FIGURE 1
Phenotypic detection of NK and NKT cells. NKT cells existed prominently in the liver compared with those in the spleen and PBL of secondary extrahepatic obliteration of only left gastric vein due to pancreatitis (A) and donor patient (B). CD56+T cells and CD161+T cells were especially abundant in the liver compared with those in PBL or spleen. And also they were abundant compared with CD57+T cells in the liver.

one case of hypercitrullinemia. Four patients underwent LRDLT with a left-lobe graft, the other three with a right-lobe graft. The man with alcoholic liver cirrhosis received a right-lobe graft and the citrullinemia patient a left-lobe graft by auxiliary orthotopic partial liver transplantation (APOLT). Moreover a patient of secondary extrahepatic portal venous obstruction (EHO) at the left gastric vein for pancreatitis who underwent left gastric venous caval shunt with splenectomy for rupture of solitary gastric varices, was examined by immunological analysis of lymphocytes in the liver, spleen, and peripheral blood. The study was approved by the Ethics Committee of the Niigata University, School of Medicine and was conducted according to the principles of the Second Declaration of Helsinki. All participants provided written informed consent.

Cell Preparation

Liver specimens were obtained by open biopsy at the exploration of donor operation: after the perfusion of graft liver with HTK solution (Bretschneider solution), and almost one hour after reperfusion of hepatic circulation in LRDLT. To prepare lymphocytes from liver and splenic tissue the samples were minced (9), then treated with collagenase (Wako, Osaka, Japan) (1.0mg/mL) and trypsin inhibitor (Sigma, St.Louise, MO, USA) (0.1mg/mL) at 37°C for 20min. Treated samples of the liver and spleen were pressed through 200-gauge stainless mesh and suspended in RPMI-

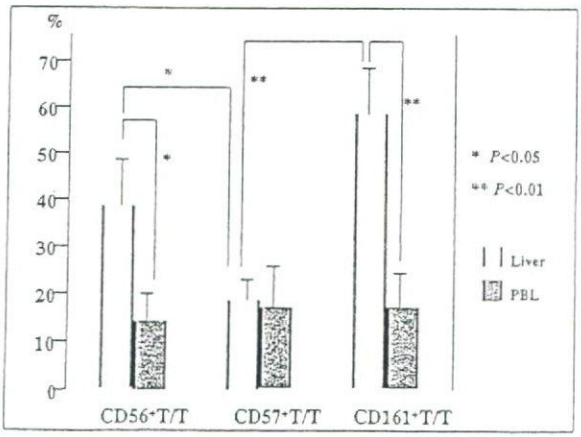

FIGURE 2 The proportion of NKT cells among CD3+T cells in donors of LRDLT (n=7).
CD56+T cells and CD161+T cells were abundant in the liver (39.3±11.2%, 58.3±6.0%) compared with those in PBL (13.4±6.5%, 18.7±10.4%). And also they were abundant compared with CD57+T cells in the liver.

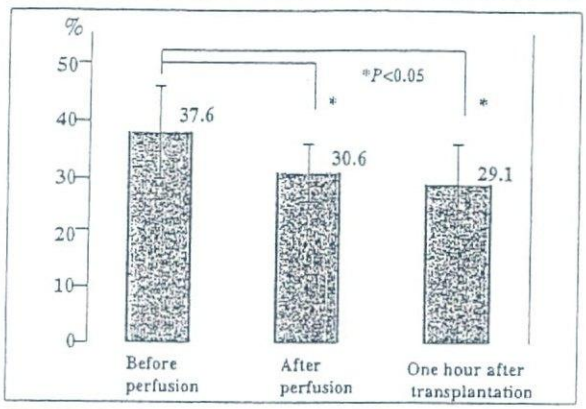

FIGURE 3 Changes of the proportion of thymus-derived cells in the graft liver by the perfusion of HTK solution in LRDLT. Thymus-derived cells of CD56+T cells in the graft before perfusion (37.6±10.3%) decreased immediately after perfusion by HTK solution (30.6±7.4%) and one hour after transplantation (29.1±8.9%) with statistical significance.

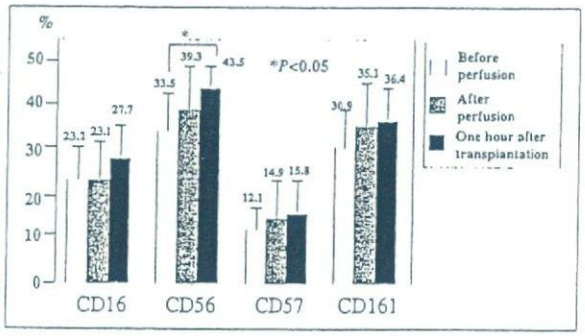

FIGURE 4 Changes of NK cells in the graft liver by the perfusion of HTK solution in LRDLT. All kinds of NK cells in the graft tended to increase immediately after perfusion by HTK solution and one hour after transplantation. Especially, CD56+T cells in the graft increased one hour after transplantation (33.5±10.2% *vs.* 43.5±5.3%) with statistical significance.

1640 medium with 5% fetal calf serum. The suspension was layered over Ficoll-Paque [1.077] (Pharmacia Biotech, Uppsala, Sweden) gradients and centrifuged at 650*g* for 20min, then mononuclear cells (MNC) were collected from the interface.

Peripheral blood lymphocytes (PBL) were isolated using Ficoll-Paque gradients.

Antibodies and Flow Cytometry

The surface phenotype of MNC was analyzed using FITC-, PE-, or PerCP-labelled MoAbs. MNC were conjugated and separated to only lymphocytes by use of anti-CD45 antibody. CD3 (NU-T3) was obtained from Nichirei (Tokyo, Japan). CD16 (Leu-11), CD56 (Leu-19), CD57 (Leu-7), CD161 (DX12) were obtained from Becton Dickinson (Mountain View, CA, USA). Flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA, USA).

Statistics

Values are expressed as mean ±s.d. Student's *t*-test was used, and *P*-values less than 0.05 were considered to be significant.

RESULTS

1. Phenotypic Detection of NK and NKT Cells in EHO Patient and Donors

NKT cells existed prominently in the liver compared with those in the spleen and PBL of EHO and donor patient (**Figure 1A and B**). They also were more numerous in the liver of the donors with the statistical significance. We investigated the proportion of CD56+T cells, CD57+T cells and CD161+T cells among T cells of liver and PBL from donors (n=7), respectively. CD56+T cells and CD161+T cells were abundant in the liver (39.3±11.2%, 58.3±6.0%) compared with those in PBL (13.4±6.5%, 18.7±10.4%). And also they were abundant compared with CD57+T cells in the liver (**Figure 2**).

2. Changes of Thymus-derived Cells in the Graft Liver by the Perfusion of HTK Solution in LRDLT

Thymus-derived cells of CD56+T cells in the graft before perfusion (37.6±10.3%) decreased immediately after perfusion of HTK solution (30.6±7.4%) and one hour after transplantation (29.1±8.9%) with statistical significance (**Figure 3**).

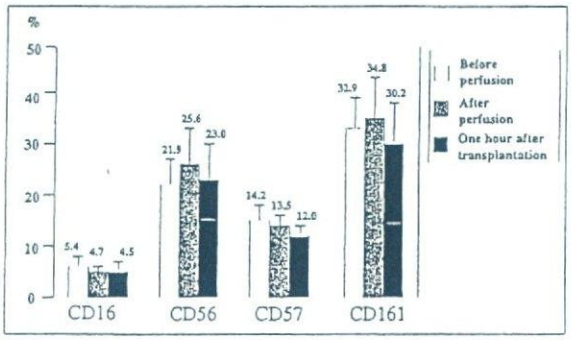

FIGURE 5 Changes of NKT cells in the graft liver by the perfusion of HTK solution in LRDLT. CD56+T and CD161+T cells in the graft liver tended to increase immediately after perfusion by HTK solution without statistical significance. However, CD16+T cells and CD57+T cells in the graft liver tended to decrease immediately after perfusion without statistical significance.

