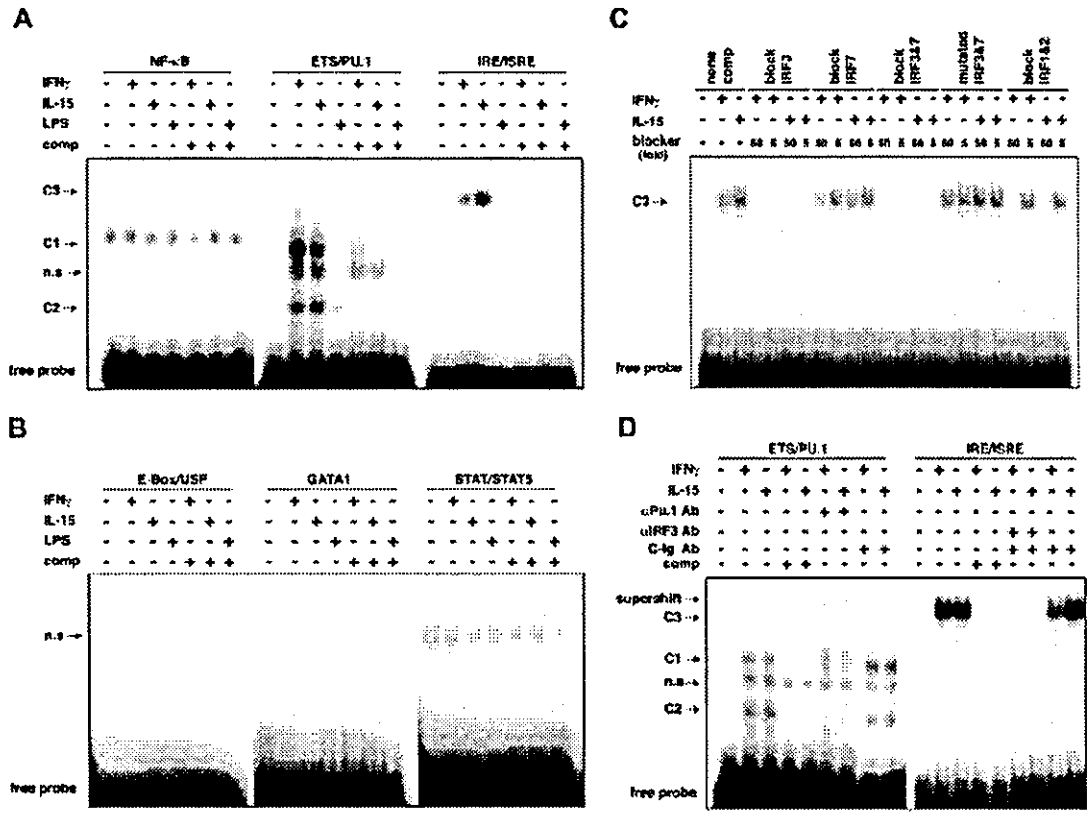

Figure 3. Specific protein binding of ETS/PU.1 and IRE/ISRE consensus sequences of IL12RB1 promoter. (A-B) RAW264.7 cells were left untreated or were treated with IFN-γ, IL-15, or LPS for 45 minutes, and the nuclear extracts were prepared for EMSA. Two micrograms nuclear extract was incubated with ^{32}P-labeled oligonucleotides complementary to the indicated consensus elements. DNA-protein complexes were separated on a 6% polyacrylamide gel. C1 and C2 indicate protein complex binding to ETS/PU.1, C3, protein complex binding to IRE/ISRE consensus sequence; n.s, nonspecific complex; comp, EMSA binding reaction containing 20-fold excess of the specific cold competitor. (C) Identification of IRF binding to the IRE/ISRE site of the IL12RB1 promoter. Cold oligonucleotides used in the competition assays are indicated. None comp indicates EMSA binding reaction that contained no competitor. (D) Supershift assay using anti-PU.1 antibody (αPU.1 Ab), anti-IRF3 antibody (αIRF3 Ab), or control isotype antibody (C-Ig Ab). EMSA was carried out as in panel A. For supershift experiments, nuclear extracts were preincubated with 2 μg indicated antibody for 1 hour before ^{32}P-labeled probe was added. Modified DNA-protein complexes are indicated.

shown in Figure 3C, protein binding to the IRE/ISRE consensus element of the IL12RB1 gene was completely abrogated by *both* high and low molar excesses of the unlabeled blocker specific for IRF3. The protein complex was also efficiently decreased by unlabeled IRF3/7 blocker. In contrast, only high molar excesses of unlabeled IRF7 and IRF1/2 blockers could block the protein binding, and the protein complex could not be blocked by the mutated form of IRF3/7 blocker. We then addressed whether an antibody against IRF3 could modify the DNA–protein complex binding to the IRE/ISRE element of the IL12RB1 gene. As shown in Figure 3D, the inducible DNA–protein complex formation was effectively inhibited by the anti-IRF3 antibody, indicating that this DNA–protein complex contained IRF3 at least as a component.

Next, to identify the protein binding to the ETS/PU.1 consensus element of the IL12RB1 gene, we applied an anti-PU.1 antibody to the EMSA. As shown in Figure 3D, DNA–protein complexes containing the ETS/PU.1 consensus element were effectively supershifted by the anti-PU.1 antibody, whereas the antibody did not modify the DNA–protein binding of IRE/ISRE sequence (data not shown). Thus, the binding of IRF3 and PU.1 appeared mutually independent. The identities of multiple DNA–protein complexes containing PU.1 are not certain. Although PU.1 can form complexes with various proteins, including IRFs and CBP, we failed to observe supershift or inhibition of the complexes with anti-IRF3 (Figure 3D) or anti-CBP (data not shown). It is possible that proteins other than IRF3 and CBP are involved in the complexes.

IL-15 and IFN-γ synergistically activated IL12RB1 gene transcription

Our data indicated that IL-15 and IFN-γ induced IL12RB1 promoter activation through ETS/PU.1 and IRE/ISRE elements (Figures 2, 3). To investigate the possible synergism between these 2 cytokines, we stimulated RAW264.7 cells with IFN-γ in combination with IL-15 and found that the combination showed a significantly synergistic effect (Figure 4A).[30] Interestingly, TSA, a specific inhibitor of histone deacetylases, also showed synergistic IL12RB1 mRNA induction in combination with IFN-γ. In contrast, the synergy was less significant for TSA plus IL-15 (Figure 4A).

