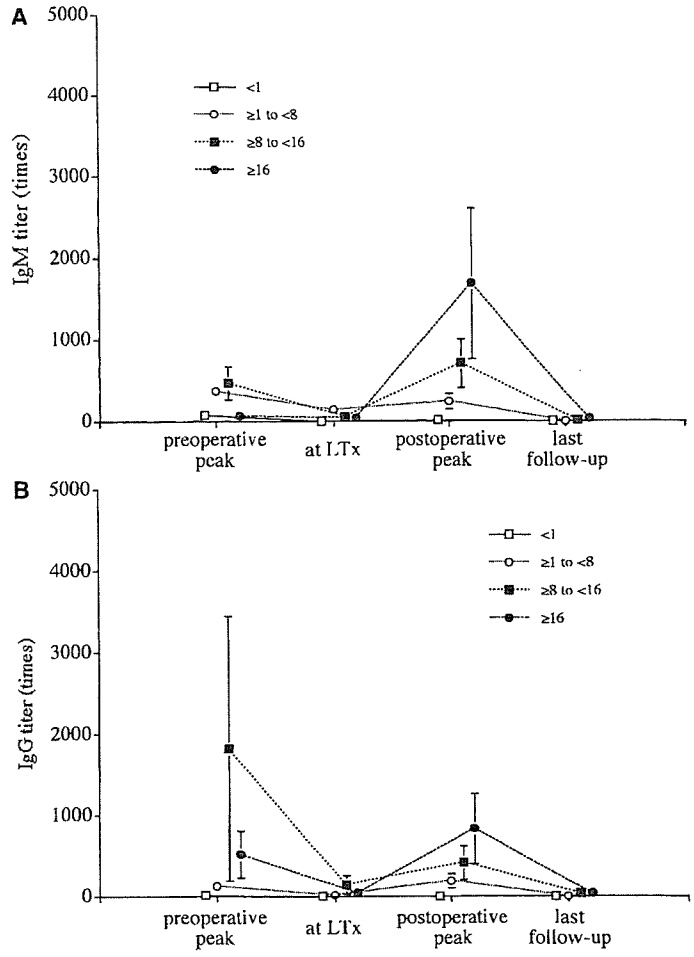

FIGURE 3. (A) Anti-ABO IgM titers among the four age groups (<1 year old, ≥1–>8 years old, ≥8–<16 years old, and ≥16 years old). (B) Anti-ABO IgG titers among the same four age groups. LFU, Last follow-up. The LFU means immediately before death in dead patients or the last measurements in the outpatient clinic in patients who survived.

In the reported study, only recipients in the 1-year age group presented low pre- and post-Tx anti-ABO Ab titers. All age groups 1 year had high pre-Tx anti-ABO Ab titers or significantly increased post-Tx titers (Figs. 3–5). In contrast, recipients in the 8 to 16 age group showed high anti-ABO Ab titers both before and after Tx. Interestingly, recipients in the 16 age group had a potential to increase anti-ABO Ab titers after Tx, even though preoperative anti-ABO Ab titers were low. High Ab titers were correlated with development of severe post-Tx complications (i.e., hepatic necrosis and IHBC). Anti-ABO Ab titers were shown to decrease to low levels in all long-term surviving patients, possibly because of tolerization of the host B and plasma cells to the donor antigens as reported in heart Tx (*23*).

The onset of hepatic necrosis ranged between 2 and 21 days post-Tx and corresponded to a hyperacute or acute Ab-mediated rejection phenomenon. The histopathologic findings were those of hyperacute rejection, but we failed to demonstrate deposition of IgG and IgM. There was no successful immunosuppressive protocol to successfully prevent or treat hepatic necrosis in our series. Although plasmapheresis was