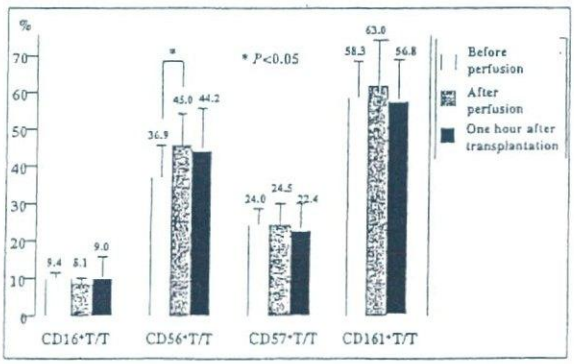

FIGURE 6 Changes of NKT cells among CD3+T cells in the graft liver by the perfusion of HTK solution in LRDLT. CD56+T cells and CD161+T cells among CD3+T cells in the graft liver tended to increase immediately after perfusion and decrease one hour after transplantation. Especially, CD56+T cells among CD3+T cells increased with statistical significance (36.9±9.1% *vs.* 45.0±7.8%).

3. Changes of NK Cells in the Graft Liver by the Perfusion of HTK Solution in LRDLT

All kinds of NK cells in the graft tended to increase immediately after perfusion by HTK solution and one hour after transplantation. Especially, CD56+T cells in the graft increased one hour after transplantation (33.5±10.2% *vs.* 43.5±5.3%) with statistical significance (**Figure 4**).

4. Changes of NKT Cells in the Graft Liver by the Perfusion of HTK Solution in LRDLT

CD56+T and CD161+T cells in the graft liver tended to increase immediately after perfusion by HTK solution without statistical significance. However, CD16+T cells and CD57+T cells in the graft liver tended to decrease immediately after perfusion without statistical significance (**Figure 5**).

5. Changes of NKT Cells among CD3+T Cells in the Graft Liver by the Perfusion of HTK Solution in LRDLT

CD56+T cells and CD161+T cells among CD3+T cells in the graft liver tended to increase immediately after perfusion and decrease one hour after transplantation. Especially, CD56+T cells among CD3+T cells increased with statistical significance (36.9±9.1% *vs.* 45.0±7.8%) (**Figure 6**).

DISCUSSION

A powerful paradigm has emerged in which leukocyte binding to the endothelium is explained by a three- or four-step process through the selectin family, integrin family, and related proteins (10). Meanwhile it has been reported that shear stress directly influences the mRNA expression of ICAM-1, CD44, and VCAM-1 on endothelial cells (4,5,11-13). Moreover, it has also been demonstrated that increased shear stress suppresses the accumulation of leukocytes onto the endothelium (14). Antibodies immunobilized on the wall of a flow chamber can also support leukocyte rolling in a shear flow. IgM mAb to Lewis (CD15) and sialyl LewisX (CD15s), which are carbohydrate antigens related to selectin ligands, plus monoclonal antibody to CD48 and CD59, are able to mediate such rolling. In contrast, IgM and IgG mAb to L-selectin (CD62L), LFA-1 (CD11a), CD43, ICAM3 (CD50), CD8, and CD45 only mediate firm adhesion. Antibodies supported rolling only within a restricted range of site densities and wall shear stress, outside of which firm adhesion or detachment occurred (15). We have demonstrated and hypothesized, therefore, that the elevation of shear stress after partial hepatectomy also affect the leukocyte binding to sinusoidal endothelial cells (SEC) after partial hepatectomy (5,6,16).

Furthermore, we have postulated the existence of two types of intrahepatic leukocytes; one type such as NKT cells (17-20) or macrophage as resident leukocyte would tend to stay associated with SEC, while the other such as thymus-derived leukocyte as passenger leukocytes would not. We have confirmed this hypothesis in the experiment of perfused liver in mice (8). In a series of recent studies, we have shown that the adult liver is one of the hematopoietic organs in mice, mainly producing extrathymic T cells [i.e., interleukin-2 receptor β chain (IL-2Rβ) + intermediate T-cell receptor cells (TCRint cells)] and granulocytes from their own pre-existing precursor cells (i.e., c-kit+Lin- stem cells) (21). Such TCRint cells and NK1.1T cells tended to stay in the liver against perfused solution (8). Conversely, thymic T cells increased in the irrigated solution compared with NKT cells. In the nude mice, this phenomenon was more prominent. In the present study, we especially paid attention to NKT cells. There is heterogeneicity in the human's NKT cells, therefore, we investigated the influences of perfusion and reperfusion against CD16+, CD56+, CD57+, and CD161+NK cells and NKT cells.

All types of NK cells increased after the perfusion by HTK solution and one hour after reperfusion of blood. CD56+NK cells, especially, increased in the graft liver.

CD56+T cells and CD161+T cells among the several human NKT cells are more numerous in the liver. CD56+ and CD161+ NKT cells almost overlapped each other in the liver. Conversely, CD57+NKT cells almost did not overlap with CD56+ and CD161+NKT cells. CD57+NKT cells are more numerous in the peripheral blood compared with CD56+ and CD161+ NKT cells. CD56+NKT cells significantly tended to stay in the graft liver against the perfusion of HTK solution compared with CD57+NKT cells. CD57+NKT cells tended to wash out from the liver into the systemic circulation. Moreover, thymus-derived T cells tended to wash out from the graft liver. We have demonstrated that the proportion of donor leukocytes in the systemic circulation immediately after LRDLT examined by short tandem repeat method was more than 10% (22). This finding may support the idea that wall shear stress influences the adhesion of intrahepatic leukocytes to SEC and the wash out of intrahepatic leukocytes from the graft liver to the systemic circulation as allo-antigen presenting cells.

Furthermore, the above findings may suggest that CD56+NKT cells are more concerned with local immunity and CD57+NKT cells partake and regulate systemic immunity. CD56+NKT cells might regulate the information from the portal vein into the graft liver. CD57+NKT cells might obtain intrahepatic instructions in the graft liver and wash out from the liver by increased shear stress and modulate the systemic immunity against thymus-derived immunity. Interestingly, we have reported that intrahepatic CD56+NKT cells strongly expressed CD28 costimulatory molecules compared with peripheral CD56+NKT cells (65.6±20.3% *vs.* 38.5±24.7%, $p<0.05$) (23,24). We think these mechanisms are important to allo-immunity.

Almost all of the intrahepatic donor leukocytes changed from donor type to recipient type within one week (23). We have reported that donor specific transfusion via portal vein was effective for the graft acceptance and gave successful reduction of immunosup-

pressants (22-27). Moreover we have confirmed that the donor type of CD56+NKT cells existed in the graft liver even 6 weeks after transplantation as macrochimerism (25). CD56+NKT cells may participate in the portal tolerance or oral tolerance as resident leukocytes accompanied with Kupffer cells. In conclusion, our present study may contribute to further understanding transplantation immunology. We are currently studying the functions and influences of dendritic cells in the graft liver following LRDLT.

ACKNOWLEDGEMENTS

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ELSEVIER

Rapid Progressive Hepatitis C After Liver Transplantation: A Case Report

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ABSTRACT

A 56-year-old man on hemodialysis for 3 years because of chronic renal failure underwent living related donor liver transplantation (LRDLT) and splenectomy using the right hepatic lobe for liver cirrhosis type C (genotype 1b) with hepatocellular carcinoma. At 69 postoperative days (POD), he displayed a high fever and his blood transaminase and total bilirubin were increased. Based on finding in his liver biopsy, we diagnosed rapid recurrence of progressive hepatitis C after LRDLT, so we administered IFNβ. Thereafter his liver function returned to normal and his HCV-mRNA decreased to 1200 kcopy/mL. We inferred that hemodialysis and splenectomy decreased his immunity, allowing rapidly progressive hepatitis C recurrence after LRDLT.

MANY CASES OF hepatitis C recur after LRDLT for liver cirrhosis. The liver functions worsen slowly relative to that seen with hepatitis B. Some cases have been reported during the early period post–liver transplantation, namely about 4 to 16 weeks. Liver transplant recipients show icterus and the histopathological findings of bile stasis. After that, patients may experience liver failure over 3 to 9 months, which worsens rapidly.[1] We experienced a case of rapidly progressive hepatitis C recurring 2 months after LRDLT.