To analyze the synergistic effects on IL12RB1 promoter–driven luciferase activity, we used RAW264.7 cells stably integrated with the pGL3-2508 luciferase construct. The fold inductions by IFN-γ/IL-15 cotreatment were significantly higher than those by stimulation with each cytokine (Figure 4B). A similar synergistic effect was observed for costimulation with IFN-γ and TSA. In contrast, no synergistic effect was observed between TSA and IL-15.

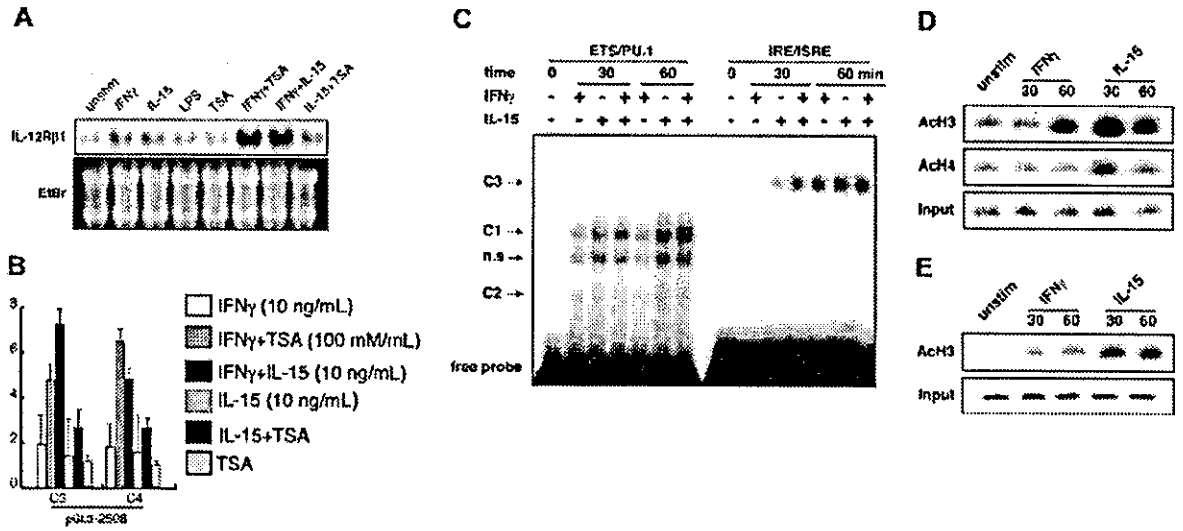

Figure 4. IL-15, synergistically with IFN-γ, activates IL12RB1 gene transcription. (A) RAW264.7 cells were left untreated (unstim) or were stimulated with either of the following: 1 μg/mL LPS, 10 ng/mL IL-15, 10 ng/mL IFN-γ, or combination IL-15 and IFN-γ for 4 hours. In some experiments, cells were pretreated with 100 mM TSA before stimulation. Total RNA was prepared for Northern blot analysis. Gene expression of IL12RB1 and a picture of the ethidium bromide (EtBr)–stained gel are shown. (B) RAW264.7 cells stably integrated with pGL3-2508 luciferase construct were prepared as described for Figure 2B. Cells were left untreated or were treated with 10 ng/mL IFN-γ, 10 ng/mL IL-15, combination IFN-γ and IL-15 for 6 hours with or without the addition of 100 mM TSA before lysate preparation and luciferase assays. Basal luciferase activity of untreated cells was generally similar among all the examined clones. Fold inductions by IFN-γ or IL-15 are expressed as described for Figure 2B. (C) IL-15 and IFN-γ synergistically enhanced protein binding to IRE/ISRE. RAW264.7 cells were left untreated or were treated with IFN-γ, IL-15, or combination IFN-γ and IL-15 for 30 or 60 minutes, and nuclear extracts were prepared for EMSA. EMSA was carried out as described in Figure 3A; inducible protein binding is shown. (D) Acetylation of histone H3 at the IL12RB1 gene is induced by IFN-γ and IL-15. RAW264.7 cells were left untreated (unstim) or were treated with 10 ng/mL IFN-γ or 10 ng/mL IL-15 for the indicated time. After treatment, chromatin was extracted and immunoprecipitated with anti–acetyl-histone H3 or anti–acetyl-histone H4. PCR analyses of DNA products from immunoprecipitation were carried out as described in "Materials and methods." (E) Mouse peritoneal macrophages were prepared as previously described,[30] and the ChIP assays were carried out as described for Figure 4D. Acetylation levels of histone H3 at the IL12RB1 gene are shown.

We further analyzed the effects of combination IFN-γ and IL-15 on the kinetics of protein binding to the IL12RB1 promoter. As shown in Figure 4C, protein binding to ETS/PU.1 was similarly induced by IFN-γ and IL-15 stimulation. No synergistic increase of protein binding was detected when cells were stimulated with a combination of IL-15 and IFN-γ. In contrast, significant synergy was observed for the content of protein binding to the IRE/ISRE consensus element. Specifically, a synergistic effect on protein binding to IRE/ISRE was more evident at 30 minutes of stimulation than at 60 minutes.

Because IL-15 and TSA similarly activated IL12RB1 gene transcription in synergy with IFN-γ, we next investigate possible histone modification by IL-15 in the regulation of IL12RB1 gene. We applied the ChIP assay using IL12RB1 promoter-specific primers. As shown in Figure 4D-E, stimulation of RAW264.7 cells or mouse peritoneal macrophages with IL-15 rapidly induced the acetylation of histone H3 at the IL12RB1 promoter, whereas acetylation by IFN-γ stimulation was slower and less significant. On the other hand, only IL-15 induced the acetylation of histone H4.