CASE REPORT

A 56-year-old man on hemodialysis for 3 years because of chronic renal failure underwent splenectomy and LRDLT using right hepatic lobe for liver cirrhosis type C (genotype 1b) with hepatocellular carcinoma. His HCV-mRNA level was 65,000 kcopy/mL preoperatively. Postoperatively we administered methylpredonisolone for 2 days after LRDLT and after day 3, only FK506. Because he had bile leakage and a slight fever we kept his FK506 trough level below 1.5 ng/mL after day 16. On day 69, his transaminase level reached on 200 IU/L. A liver biopsy showed neutrophils and monocytes infiltrating periportal vein areas with angitis of the portal veins. We considered acute rejection and started steroid pulse therapy but his liver dysfunction did not recover. His blood total bilirubin reached 8.6 mg/dL. Again we performed a liver biopsy, showing hepatocyte regeneration and apoptosis. The hepatitis C had recurred so we prescribed IFN β. At this point his HCV-mRNA was 25,000 kcopy/mL, but decreased to 1200 kcopy/mL after the therapy. The blood data showed near normal levels of transaminase and total bilirubin levels. We inferred that hemodialysis and splenectomy had decreased his immunity with rapidly progressive hepatitis C recurring after LRDLT.

DISCUSSION

Among 100 LRDLT cases in Japan, include 6% for viral hepatitis until July 2002. Chronic viral cirrhosis is the most common disease for LRDLT in the world.[2] The factor affecting the prognosis after LRDLT is estimating the amount of virus. Genotype 1b occurs early with liver dysfunction often because of the large amount of virus. And immunosuppresants increase hepatitis C virus, it HCV-mRNA increases 10- to 20-fold after LRDLT.[3] But to present nobody has described a difference between immunosuppressant drugs. In our case, the patient had previously started hemodialysis 3 years prior due to chronic renal failure. We performed splenectomy to decrease the portal vein blood pressure. We inferred that hemodialysis and splenectomy reduced his immunity, allowing rapidly progressive hepatitis C after LRDLT.

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インターフェロン抵抗性に関与する宿主免疫関連因子

山際　訓*　市田隆文**

索引用語：インターフェロン抵抗性，免疫関連因子，遺伝子多型，遺伝子発現解析

1 はじめに

C型慢性肝炎に対してはインターフェロン（IFN）とリバビリン（RBV）併用療法が現在最も有効な治療法である．しかし，これら併用療法が標準治療として普及するにつれて治療効果の改善がより期待されているものの，難治例である遺伝子型1b型かつ高ウイルス量の症例におけるウイルス学的著効（sustained virological response；SVR）率は50％強にすぎない．このようなIFN治療効果を規定する因子として，ウイルス側因子と使用薬剤側因子が重要であることが明らかとなっている．しかしながら，同じIFN＋RBV併用療法によりウイルス側因子からは難治例と予測される症例であってもSVRとなる症例があるのに対し，遺伝子型2a型かつ低ウイルス量であってもSVRに至らない症例など，治療効果を規定する因子が宿主側にも存在することが示唆される．したがって，現行以上の治療効果の改善には免疫応答を含めた宿主側因子の解析と理解が重要であることに間違いはないであろう．本稿では，IFN治療効果を規定する宿主側応答因子，特に免疫関連因子に関する最近の知見とともに，治療抵抗性機序の解明を目指した筆者らの研究成績を解説する．

2 HCV感染に対する免疫応答

ウイルス感染の初期免疫応答においてはnatural killer（NK）細胞，natural killer T（NKT）細胞が強力なエフェクター細胞として抗原非特異的な反応によりウイルス感染細胞の排除を行う．また，ウイルス感染細胞がIFN-α/βを，一方樹状細胞（dendritic cells；DC）がIFN-αをそれぞれ産生しウイルス増殖を抑制する．そして，早期にウイルス感染が制御できない場合には，引き続いて中和抗体および細胞傷害性T細胞（cytotoxic T lymphocytes；CTL）が誘導されてウイルス排除に働く．このような獲得免疫応答はDCやKupffer細胞などの抗原提示細胞によるウイ

Satoshi YAMAGIWA et al：Immunological factors associated with a poor response to interferon treatment for chronic hepatitis C
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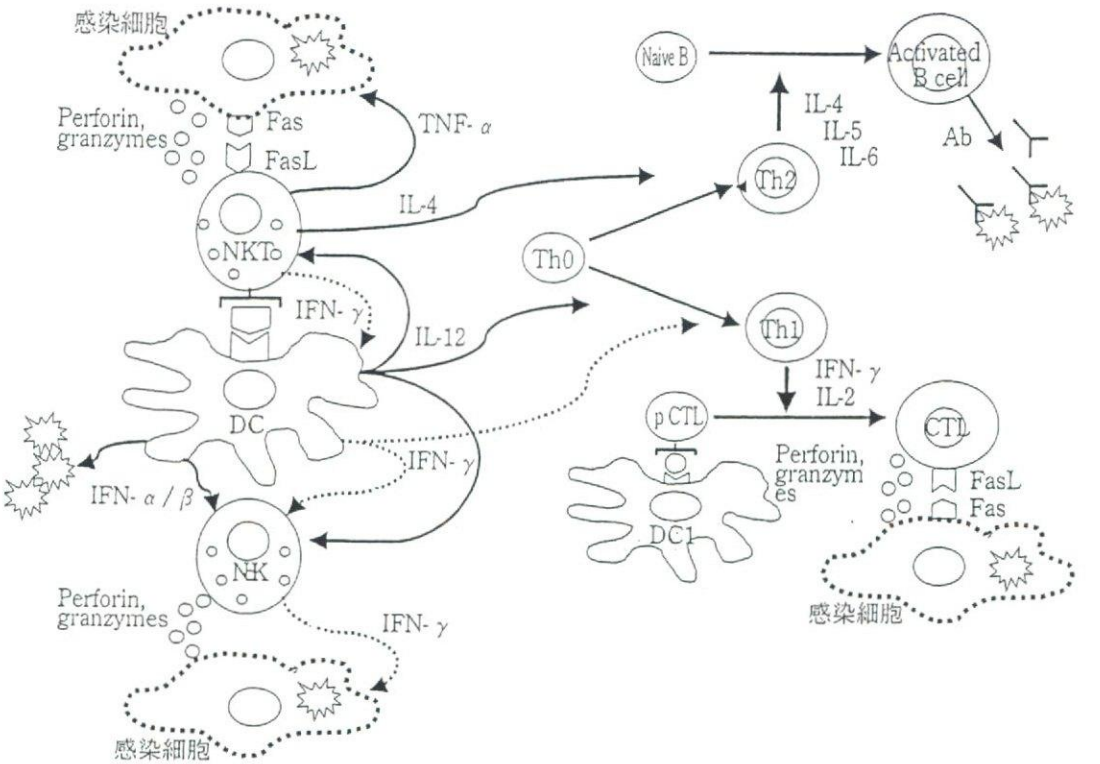

図1　HCV感染における免疫応答

ルス抗原提示時のT細胞活性化動態により規定されるが，特にNKT細胞はTh1やTh2サイトカインをともに産生し得るため，自然免疫系における初期刺激の質的差異により抗原特異的な獲得免疫応答の方向性が制御されている可能性が示唆されている（図1）[1,2]．

C型肝炎ウイルス（hepatitis C virus；HCV）感染においてもこの初期免疫応答の重要性が報告されており，HCV特異的Th1応答が強い場合は一過性感染で治癒するという報告[3]や，Th1応答は急性肝炎患者で強く反応する，あるいはTh2応答はむしろ慢性肝炎患者で強い[4]などの報告から，HCVの生体からの排除にはTh1応答が重要と考えられている．C型慢性肝炎においてはT細胞活性化に重要であるDCの機能不全が存在し，ウイルス排除に働くT細胞の活性化が不十分なため肝炎の慢性化につながっている可能性が示唆されている[5~7]．また，筆者らの検討を含めてC型慢性肝炎症例の肝臓内ではNK細胞やNKT細胞の関与する先天免疫応答の低下が示唆されており[8~12]，このような状況はウイルス感染細胞の排除の低下をきたす免疫環境にあると考えられる．そのような免疫環境下にあるC型慢性肝炎に対するIFN-α，RBVの治療効果の発現は，直接的な抗ウイルス作用に加えて，免疫系への修飾作用とくにTh1応答優位な免疫応答の誘導が関与していることが推測されている．

3　治療反応性に関与する免疫関連因子

1．リンパ球分画

IFN単独療法の奏効率が治療中のHCV特異的Th1細胞とCD8陽性細胞（CTL）の増加と関連することが報告されている[13,14]．同様にIFN＋RBV併用療法においてもSR例で治療中の末梢血中にHCV特異的なTh1細胞とCD8陽性細胞の有意な増加が認められたのに対し，NR例ではHCV特異的なTh2細胞が有意に増加することが報告されている