Activation of p38 MAPK is necessary for the transcriptional induction of IL12RB1

Previous studies have indicated p38 MAPK in association with the phospho-acetylation of histone H3 and the transcription of a subset of stimulus-induced cytokine and chemokine genes.[31] To investigate whether p38 MAPK activation is involved in IL12RB1 gene induction in macrophages, we first examined p38 MAPK activation in RAW264.7 cells after IFN-γ and IL-15 stimulation. LPS stimulation was also carried out as a positive control. As shown in Figure 5A,[32] treatment of RAW264.7 cells with IL-15 or LPS induced rapid phosphorylation of p38 MAPK within 30 minutes. In contrast, p38 MAPK phosphorylation by IFN-γ was slower, requiring 60 minutes for detectable phosphorylation. We also investigated JNK phosphorylation and found that, unlike LPS, neither IFN-γ nor IL-15 could induce detectable phosphorylation of JNK.

Pretreatment of RAW264.7 cells with 50 μM SB203580, a specific p38 MAPK inhibitor, effectively abrogated p38 phosphorylation by IFN-γ and IL-15 (data not shown). As shown in Figure 5B, pretreatment with SB203580 significantly inhibited IL12RB1 promoter-driven luciferase activity by IFN-γ and IL-15. In contrast, a JNK-specific inhibitor, SP600125, showed no inhibitory effect. Consistently, IL12RB1 mRNA increase by IFN-γ and IL-15 in RAW264.7 cells was effectively inhibited by SB203580 but not by SP600125 (data not shown). We also examined the acetylation of histone H3 in the presence and absence of SB203580 by ChIP. As shown in Figure 5C, pretreatment of RAW264.7 with the p38 MAPK inhibitor significantly reduced the acetylation level of histone H3 at the IL12RB1 promoter. In agreement with these data, SB203580 pretreatment completely abrogated phosphorylation of histone H3 (Figure 5D).

Roles of CBP and IRFs in IL12RB1 transcription

We were interested in determining the possible involvement of cyclic adenosine monophosphate (cAMP)–responsive element-binding protein (CREB)–binding protein (CBP), which is an endogenous histone acetyltransferase (HAT), in IL12RB1 gene activation. For this purpose, RAW264.7 cells were stably integrated with pGL3-3875 and a CBP expression plasmid. Several G418-resistant clones were obtained. As confirmed by Western blotting, the HA-tagged CBP expression levels varied among these

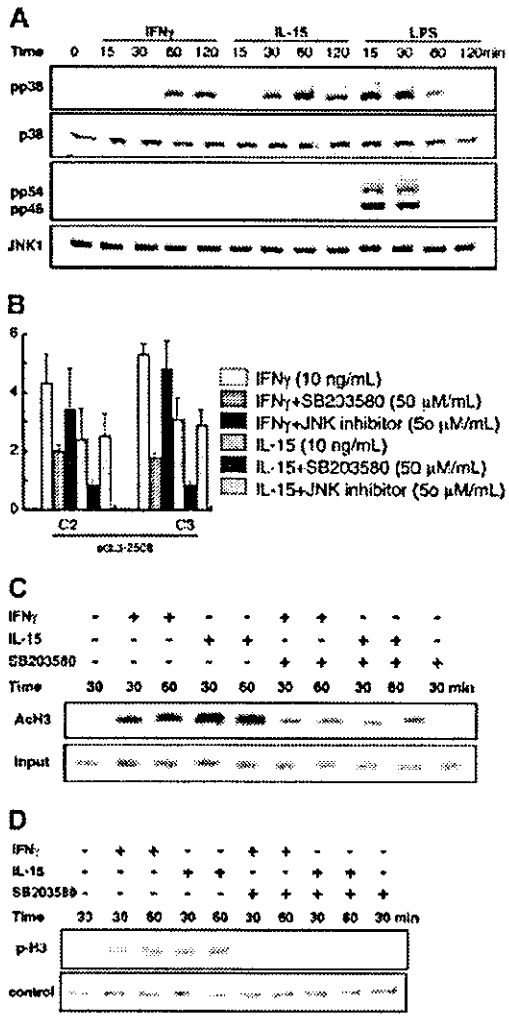

Figure 5. p38 MAPK is involved in the transcriptional regulation of IL12RB1. (A) RAW264.7 cells were treated with 10 ng/mL IFN-γ, 10 ng/mL IL-15, or 1 μg/mL LPS for the indicated time before total cellular lysate preparation. The phosphorylation of p38 (pp38) or JNK (pp54 and pp46) was detected by Western blot analysis using polyclonal antibody (pAb) specific for the phosphorylated p38 or mAb specific for the phosphorylated JNK. As a control, 10% of each lysate was used to detect p38 or JNK using anti-p38 or anti-JNK1 pAb, respectively. (B) RAW264.7 cells stably integrated with pGL3-2508 luciferase construct were prepared as described for Figure 2B. Cells were left untreated or were pretreated with 50 μM SB203580 or 50 μM SP600125 for 30 minutes before stimulation with 10 ng/mL IFN-γ or 10 ng/mL IL-15 for an additional 8 hours. Lysates were prepared for luciferase assay. Basal luciferase activity of untreated cells was generally similar among all the examined clones, and the fold inductions are expressed as described for Figure 2B. (C) RAW264.7 cells were pretreated with SB203580 for 30 minutes before stimulation with 10 ng/mL IFN-γ or 10 ng/mL IL-15 for the indicated time. The acetylation of histone H3 was analyzed as described for Figure 4E. (D) RAW264.7 cells were treated as described in panel C. Nuclear lysates were prepared as previously described.[32] The phosphorylation of histone H3 (p-H3) was detected by Western blot analyses using an antibody specific for the phosphorylated histone H3. As a control, 10% of each lysate was used to measure protein levels by Coomassie staining.

clones (Figure 6A). Using these stably integrated clones, we found that overexpression of CBP strongly enhanced IL12RB1 promoter-driven luciferase activity after IFN-γ treatment, whereas CBP expression had no effect on the promoter activation by IL-15 (Figure 6B). In addition, the inducible effect of CBP on IFN-γ–driven promoter activation correlated with its expression levels among the clones (Figure 6A-B).