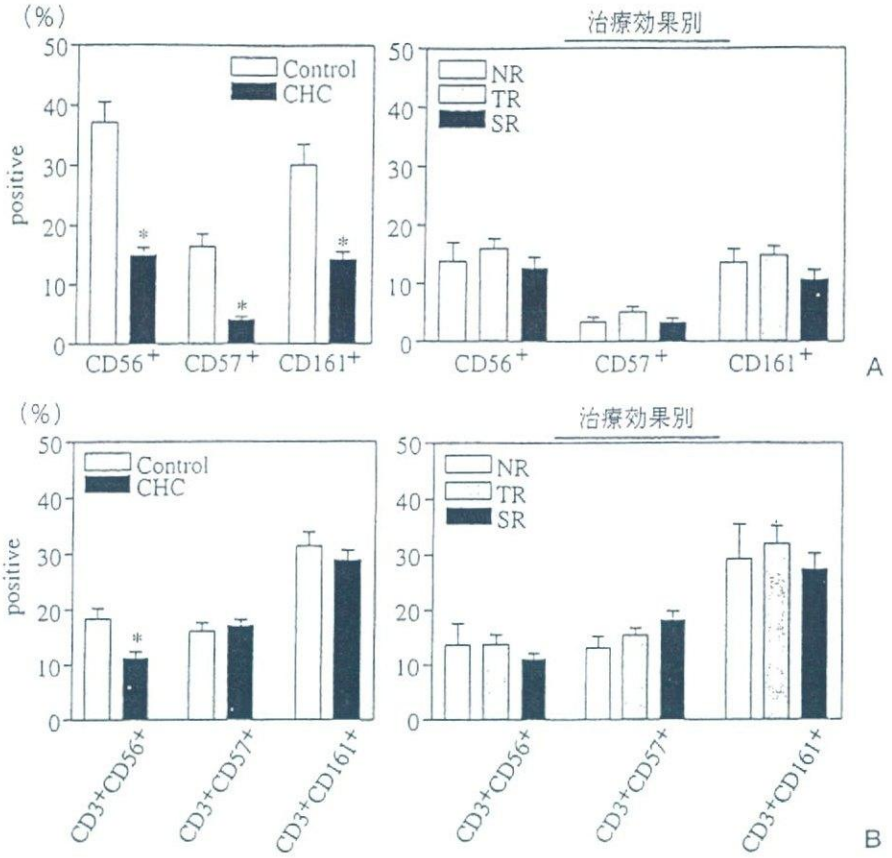

図2

A：正常肝およびC型慢性肝炎の肝内NK細胞陽性率

慢性肝炎の肝内ではNK細胞の有意な減少が認められたが，治療効果別に比較した場合，有意な変化は認められなかった．Mean ± SEを示す．*$p < 0.01$

B：正常肝およびC型慢性肝炎の肝内NKT細胞陽性率

慢性肝炎の肝内ではCD56陽性NKT細胞の有意な減少が認められたが，治療効果別に比較した場合，有意な変化は認められなかった．Mean ± SEを示す．*$p < 0.05$

[15,16]．Vrolijkらは治療前の肝組織中とくに門脈域におけるCD8陽性細胞数がIFN＋RBV併用療法におけるSR例で有意に高値であったと報告しており[17]，治療前や治療中におけるCD8陽性細胞の増加が治療効果と関連することが示唆されている．また，HCV遺伝子型3a型の症例のみの検討ではあるものの，Kupffer細胞におけるCD8陽性細胞に抗原を提示するMHC class I分子の発現がIFN＋RBV併用療法のSR例で有意に増加していたという報告もある[18]．

CTLとともにウイルス感染肝細胞の排除に重要と考えられるNK細胞，NKT細胞について，筆者らはIFN＋RBV併用療法の治療前後における肝組織および末梢血について解析を行い治療効果との関連について検討した．図2に示すように，治療前の肝組織中では治療効果別に比較した場合に有意な変化は認められなかったが，NK細胞，NKT細胞比率ともSR例でNR例よりも減少傾向にあるものがあった．一方，治療前後での変化をみるとSR例では治療後の肝組織中のCD161陽性NK細胞とNKT細胞の有意な増加が認められ，治療前に認められた自然免疫担当細胞の減少が改善したことを示唆する変化が認められたのに対して，NR症例では変

化が認められなかった[12]．これらの結果はCD8陽性細胞と同様にIFN＋RBV併用療法によるTh1応答の増強によるNK細胞，NKT細胞の増加がウイルス排除に重要であることを示唆すると考えている．

2．サイトカイン

インターロイキン10(IL-10)のプロモーター遺伝子多型がIFN単独療法における初期反応性と関連していることが報告され[19]，またYeeらはそのようなIL-10プロモーター遺伝子多型がIFN＋RBV併用療法においても治療効果に関係すると報告している[20]．IL-10はTh2サイトカインであり，Th1サイトカイン産生を抑制するとともに肝臓におけるIFN-αによるsignal transducer and activator of transcription 1(STAT-1)活性化を抑制する[21]．IFN単独療法のNR例で血清中IL-10が高値であることや，SR例でIL-10産生がNR例と比べて低値であることが報告されていることから[22,23]，プロモーター遺伝子多型に由来するIL-10産生低下がIFN治療効果に関与している可能性が示唆される．Mangiaらは IL-10ハプロタイプの違いがHCV感染の持続化に関与し，急性感染後のウイルス排除の予測因子となるが治療効果との関連は認められなかったと報告している[24]．彼らはIFN-γやINF-αの遺伝子多型もIFN単独療法の治療効果に関連しなかったと報告している．

3．ケモカイン

HCV排除には細胞性免疫を賦活するTh1応答が重要であるが，Th1応答はIFN-γなどのサイトカイン産生とともにケモカイン受容体の一種であるCC-chemokine receptor 5(CCR5)のT細胞や単球における発現を増強する．CCR5はCCケモカインであるmacrophage-inflammatory protein(MIP)-1α，MIP-1βおよびregulated upon activation, normal T cell expressed and secreted(RANTES)のレセプターであり，活性化T細胞，特にTh1細胞とCD8陽性細胞や単球の遊走に関与している．C型慢性肝炎の肝組織中リンパ球にはCCR5の高発現が認められている[25]．これまでに，CCR5のプロモーターsingle nucleotide polymorphisms(SNPs)(59029-A allele)がIFN単独療法の奏功率に関与しているという報告[26,27]や，特に機能的なCCR5発現が欠損する32-base pair deletion(Δ32)をきたす変異がIFN単独治療の奏効率と関連していることが報告されている[28]．またWasmuthらはリガンドであるRANTESのSNPs(403 G/A, Int1.1 T/C, 3'222 T/C)を解析し，RANTESハプロタイプ(Int1.1 Cと3'222 C)がIFN＋RBV併用療法を施行された遺伝子型1および4型の症例の奏効率に関係すると報告している[29]．CCR5とそのリガンドの遺伝子変異はTh1細胞やCD8陽性細胞などの肝臓への遊走を低下させ，IFN治療によるTh1応答賦活作用の低下をきたし，その結果，治療抵抗性を示すという機序が示唆される．しかしながら，IFN＋RBV療法においてはCCR5-Δ32は治療効果に関与していないという報告[28,30]もあるなど，現在のところCCR5がINF＋RBV治療抵抗性に関与しているかどうかはコンセンサスが得られていない．RBVのTh1賦活作用がCCR5-Δ32によるTh1応答低下を埋め合わせている可能性もあるが，人種間の違いなどを含めて今後の検討が待たれる．

4．IFNレセプター

IFNはサイトカインであり，細胞表面のIFNレセプター(INF-αレセプターであるIFNAR1とIFN-α/βレセプターであるIFNAR2)と結合することによりその作用を

表1　IFN＋RBV治療前後における肝臓内IP-10遺伝子発現解析結果

Case		1	2	3	4	5	6	7
治療効果		NR	NR	TR	SR	SR	SR	SR
IP-10	治療前	26.52	136.8	14.21	14.87	24.79	16.47	10.63
	治療後	21.72	14.82	2.95	1.30	2.34	1.64	3.05

NR症例において治療後も肝臓内IP-10遺伝子発現の増加が持続していた．

表2　IFN＋RBV治療前の肝臓発現遺伝子解析に基づく治療効果予測

	正解数/総数	正解率
NR症例	5/6	83.33 %
SR/TR症例	27/29	93.10 %

IFN＋RBV治療前の肝臓発現遺伝子解析結果をもとにMahalanobis Distance（MD値）を用いたアルゴリズムにより治療効果を予測した．NR例，SR/TR例とも高率に予測可能であった．