Although our EMSA data have indicated that IRF3 is responsible for IL12RB1 gene activation (Figure 3B-C), mechanisms regarding how IRF3 regulates IL12RB1 gene transcription remain to be elucidated. We examined mRNA levels of IRFs in RAW264.7 cells after IFN-γ or IL-15 stimulation. Given that LPS stimulation is known to up-regulate gene expression of several IRF family members, LPS was used as a positive control. Northern blot analysis showed that gene expression of IRF1, IRF2, and IRF7 was induced by IFN-γ, but not with IL-15 stimulation (Figure 6C). Neither IL-15 nor IFN-γ could induce the IRF3 mRNA level, suggesting that IRF3 regulates IL12RB1 gene activation through posttranscriptional mechanisms. The studies on viral infection–responsive genes indicated that serine/threonine phosphorylation of IRF3 is associated with its nuclear translocation, DNA binding, and holocomplex formation with CBP.[33-35] We investigated the serine-phosphorylation levels of IRF3 after IFN-γ and IL-15 stimulation of RAW264.7 cells. As shown in Figure 6D, IL-15

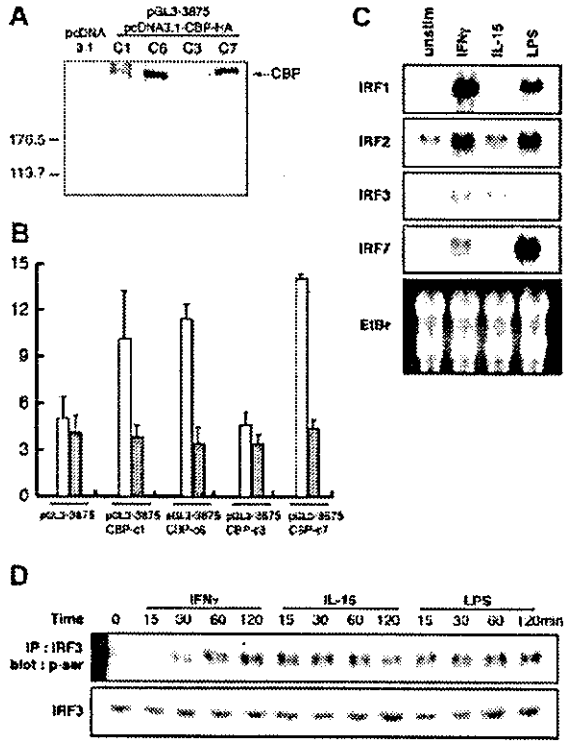

Figure 6. Effects of CBP expression on IL12RB1 promoter activity and IRF3 phosphorylation. (A) RAW264.7 cells were stably integrated with pGL3-3875 of IL12RB1 luciferase construct, together with CBP-HA expression plasmid. Total cellular lysates were prepared from stably integrated clones, and the expression levels of CBP were analyzed by Western blotting using anti-HA mAb. (B) Overexpression of a coactivator protein CBP increases IFN-γ–mediated IL12RB1 transcription. The stably integrated clones shown in panel A were cultured and plated as described for Figure 2B. Cells were left untreated or were treated with either of the following: 10 ng/mL IFN-γ (▨), 10 ng/mL IL-15 (□), or 1 μg/mL LPS (not shown) for 8 hours before lysate preparation and luciferase assays. Basal luciferase activity of untreated cells was generally similar among all the examined clones. Fold inductions are shown. (C) Neither IFN-γ nor IL-15 induces gene expression of IRF3. RAW264.7 cells were left untreated (unstim) or were treated for 4 hours with 1 μg/mL LPS, 10 ng/mL IL-15, or 10 ng/mL IFN-γ. Total RNA was prepared for Northern blot analysis using IRF1, IRF2, IRF3, and IRF7 cDNA probes. Gene expression of the indicated gene and a picture of ethidium bromide (EtBr) are shown. (D) RAW264.7 cells were treated as described for Figure 5A. Total cellular lysates were prepared and immunoprecipitated with anti-IRF3 pAb. The phosphorylation of IRF3 was detected by Western blot analysis using antiphosphoserine mAb. As a control, 10% of each lysate was used to detect IRF3 by anti-IRF3 pAb.

rapidly induced IRF3 phosphorylation, whereas IFN-γ induced the phosphorylation with a much longer time course.

Discussion

IRFs are a family of transcription factors first identified as the regulators of IFNA and IFNB gene expression.[36,37] Among the 9 IRF members, IRF1, IRF3, and IRF-9 function as transcriptional activators, and the others can function as repressors or as activators/repressors.[38] Although all IRF proteins recognize the core IRE/ISRE consensus sequence, each IRF has a clear sequence preference, enabling some oligonucleotides to be used as specific competitors for individual IRF.[28] In our EMSA competition assays, an oligonucleotide specific for IRF3 completely abrogated protein binding to the IRE/ISRE element of the IL12RB1 gene. Involvement of IRF3 was also confirmed by the inhibition of the DNA–protein binding with a specific anti-IRF3 antibody (Figure 3C). On the other hand, competitive oligonucleotides for IRF1/2 and IRF7 partially blocked the complex formation (Figure 3C). These IRFs have DNA binding properties similar to those of IRF3, suggesting other IRF(s) might share the IRE/ISRE binding to the IL12RB1 promoter with IRF3. It is also possible that IRF7 forms a heterodimer complex with IRF3 on the IL12RB1 promoter, a phenomenon reported for the IFNB and certain IFNA family genes.[39] However, mRNA for IRF1, IRF2, and IRF7 was induced significantly by IFN-γ but not IL-15 in RAW264.7 cells (Figure 6C), and modification of the DNA–protein binding by anti-IRF3 antibody was greater than 90% (data not shown), indicating that IRF3 is at least contained in most DNA-protein complexes.

In quiescent cells, IRF3 resides in the cytoplasm as an inactive form. IRF3 is activated by virus infection, LPS, and inflammatory cytokines through phosphorylation at multiple serine/threonine residues. Phosphorylation allows conformational changes and promotes the translocation of IRF3 from cytoplasm into the nucleus.[40] Although mere nuclear translocation does not directly confer its transcriptional activity, increased DNA-binding affinity of the phosphorylated IRF3 results in transcriptional activation. In our cell system, IL-15 and IFN-γ did not increase *IRF3* gene expression, but it did increase the IRF3 phosphorylation level (Figure 6D).