発現する．IFN治療効果と肝組織内IFNレセプター発現量との関連についてはいくつかの報告があり，またIFNAR1遺伝子のプロモーター領域のマイクロサテライト多型がIFN効果予測因子になると報告されている[31]．Hijikataらは IFNにより誘導される蛋白であるMxAのプロモーターSNPsがIFN治療感受性と関連すると報告している[32]．IFNレセプターとIFN誘導遺伝子はIFN治療の反応性に関与する重要な因子であることが示唆されている[33]．

5．その他の因子

活性化T細胞に発現しT細胞機能の抑制に関与するco-stimulatory moleculeであるcytotoxic T lymphocyte antigen-4（CTLA-4）のSNPs（319 C/Tと 49 A/G）解析により，CTLA4ハプロタイプ（－318C-49G）がHCV遺伝子型1型のIFN＋RBV併用療法におけるSR例と関連していることが報告されている[34]．ハプロタイプの違いはCTLA-4の発現低下をきたしT細胞機能の活性化に関与するとともに，Th1/Th2バランスのシフトにも関与する可能性が示唆されている[34]．

4　肝臓発現遺伝子解析と治療効果予測

これまでに述べたように，筆者らはIFN＋RBV治療前後での肝臓内リンパ球の解析を行うとともに，治療前後の肝臓を用いた網羅的遺伝子発現解析を行い，治療による生体側因子の変動を解析するとともに，治療前の解析結果による治療効果予測を行った．方法は既報[35]のように肝組織よりRNAを抽出・増幅後，肝臓発現遺伝子から選択し機能別に分類した優位発現遺伝子を搭載したcDNAチップによる解析を行い，得られた発現データを用いてプロファイリング解析と診断アルゴリズムによる治療効果予測を試みた．

IFN＋RBV治療前後で解析可能であり，かつ治療効果が判定された遺伝子型1b型症例7例の，肝臓内リンパ球変動との関連が示唆される免疫応答関連遺伝子群の変化をみると，特に活性化T細胞やNK細胞に対する遊走活性をもつケモカインであるIFN-gamma inducible chemokine IP-10（IP-10）がNR症例で治療前の発現が特に高値で，治療後にも高値が持続していた（表1）．Narumiらは血清中のIP-10が低値の症例ほどIFN単独療法に対する反応性が高く，著効例では治療後に正常人のレベルまで低下するのに対し，無効例では治療後も高値が持続すること

Case	1	2	3	4	5	6	7
治療前 MD 値	24.75	113.00	6.10	1.49	0.52	4.12	2.11
効果予測	NR	NR	SR/TR	SR/TR	SR/TR	SR/TR	SR/TR
治療後 MD 値	14.27	13.52	6.63	0.48	1.33	0.60	0.29
治療結果	NR	NR	TR	SR	SR	SR	SR

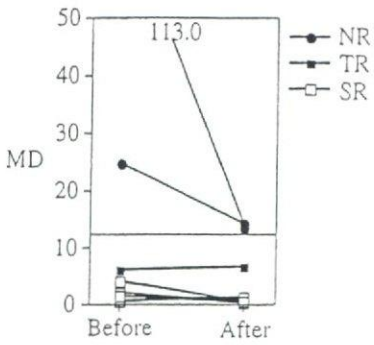

図3　IFN＋RBV 治療前後における肝臓発現遺伝子解析結果
IFN＋RBV 治療前後での肝臓発現遺伝子解析結果をもとに Mahalanobis Distance（MD 値）の変動を解析した．NR 例も治療後は MD 値の減少を認めた．

を報告しており[36]，筆者らの検討からも同様に IP-10 と IFN 治療抵抗性との関連が示唆された．また，治療反応性の違いに対する IFN 反応関連遺伝子群の関与も示唆された．

一方，治療効果予測は Mahalanobis Distance（MD 値）を用いたアルゴリズムにより行った．MD 値は既知の SR/TR 群データベースを基準データとして特性基準空間を作成し，これに対し対象となるサンプルのデータによる対象空間との乖離度を算出したもので MD 値が大きくなるほど基準となる SR/TR 群とは異なる特性を持つことになる．今回のアルゴリズムでは MD 値 13 以上を NR，それ以下を SR または TR と判定した．そのような診断アルゴリズムによる効果予測を基準空間作成には使われていない cross validation サンプル 35 検体で行ったところ，NR 予測は 6 検体中 5 例が的中し正解率は 83％に，SR または TR 予測の正解率は 93％と高率であった（表 2）．さらに，同一症例において治療前後で MD 値の変化をみると治療後に MD 値の減少している症例が多く，特に NR 症例においても治療後の MD 値は治療前と比べ明らかに減少しており，その遺伝子発現は SR/TR 症例の特性に近くなっていることが示唆された（図 3）．検討した症例数が少なくさらに検討が必要であるが，今回検討した遺伝子群のみの解析では NR 例の予測はある程度期待されるものの，治療後の再燃例の予測は困難であり，再燃例の予測にもつながる治療抵抗性を示す機序の解明を目指したさらなる検討が必要である．

5　おわりに

IFN とリバビリン併用療法によっても治療抵抗性を示す症例に関連する宿主側因子について，免疫関連因子を中心に概説した．遺伝子多型を含めてさまざまな因子が報告されているが，免疫関連因子をまとめると"IFN と RBV の併用によっても肝臓内に Th1 優位な細胞性免疫応答が十分に賦活されない”症例が治療に抵抗性を示すことが示唆された．そのような細胞性免疫賦活が誘導されない要因としてゲノムレベルでの個体差の検討とともに，樹状細胞の機能不全のような HCV ウイルス自体による原因の解明が重要である．現時点では今後も IFN が治療の中心になると考えられ，更なる治療効果の改善のためには，そのような治療抵抗例においても Th1 応答を誘導しうる機序を解明し，新たな薬剤との併用や免疫賦活療法など新たな治療法の開発が急務であると考えられた．

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Influence of serum from rats with fulminant hepatic failure on hepatocytes in a bioartificial liver system

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ABSTRACT: Fulminant hepatic failure (FHF) is a life-threatening condition marked by many excessively increased unmetabolized toxins and growth factors. Recently developed bioartificial liver (BAL) systems containing hepatocytes can be used to treat patients with FHF. However, the behavior of these hepatocytes on exposure to FHF serum in vitro *remains unclear. In the present study, we used FHF rat models and the sera from these rats (i.e., FHF serum) contained elevated inflammatory cytokines (TNF-α, IL-1ß, and IL-6), HGF, and TGF-ß1. In addition, 1x10⁸ hepatocytes were harvested from the livers of inbred rats and incubated with microcarrier beads. Four hours later, the hepatocyte-coated beads were inoculated into a hollow-fiber module (=BAL system). FHF serum or normal control serum circulated for 6 hours through the BAL system. Expressions of mRNA for albumin, GST A1, CYP 1A2, OTC and c-fos were investigated by RT-PCR, and PCNA staining was performed before and after perfusion. The expressions of albumin, GST A1, and CYP 1A2 mRNAs were markedly decreased, whereas those of OTC and c-fos were modestly decreased. PCNA positive cells were low and showed no difference between FHF and normal serum-exposed hepatocytes. In conclusion, the exposure of hepatocytes to hypercytokinemia, including inflammatory cytokines and positive and negative growth factors, caused a loss in liver specific functions. This environment also failed to facilitate hepatocyte regeneration. (Int J Artif Organs 2004; 27: 303-10)*

KEY WORDS: Fulminant hepatic failure, Hepatocyte, mRNA, Cytokine, Rat

INTRODUCTION

Fulminant hepatic failure (FHF) is a life-threatening condition that stems from the loss of metabolic functions. Patients are exposed to various hepatotoxic substances, resulting in coagulopathy, encephalopathy, cerebral edema, and multiple organ failure (1).

Orthotopic liver transplantation has become the established therapy for FHF; however, owing to organ shortage and rapid progression of liver failure, many people lose the chance to recover (2, 3).

Over the last decade, several bioartificial liver (BAL) support systems have been developed for the purpose of bridging patients with FHF to liver transplantation or to regeneration of the diseased liver. These systems utilize viable hepatocytes from humans, human cell lines, rabbits and pigs (4-10). Some of them have been clinically tested and proved to have some efficacy (8-10). The inoculated hepatocytes seem to be active metabolically and to perform detoxification in a circuit. Nevertheless the effect of FHF serum on hepatocyte function remains unclear.