PU.1 appears to be another important transcription factor regulating IL12RB1 transcription (Figure 3D). PU.1 is a member of the ETS family, which is specifically expressed in B cells, neutrophils, mast cells, and macrophages.[41,42] Like other ETS proteins, PU.1 contains a conserved ETS domain that confers its sequence-specific DNA binding to the 5′-C/AGGAA/T core sequence.[41] PU.1 is a transcription factor involved in the regulation of a myriad of genes in the cell types listed here.[43] Interestingly, as in the *IL-12βIR* gene, PU.1 plays a pivotal role in up-regulating *IL-12 p40* gene expression in macrophages.[44] Thus, our data indicated that activation of PU.1 increased IL-12–mediated signals in macrophages by up-regulating IL-12 p40 and IL12RB1 expression. It is unclear how the PU.1 pathway is activated by IFN-γ and IL-15. PU.1 activation through the formation of heterocomplexes with proteins such as IRFs and CBP has previously been reported. However, we could not detect heterocomplexes of PU.1 with IRF3 or CBP in RAW264.7 cells stimulated with IFN-γ or IL-15 (Figure 3D; data not shown). It is possible that these cytokines induce the association of PU.1 with the IL12RB1 gene promoter region through chromatin remodeling.

Our data indicated that IL-15 acted in concert with IFN-γ in the up-regulation of IL12RB1 mRNA and promoter activity in RAW264.7 cells (Figure 4A-C). This finding is compatible with previous reports on the role of IL-15. For example, decreased IL12RB1 expression and diminished IL-12 responsiveness are evident in IRF-1$^{-/-}$ mice,[15] in which IL-15 expression is severely damaged.[45] Moreover, splenic DCs and peritoneal macrophages from IL-15$^{-/-}$ mice produced significantly less IFN-γ than their control counterparts in response to IL-12, suggesting IL-15 directly affects APCs for IL-12 responsiveness.[19] Furthermore, IL12RB1 mRNA expression is significantly reduced in APCs from IL-15$^{-/-}$, γc$^{-/-}$RAG-2$^{-/-}$, and IL-2Rβ$^{-/-}$RAG-2$^{-/-}$ mice,[19] indicating IL-15 is responsible for IL12RB1 expression in APCs in a T cell–independent manner. These lines of evidence, together with our current finding, strongly indicated that IL-15 is pivotal in the generation of T_H1-type immune responses by directly inducing IL12RB1 expression in APCs.

Combination IL-15 and IFN-γ treatment induced significant protein binding to the IRE/ISRE consensus sequence more quickly than treatment with IL-15 or IFN-γ alone (Figure 4C). The combination did not have any synergistic effects on PU.1 binding (Figure 4C). These data indicated that IL-15 cooperates with IFN-γ for IL12RB1 promoter activation by increasing protein binding to the IRE/ISRE element. Notably, as for the synergy with IFN-γ, IL-15 was similar to TSA (Figure 4A-C). The combination of IL-15 and TSA, on the other hand, showed no synergic effect. Because TSA is a well-known inhibitor of histone deacetylases, we sought to investigate the possibility that IL-15 may induce histone acetylation and chromatin remodeling of the IL12RB1 gene. ChIP assays showed that IFN-γ and IL-15 stimulation potently induced the acetylation of histone H3 at the IL12RB1 promoter. However, IFN-γ–induced acetylation was weaker and significantly slower than that by IL-15 (Figure 4D). These data indicated that the synergistic effect of IL-15 may be mediated by the rapid remodeling of the IL12RB1 gene promoter region into a more accessible structure for IRFs.

Histone H3 acetylation is closely related to its phosphorylation, and a recent report indicated that p38 MAPK is responsible for phospho-acetylation of histone H3 on a subset of stimulus-induced cytokine and chemokine genes.[31] Our previous report also revealed that p38 MAPK is involved in phospho-acetylation and chromatin remodeling at *mkp-m*, a MAPK phosphatase gene.[21] Our current data indicated that IL12RB1 appears to be another example of the genes regulated by p38 MAPK because pretreatment with SB203580, a specific inhibitor of p38 MAPK, significantly inhibited promoter responsiveness to IFN-γ and IL-15 (Figure 5B). Consistently, SB203580 effectively inhibited acetylation and phosphorylation of histone H3 induced by IFN-γ and IL-15 (Figure 5C-D). SB203580 inhibited IL12RB1 promoter activation by IL-15 more efficiently (almost completely abrogated) than that by IFN-γ, which seems consistent with the finding that IL-15 more potently induced histone H3 acetylation at the IL12RB1 gene (Figures 4D, 5D).

Conversely, however, activated p38 MAPK alone is not sufficient for IL12RB1 mRNA induction, given that LPS, an efficient activator of p38 MAPK (Figure 5A), only marginally induced IL12RB1 mRNA expression (Figure 1A). Interestingly, LPS-mediated p38 MAPK phosphorylation is temporary; it lasts only for 30 minutes (Figure 5A). Thus, it is possible that sustained activation of p38 MAPK may be required for the efficient transcriptional induction of IL12RB1. Additionally, IL-15 induced p38 MAPK phosphorylation more quickly than IFN-γ, which is

consistent with the different time course of histone H3 acetylation by these 2 cytokines (Figure 5A). On the other hand, neither IFN-γ nor IL-15 induced JNK phosphorylation in RAW264.7 cells (Figure 5A), indicating that JNK activation is not necessary in the induction of IL12RB1 gene expression. Consistently, SP600125, a specific JNK inhibitor, failed to inhibit IL12RB1 promoter activation by IFN-γ or IL-15 (Figure 5B).

A transcriptional coactivator protein, CBP, is involved in the transcriptional regulation of various genes by providing a scaffold for transcription factors and modifying chromatin structure through its HAT activity.[46-48] We demonstrated that the overexpression of CBP protein strongly enhanced IFN-γ–mediated IL12RB1 promoter activities. In contrast, IL-15–driven IL12RB1 promoter was not affected by CBP expression. Because IL-15 induces histone H3 acetylation in the IL12RB1 gene more potently than IFN-γ, it seems possible that IL-15 stimulation alone induced sufficient chromatin remodeling so that a further increase of HAT activity by CBP had no significant effects. The interaction between CBP and other transcription factors, including PU.1 and IRF3, is known to be involved in the transcriptional regulation of various genes.[49] Notably, the phosphorylation of IRF3 promoted increased affinity for CBP, resulting in transcriptional activation.

In conclusion, we showed that IL-15 and IFN-γ stimulation of macrophages induce PU.1 and phosphorylated IRF3 binding to ETS/PU.1 and IRE/ISRE consensus motifs of the IL12RB1 promoter, respectively. IL-15 not only induces IL12RB1 mRNA by itself, it synergizes with IFN-γ to induce higher levels of IL12RB1 gene transcription. Understanding the roles of IL-15 and IFN-γ in the activation of IL12RB1 may promote our knowledge of host defense mechanisms against microbial infection.

Acknowledgments

We thank Ms Itano and Ms Nishikawa for their technical assistance.

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Original Paper

Acute Renal Failure during the Early Postoperative Period in Adult Living-related Donor Liver Transplantation

Satoshi Yamamoto MD[1], Yoshinobu Sato MD[1], Takafumi Ichida MD[2], Isao Kurosaki MD[1]
Hideki Nakatsuka MD[1], Katsuyoshi Hatakeyama MD[1]
[1]Division of Digestive and General Surgery, [2]Division of Gastroenterology and Hepatology
Niigata Graduate School of Medical and Dental Sciences, Niigata, Japan
Corresponding Author: Dr. Yoshinobu Sato, Division of Digestive and General Surgery
Niigata Graduate School of Medical and Dental Sciences, Asahimachi-dori 1-757, Niigata 951-8510, Japan
Tel: +81 (0)25 227 2228, Fax: +81 (0)25 227 0779, E-mail: sugarman@med.niigata-u.ac.jp

ABSTRACT

Background/Aims: Acute renal failure after liver transplantation can occur in some and is an important postoperative complication. Our goal is to clarify the risk factors of acute renal failure after living-related donor liver transplantation (LDLT).

Methodology: From March 1999 to August 2000, ten consecutive patients were investigated the changes of the systemic hemodynamics and the renal function. They were classified into Group A (Creatinine (Cre) was over 2.0mg/dL) and B (Cre was below 2.0mg/dL). Retrospective variables were examined with two groups A and B being compared.

Results: In both groups, Cardiac Index (CI) was above standard levels. However, the CI levels in Group B were significantly higher than those in Group A (p=0.031). The early postoperative transaminase levels were significantly higher in Group A than in Group B (p=0.049) and graft liver volume/recipient body weight ratio was significantly smaller in Group A than in Group B (p=0.016).

Conclusions: Our study suggests that small-for-size graft or hypovolemia, resulting in the delay of the recovery of graft liver function, may be an important cause of acute renal failure during the early postoperative period in adult LDLT.

KEY WORDS: Systemic hemodynamics; Renal function; Living-related donor liver transplantation (LDLT)

ABBREVIATIONS: Living-Related Donor Liver Transplantation (LDLT); Creatinine (Cre); Cardiac Index (CI); Orthotopic Liver Transplantation (OLT); Hepatocellular Carcinoma (HCC); Prednisolone (PSL); Methylprednisolone (MP); Donor Whole Blood Transfusion (DST); Acute Cellular Rejection (ACR); Cardiac Output (CO); Urinary Output (UO); Creatinine Clearance (Ccr); Platelets (Plt); Prothrombin Time (PT); Aspartate Aminotransferase (AST); Alanine Aminotransferase (ALT); Total Bilirubin (TB); Total Protein (TP); Graft Liver Volume/Recipient Body Weight Ratio (GRWR); Arterial Ketone Body Ratio (AKBR); Blood Urea Nitrogen (BUN)

INTRODUCTION

Several studies have been performed in patients with a terminal state of liver disease, to evaluate the relationship between systemic hemodynamics and renal function (1-6). It has been reported that patients with severe liver failure are in a hyperdynamic state and that the splanchnic and systemic hemodynamics may affect the balance between vasoconstricting and vasodilating factors, and induce marked irreversible sodium and water retention, leading to refractory ascites, a progressive rise in plasma creatinine (Cre) levels, and reduction of renal clearances (7-9). Orthotopic liver transplantation (OLT) is intended to restore normal liver function, hemodynamics and hepatorenal syndrome. Although this is achieved in most patients, acute renal failure can occasionally occur in the perioperative period and is an important postoperative complication, affecting patient's survival. The changes of systemic hemodynamics and renal functions after OLT, especially after living-related donor liver transplantation (LDLT), have rarely been investigated. The purpose of the present study was to investigate them and to clarify the risk factors of acute renal failure during early postoperative period in adult patients who underwent LDLT for severe liver failure.

Hepato-Gastroenterology 2004; 51:1815-1819

METHODOLOGY

Patients

From March 1999 to August 2000, thirteen adult patients with liver disease underwent LRPLT. Of these 13 patients, the changes of the systemic hemodynamics and the renal function in 10 consectutive patients were investigated. At the time of transplantation, all patients suffered from end-stage liver disease: HCV hepatitis with hepatocellular carcinoma (HCC) (two patients), HBV hepatitis (one patient), primary biliary cirrhosis (three patients), subacute fulminant hepatitis (two patients), Budd-Chiari syndrome (one patient), Alcoholic liver cirrhosis (one patient). The mean age was 55.1±3.2 years old (range 34-68 yr) **(Table 1)**. Three patients were excluded from this study. Two had long experienced renal failure which needed dialysis, and the other died due to cerebral hemorrhage in the early postoperative period.

Surgery

All patients underwent standard LDLT (10,11). There were 6 right lobe grafts, 3 left lobe grafts, and 1 left and caudate lobe graft. All of the grafts were flushed and preserved with histidine-tryptophan-ketoglutarate solution (Dr. Franz Koler Chemie, Alsbach-hanlein, Germany). Graft weight ranged from

TABLE 1 Characteristic and Demographic Data of LDLT Patients

Total patients	10
Mean age	55.1±3.2 yr
Sex (male/female)	5M/5F
Indication pf LRPLT	
Primary biliary cirrhosis	3
Subacute fulminant hepatitis	2
Liver cirrhosis (C) with HCC	2
Liver cirrhosis (B)	1
Liver cirrhosis (alcohol)	1
Budd-Chiari syndrome	1
Child-Pugh score	
A	0
B	3
C	7
UNOS status	
1	2
2A	3
2B	4
3	1
Immunosuppression	
FK506+prednisolone	6
FK506+prednisolone+azathioprine	4
Intraportal DST	
DST	3
no DST	7

0.64 to 1.30% of recipient body weight.