It is well known that FHF serum contains many regulating factors such as inflammatory cytokines (TNF-α, IL-1ß, and IL-6), hepatocyte growth factor (HGF), and transforming growth factor-ß1 (TGF-ß1) as well as toxins (11-14). During clinical treatment, hepatocytes in the bioreactor are directly or indirectly exposed to FHF serum. Although several *in vitro* studies on interaction between FHF serum and hepatocytes have been reported, their results are not consistent (15-19). Moreover, only a few studies have investigated effects on hepatocytes in a BAL system (20).

In the present study, we evaluated rat hepatocyte changes in response to perfusion with FHF serum, focusing on liver gene expression.

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MATERIALS AND METHODS

All study protocols were reviewed and approved by the University of Nagasaki Research Animal Resources and Animal Care Committee, and met both institutional and national guidelines.

Induction of fulminant hepatic failure

Male Lewis rats (SLC, Shizuoka) weighing 300 to 350g were used in this study. The animals were housed in plastic cages in a temperature and humidity-controlled room with 12-hour light/dark cycle, and were given standard rat chow and water. The fulminant hepatic failure rat was prepared according to the method described by one of the authors (S. E.) (21). Briefly, the common pedicle to the right liver lobes (24% liver) was ligated, and the two anterior liver lobes (68% liver) were removed using the standard Higgins and Anderson technique (22). After 18 hours, whole blood was harvested by aortic puncture, and FHF serum was collected. Normal serum was collected from non-operated rats. High levels of HGF and TGF-ß1 were shown in this model.

Hepatocyte isolation

Isolated hepatocytes were prepared by perfusion of inbred Lewis rats (150-200 g). Livers were treated with collagenase using a modification of the method described by Selgen (23). After enrichment through a Percoll gradient (Amersham Biosciences Corp., Piscataway, NJ), hepatocytes viability was nearly 90% by trypan blue exclusion.

Preparation of the bioreactor and perfusion model

$1x10^8$ hepatocytes were incubated with collagen-coated dextran microcarrier beads (Cytodex3, Amersham Biosciences) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum at 37°C in 95% air and 5% CO_2. Four hours later, the microcarrier-attached hepatocytes were inoculated into the extra-fiber space of a hollow-fiber module (PE-G, Asahi Medical Co., Ltd, Tokyo). The hollow polyethylene fibers had an internal diameter of 330 µm, a pore size of 0.3 µm, and a membrane thickness of 50 µm. The system was composed of a pump, serum pool whose volume was 30ml, hollow fiber module, temperature controlled bath and oxygenator. The temperature of the circulated serum was maintained at 37°C.

Experimental design

Two BAL system groups were compared: the FHF BAL group (n=5 modules) and the control BAL group (n=5), in which either Lewis rat FHF serum or normal Lewis rat serum, respectively, were circulated through each module for 6 hours.

Isolation of RNA

Total ribonucleic acid (RNA) was extracted from the hepatocytes attached to the microcarriers before and after circulation, according to the instructions of the manufacturer of the Katorimox-14 RNA Isolation kit (Takara Bio Inc., Tokyo). Total RNA content was quantified by using a spectrophotometer (BioSpec-1600, Shimadzu Biotech, Kyoto).

Quantitative assessment of the relative expressions of mRNAs

For the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, a one-step RT-PCR (Takara Bio Inc., Tokyo) kit was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. Albumin, glutathione S-transferase A1 (GST A1), cytochrome P450 1A2 (CYP 1A2), ornithine transcarbamylase (OTC), and c-fos mRNAs were measured at the exponential portion of the curve, after PCR amplification. The optimal number of thermal cycles for GAPDH, albumin, GST A1, P450 1A2, OTC and c-fos mRNA was determined, respectively, 14, 12, 16, 20, 20 and 24. Forward and reverse primers are presented in Table II (24-29). A sample of mRNA (1 µg) was added to 5 mM $MgCl_2$, 1 mM dNTP, 0.8 U of RNase inhibitor, 0.1 U of AMV reverse transcriptase XL, 0.1 U of AMV-optimized Taq, and 0.2 µM 5' and 3' specific primers. PCR was performed using an automated thermal cycler (Gene Amp PCR System 9700, Applied Biosystems Japan Co., Ltd, Tokyo). The samples were denatured by heating to 94°C for 60 sec, annealed by cooling to 56-60°C for 60 sec, and extended by heating to 72°C for 90 sec. The PCR products were separated by 2.0% agarose gel electrophoresis and visualized by ethidium bromide staining. The optical density of various bands was quantified using the Scion image analysis package (Scion Corp., Frederick, MD).

Immunohistochemical staining

The hepatocytes attached to microcarriers were collected and 5 μm-thick paraffin sections were made for PCNA immunohistochemical staining with monoclonal anti-PCNA antibodies (PC-10, DakoCytomation, Carpinteria, CA) before and after serum exposure. The number of PCNA-positive hepatocytes per 100 hepatocytes on the microcarrier surface was determined.

Biochemical analysis

Serum aspartate aminotransferase (AST), total bilirubin (T. Bil), and ammonia (NH_3) were measured with commercial kits (Wako Pure Chemical Industries, Ltd., Osaka). Serum IL-1ß, IL-6, and TNF-α were measured using ELISA kits (BioSource International Inc., Camarillo, CA).

Statistical analysis

All data were expressed as a mean ± standard deviation (SD). Between-group comparisons of the data carried out by the Wilcoxon t-test. A p-value less than 0.05 was considered to indicate significant differences.

RESULTS

Cytokine levels

Apart from high levels of HGF and TGF-ß1 in the FHF rats (n=5) at 18 hours after induction, serum TNF-α (125.4 ± 27.7 pg/mL), IL-1ß (327.5 ± 160.2 pg/mL), and IL-6 (2597.8 ± 631.8 pg/mL) levels were markedly increased. In the normal rats (n=5), TNF-α, IL-1ß, and IL-6 levels were low or undetectable (Tab. I).

Liver support functions

The AST value was significantly higher after perfusion than before (3387.0 ± 94.2 IU/L, 2085.0 ± 135.4 IU/L, $p<0.05$), suggesting that hepatocyte damage had occurred in the module. In contrast, total bilirubin and ammonia levels were significantly lower after perfusion than before: T. Bil (1.31 ± 0.24 mg/dL, 1.77 ± 0.25 mg/dL, $p<0.05$) and NH_3 (294.6 ± 15.9 μg/dL, 346.4 ± 29.7 μg/dL, $p<0.05$) (Fig. 1).

mRNA expression in BAL hepatocytes

The oligonucleotide PCR primers were used to amplify rat albumin, GST A1, CYP 1A2, OTC, c-fos and GAPDH. As the housekeeping gene, GAPDH was used to determine the constitutive level of gene transcription and to control for variations in RNA recoveries from each specimen.

The albumin, GST A1, and CYP 1A2 levels decreased more after the perfusion of FHF serum than after the perfusion of normal serum. Mild decrease in OTC and c-fos expression was also seen (Fig. 2).

The mRNA expression (percent decrease) was calculated by densitometric evaluation. Even though the effect of FHF relative to normal serum on OTC (70.6 ± 20.5%, 60.0 ± 28.0%) and c-fos mRNAs (56.8 ± 12.9%, 58.6 ± 27.7%) was insignificant, albumin (88.0 ± 6.5%,

TABLE I - INFLAMMATORY CYTOKINE PROFILES[a] IN FHF RATS AND NORMAL RATS

Groups	TNF-α (pg/mL)	IL-1 ß (pg/mL)	IL-6 (pg/mL)
FHF (n=5)	125.4 ± 27.7	327.5 ± 160.2	2597.8 ± 631.8
Normal (n=5)	7.7 ± 4.9	N.D.	N.D.

In the FHF rats, serum levels of TNF-α, IL-1ß, and IL-6 were markedly increased. In contrast, in the normal rats TNF-α, IL-1ß, and IL-6 were low or undetectable. [a] 18 h after induction of FHF.

TABLE II - PRIMERS USED IN PCR REACTION

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Fragment size (bp)
Albumin	TTGCCAAGTACATGTGTGAG	TTGCCAAGTACATGTGTGAG	373
GST A1	ATGAGAAGTTTATACAAAGTCC	GATCTAAAATGCCTTCGGTG	213
CYP 1A2	GTCACCTCAGGGAATGCTGTG	GTTGACAATCTTCTCCTGAGG	236
OTC	GATAAGCATGGGACAAGAGG	CAGGTGAGTAGTCTGTCACG	247
c-fos	AGCCGACTCCTTCTCCAGCAT	CAGATAGCTGCTCTACTTTGC	298
GAPDH	TTCAACGGCACAGTCAAG	CACACCCATCACAAACAT	240

GST A1, glutathione S-transferase A1; CYP 1A2, cytochrome P450 1A2; OTC, ornithine transcarbamylase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase.

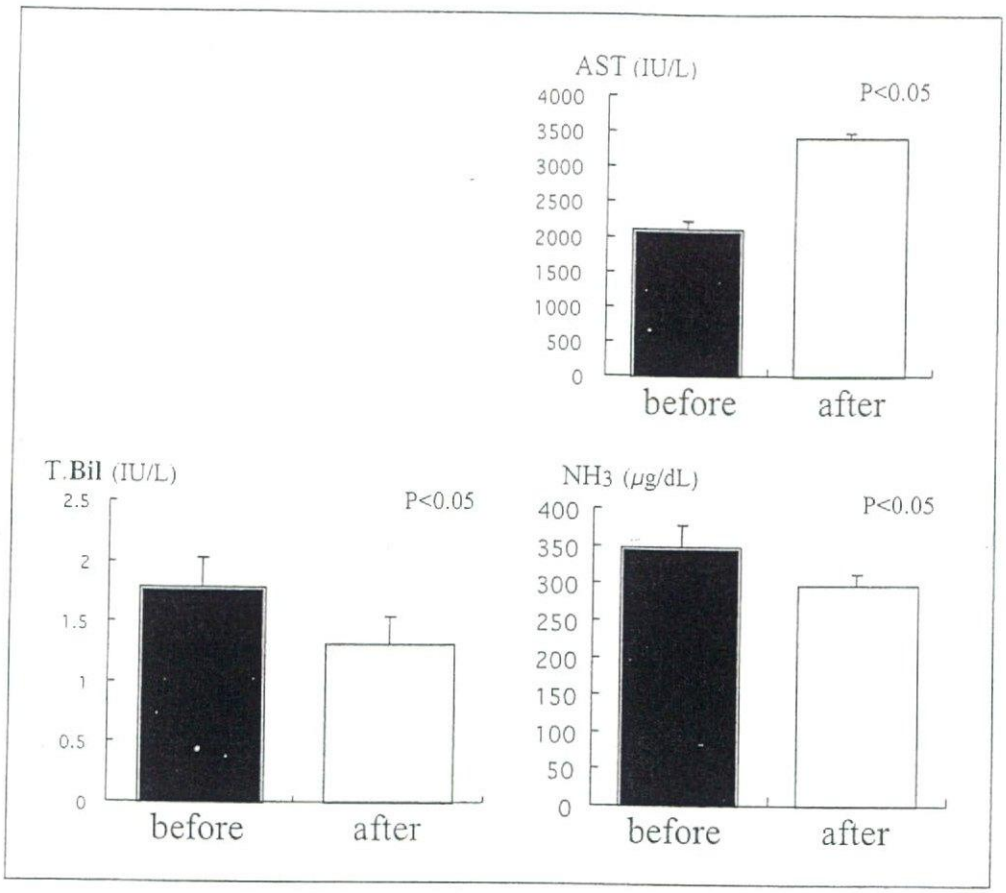

Fig. 1 - *Serum AST, T.Bil and NH_3 values before and after perfusion. The serum AST value increased significantly more after perfusion than before ($p<0.05$). In contrast, T.Bil and NH_3 values improved significantly more after perfusion than before ($p<0.05$). AST, aspartate aminotransferase; T.Bil, total bilirubin.*

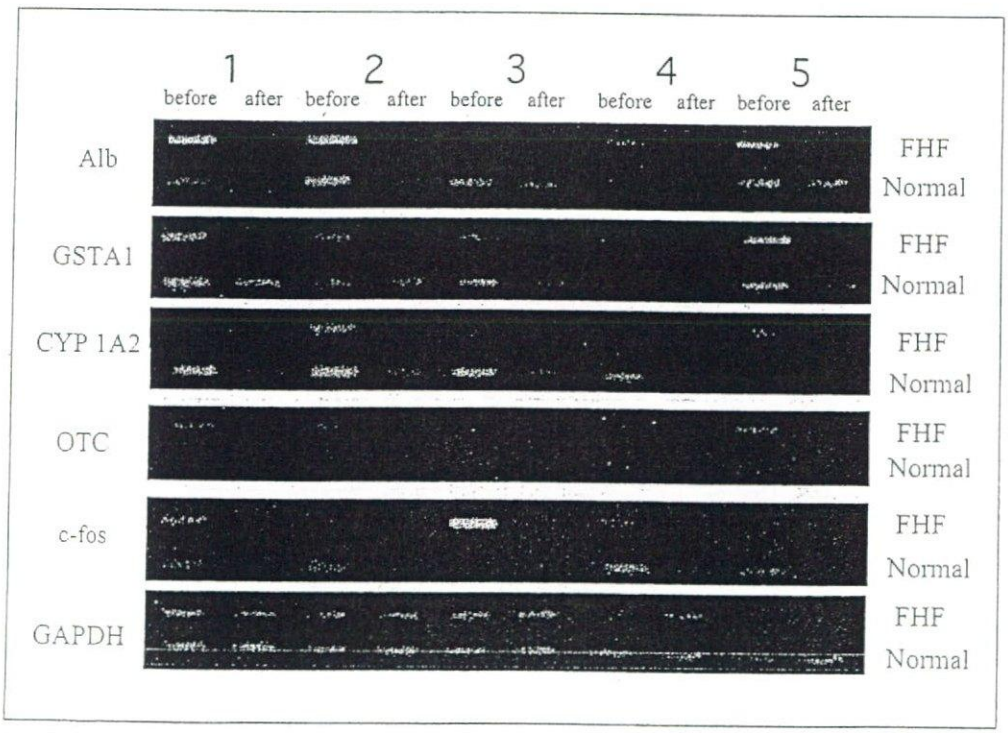

Fig. 2 - *Profile of mRNA expression, rat albumin, GST A1, CYP 1A2, OTC, c-fos, and GAPDH. The albumin, GST A1, and CYP 1A2 levels decreased more after the perfusion of FHF serum than after the perfusion of normal serum. Mild decreases in OTC and c-fos expression were also seen. Alb, Albumin; GST A1 glutathione S-translerase A1; CYP 1A2, cytochrome P450 1A2; OTC, ornithine transcarbamylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and FHF, fulminant hepatic failure.*

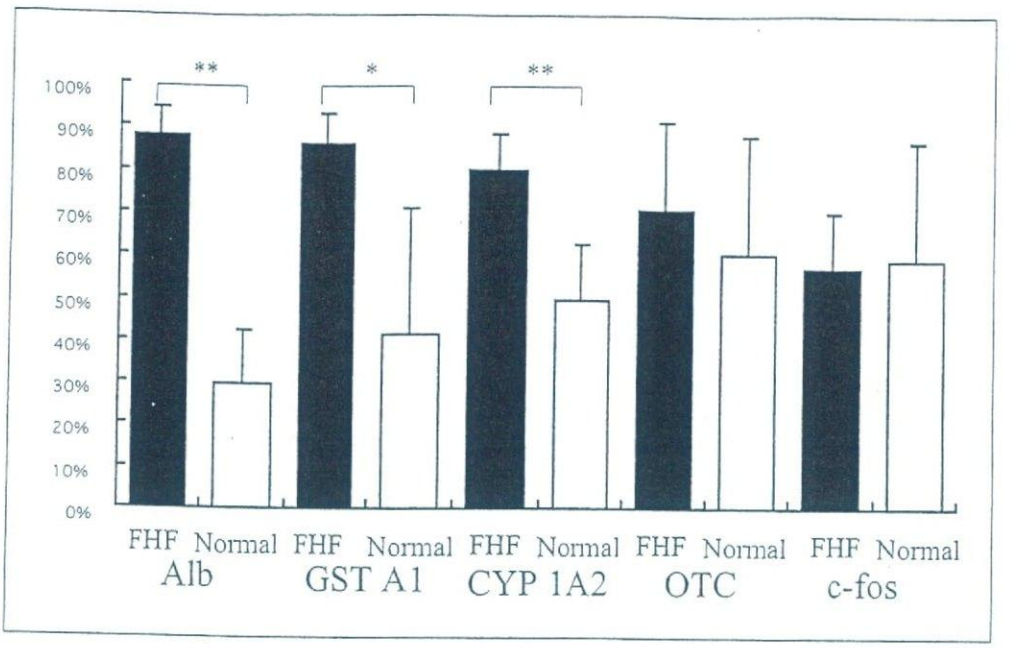

Fig. 3 - *The mRNA expression (percent decrease) was calculated by densitometric evaluation. The expressions of albumin, GST A1, CYP 1A2 were significantly decreased in hepatocytes treated with FHF serum compared to normal control cells, whereas those of OTC and c-fos were not different.*