Immunosuppression Protocol

The immunosuppression protocol was maintained by Tacrolimus (FK506) and prednisolone (PSL) in all patients. Induction therapy was begun on methylprednisolone (MP) at 10mg/kg administered intravenously (i.v) prior to portal vein and hepatic artery reperfusion, followed by MP at 10mg/kg/day, decreasing to 0.5mg/kg/day during the 7 days after LDLT. After then, MP i.v. was converted to PSL oral administration (p.o), tapered off to 0-0.3mg/kg/day until the end of the first month.

FK506 was started one day before transplantation at a dose of 2.0-3.0mg/body/day divided into two doses, except four cases with hepatic encephalopathy. The target for the posttransplant whole blood trough level of FK506 was 10-15ng/mL during the first 2 weeks and around 10ng/mL thereafter.

We have performed intraportal donor whole blood transfusion (DST) for four patients so that they could acquire the tolerance. Four patients who received intraportal DST, maintained relatively low FK506 trough levels. All of these patients were able to stop taking PSL within the first month after surgery. Moreover, they had almost no acute cellular rejection (12-14).

Clinical Acute Cellular Rejection

Clinical acute cellular rejection (ACR) was diagnosed on the bases of an increase in transaminase and/or gamma-glutamyl transpeptidase with histological evidence. Histological diagnosis and grading of ACR were performed according to the criteria proposed by Demetris, *et al.* (15).

Systemic Hemodynamics

Systemic hemodynamic studies were performed in the early postoperative period. Cardiac output (CO), right atrial pressure, and pulmonary arterial and wedged pressures were measured using a Swan-Ganz catheter. The Cardiac Index (CI) was determined as the CO/body surface area. For a week after LDLT, urinary output (UO) was calculated every 4 hours. Serum Cre and blood urea nitrogen (BUN) levels were measured approximately two or three times per day. These patients were divided into two groups; Group A (n=5): patients for whom serum Cre levels rose above 2.0mg/dL in the early postoperative period; and Group B (n=5): patients for whom serum Cre levels did not exceed 2.0mg/dL in the early postoperative period. No patient required hemodialysis (16). The clinical characters of ten consecutive patients are summarized in **Table 1.**

Risk Factors for Acute Renal Dysfunction in the Early Postoperative Period

Perioperative data for all consecutive patients were collected and stored in a database. The pretransplant recipient characteristics analyzed included: age, sex, Child-Pugh score, existence of infections, creatinine clearance (Ccr), Platelets (Plt), prothrombin time (PT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), and total protein (TP). The intraoperative variables analyzed were: surgical duration, cold ischemia time, warm ischemia time, anhepatic phase, surgical blood loss, and graft liver volume/recipient body weight ratio (GRWR). The postoperative variables analyzed were: existence of DST, arterial ketone body ratio (AKBR) at 24 hours after LRPLT, total volume of postoperative blood transfusion for 7 days, change of serum ALT, AST, T.Bil and FK506 trough levels in perioperative period.

Statistical Analysis

Statistical analysis was performed for Group A,

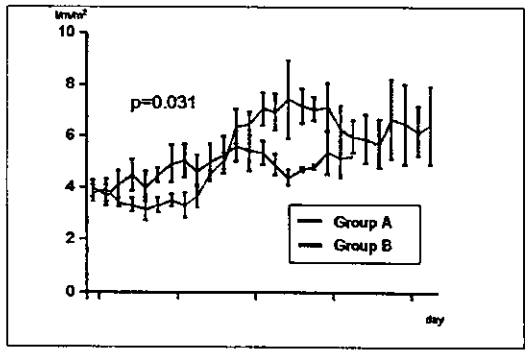

FIGURE 1 Changes of the CCl levels in the early postoperative period after adult LDLT Group A (sCre>2.0mg/dL), Group B (sCre ≤2.0mg/dL).

which had serum Cre levels over 2.0mg/dL and Group B, which did not, in the early postoperative period. The Mann-Whitney U-test and ANOVA were used for quantitative data and Pearson's chi-square or Fisher's exact test for categorical data. The difference was considered statistically significant when probabilities were less than 0.05. Data are shown as mean values±SD. Actuarial survival was determined by the Kaplan-Meier method. A comparison between survival curves was made by the Wilcoxon method (17).

RESULTS

Relationship between Systemic Hemodynamics and Urinary Output

The changes of early postoperative CI in Group A and B are shown in **Figure 1**. The CI levels rose above standard CI levels after LDLT. The mean CI level of all patients approximately 24 hours after surgery was 4.26±0.35mL/min/mm². The mean CI level in Group A and Group B were 3.68±0.52L/min/m² and 4.84±0.59L/min/m² respectively. The CI levels in Group A were significantly higher than those in Group B for approximately three days after surgery (p=0.031).

The relationships between CI and UO for each group are shown in **Figure 2**. In both groups, CI levels rose above standard CI level immediately after LDLT, but UO remained at extremely low levels for approximately 2 days after surgery and anuria continued for 12 hours in Group A. UO began rising as soon as the CI level exceeded 5.0L/min/mm², being far above normal CI levels, and after which, serum Cre and BUN began decreasing gradually. The timing was approximately 12 hours earlier in group B than in Group A.

Risk Factors for Acute Renal Failure in Early Postoperative Period

The results of univariate analysis regarding preoperative, intraoperative, and postoperative variables are shown in **Table 2**, respectively. In the analyses of preoperative characteristics, no factor, including preoperative creatinine clearance and Child-Pugh score had the significant difference between the two groups. Though the difference was not statistically significant, surgical blood loss of intraoperative variables was clearly greater in Group A than in Group B.

In the analyses of postoperative variables, the G/R ratio was significantly smaller in Group A than in Group B (p=0.042) and the levels of AST and ALT were significantly higher in Group A than in Group B (p=0.014 and p=0.048) (**Figure 3**). T.Bil and Plt levels were not statistically different between the two groups (data not shown). Regarding other factors, AKBR at approximately 24 hours after surgery, reflecting graft liver function, was lower in Group A than in Group B and postoperative blood transfusion was also clearly greater in Group A than in Group B. However, neither factor was statistically different between the two groups. Although renal dysfunction is an important side effect of immunosuppressant, the trough levels of FK506 was not also statistically different between Group A and Group B (**Figure 4**). In both groups, no patients experienced vascular, biliary, and infectious complication within a week after surgery.