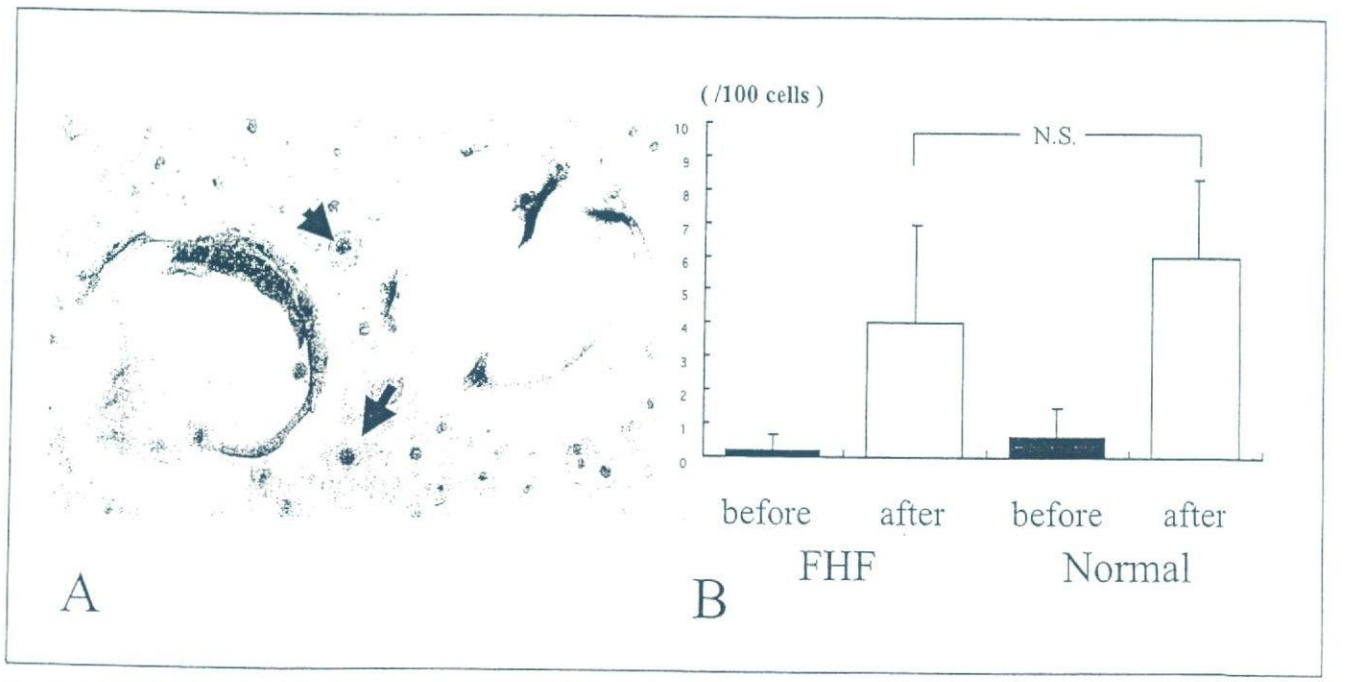

Fig. 4 - *(A) Immunohistochemical staining of hepatocytes using anti-PCNA antibody (original magnification x 100). Arrows represent positive staining. (B) PCNA positive cells were modestly increased after perfusion. However, the number of these cells was low and did not differ between FHF- and normal serum-exposed hepatocytes.*

29.6 ± 12.7%, $p<0.01$), GST A1 mRNA (86.0 ± 6.8%, 41.4 ± 29.3%, $p<0.05$) and CYP 1A2 (80.0 ± 8.4%, 49.2 ± 13.1%, $p<0.01$) levels were significantly decreased (Fig. 3).

Proliferating cell nuclear antigen (PCNA) labelling

PCNA positive cells were modestly increased after perfusion (i.e., 0.2 ± 0.4 cells before FHF vs 4.0 ± 2.9 cells

after FHF, and 0.6 ± 0.9 cells before normal serum vs 6.0 ± 2.3 cells after normal serum). However, no difference in these increases was found between the FHF and normal serum treated groups (Fig. 4).

DISCUSSION

Two opposite effects of FHF serum on hepatocytes have been reported. One report indicates that hepatocyte proliferation is increased and the other indicates it is suppressed (18, 19).

HGF, a strong growth factor of hepatocytes, is excessively increased in FHF serum. However, in patients with FHF, liver regeneration is hardly seen and also levels of HGF are reported to correlate inversely with patient prognosis (30). This finding implies the presence of some growth inhibitory factor antagonistic to HGF in FHF serum. We reported TGF-ß1 was increased in FHF patient's blood (14) and one of our animal studies showed a similar profile for HGF and TGF-ß1 and less liver regeneration in FHF than normal animals (21). Theoretically, when cells begin DNA synthesis, cell-specific functions are down-regulated during liver regeneration, while in FHF livers, neither proliferation nor specific functions are maintained. This discrepancy suggests that another factor other than HGF or TGF-ß1 is mediating this cellular derangement.

Several putative factors (we focused on inflammatory cytokines) increase in FHF livers. In fact, our model showed higher levels of TNF-α, IL-1ß, and IL-6. It was reported that inflammatory cytokines rapidly lowered the expression of major cytochrome P450 enzymes and decreased the transcription of rGST A2 *in vitro* (15, 16).

For example, IL-6 modulates hepatocyte proliferation through the signal transducer and activator of transcription (Stat3) (31). It also directly suppresses albumin secretion (17). It has been suggested that IL-6 causes hepatocytes to switch proliferation and performing other cellular functions. In an animal model of FHF, Stat3 activation suppressed an early phase of the cell cycle by inducing Stat3 inhibitors, resulting in impaired proliferation (32). In the present study, it was demonstrated that PCNA labeling is low despite the fact that immediate early gene (c-fos) expression is well preserved. The disruption of Stat3 signaling is caused by negative feed back mechanisms (32). Thus, cell proliferation is interrupted before DNA synthesis. We speculate that elevated IL-6 suppressed liver specific functions initially allow hepatocytes to enter the proliferating process in normal regeneration. In contrast, under FHF conditions, this process is interrupted by IL-6 itself and finally liver loses both its ability to proliferate and perform other functions.

In several inflammatory conditions, OTC is consistently expressed. It could be regulated by another signal pathway that does not directly connect to proliferation. In a phase I clinical trial of a BAL system at Cedars-Sinai Medical Center, treatment resulted in significant reduction of ammonia but had no effect on albumin (10). Our experiment showed similar results.

Some liver specific functions were consistently expressed in any situation; however, the majority of hepatocyte-specific functions was impaired by imbalance between growth factors and inflammatory cytokines, especially inflammatory cytokines. This imbalance may play a key regulatory role in the switch between performing cellular functions and undergoing proliferation.

To eliminate external cytokine stimuli, activated charcoals and hemodiafiltration have been developed; however, clinical outcome of patients treated by these methods is unsatisfactory (33, 34). The IL-6 pathway is considered to involve a soluble receptor that is necessary to transduce the signal (35). Such receptors are usually high molecular weight molecules that are difficult to remove from circulation using current modalities of cytokine reduction. Therefore, we consider it is important to create new hepatocyte systems that are functionally stable and hyposensitive to extra-cellular stimuli. Recent advances in cellular technology are promising.

In conclusion, several specific functions of hepatocytes were down-regulated after perfusion with FHF serum, even though the BAL system showed a better elimination capacity. Our findings suggest that the exposure of hepatocytes to hypercytokinemia, including inflammatory cytokines and positive and negative growth factors, causes a loss in liver specific functions and fails to support liver regeneration.

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