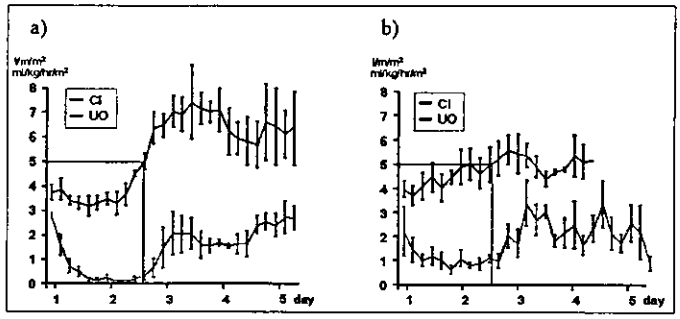

FIGURE 2 Relationship between CI levels and UO in the early postoperative period after adult LRPLT. (a) Group A (sCre>2.0mg/dL), (b) Group B (sCre≤2.0mg/dL).

TABLE 2 Univariate Analysis of Perioperative Variables

	Group A	Group B	*P*
Preoperative			
Mean age	46.2±4.2	56.4±3.7	0.058
Sex (male / female)	4/1	1/4	NS
UNOS			
At home	0	0	
In hospital	4	3	
ICU	1	2	NS
Blood combination			
Identical	3	4	
Compatible	2	1	
Incompatible	0	0	NS
Crossmatch			
Positive	0	1	
Negative	4	5	NS
Child-Pugh score (point)	10.6±0.8	11.6±1.1	0.491
Ccr (mL/min)	48.2±7.6	53.2±11.5	0.727
AST (IU/L)	120.8±21.8	65.8±20.0	0.101
ALT (IU/L)	62.4±7.7	37.0±11.4	0.101
T.Bil (mg/dL)	13.1±4.0	7.7±3.0	0.310
Plt (/mm³)	4.8±2.1	8.8±3.2	0.336
PT (%)	38.4±5.5	51.0±9.5	0.283
Intraoperative			
Surgical duration (min)	903±97.9	758±32.1	0.199
Blood loss (g)	24130±13847	9885±5168	0.363
Cold ischemic time (min)	81.8±27.6	55.6±12.8	0.442
Warm ischemic time (min)	51.6±4.0	49.0±3.2	0.318
Anhepatic phase (min)	190±69.4	164±32.6	0.613
G/R ratio (%)	0.902±0.044	1.094±0.046	0.016
Postoperative			
Immunotherapy			
FK504 prednisolone	3	1	
FK506+prednisolone +Azathioprine	2	4	NS
Intraportal DST			
DST	2	1	
No DST	3	4	NS
Blood transfusion (mL)	14422±8513	7092±4751	0.473
AKBR at 24 hr after surgery	1.48±0.25	1.16±0.23	0.381
Acute cellular rejection			
Rejection	4	0	
No rejection	1	5	NS

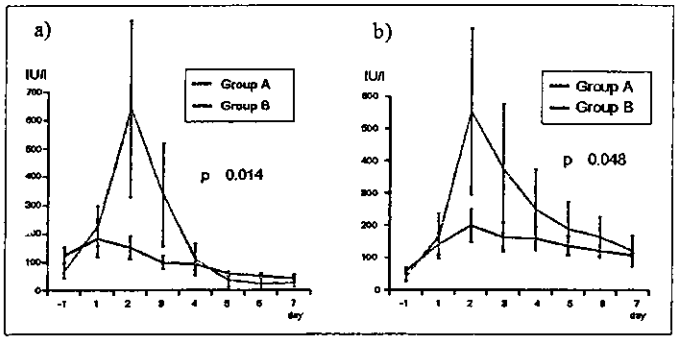

FIGURE 3 Changes of blood chemistry data in the early postoperative period. **(a)** aspartate aminotransferase (AST), **(b)** alanine aminotransferase (ALT).

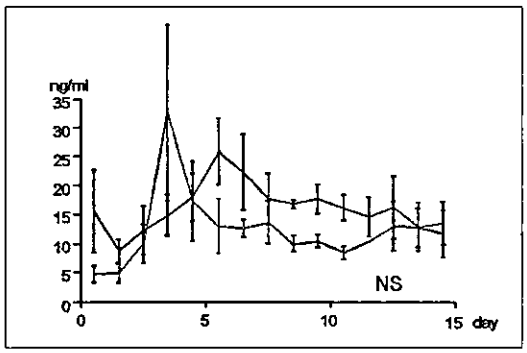

FIGURE 4 Changes of posttransplant whole blood trough level of FK506.

Incidence of Acute Cellular Rejection and Patients Survival

Four of the ten patients, all four in Group A, had, suffered from mild or moderate ACR in the postoperative period. The median age and follow-up time was 56 years old (range 40-63) and was 34 months (range 26-44), respectively. Actuarial 3-year patients survival rate was 100% and 80%, respectively and showed no statistical differences between the two groups. In Group B, one patient died because of systemic infection.

DISCUSSION

In OLT, acute renal failure can occasionally occur in the perioperative period and is an important postoperative complication (18). However, early postoperative renal function after OLT, especially after adult LDLT, has rarely been investigated. Therefore, our study focused on the relationship between systemic hemodynamics and renal function, as well the risk factors predicting this serious complication.

In agreement with several previous reports, this study documents hyperdynamic circulatory state that was sustained after LDLT for patients with severe liver failure (1-4,8).

The CI levels of all patients exceeded standard CCI levels, but the patients experienced anuria or oliguria phase for two days after surgery and in all of five patients who experienced the anuria phase, serum Cre levels rose above 2.0mg/dL in the early postoperative period. For approximately three days after surgery, the CCI levels in Group A was significantly higher than those in Group B. Surgical blood loss and postoperative blood transfusion, which had a great effect on systemic hemodynamics, were clearly larger in Group A than in Group B, neither factor was significantly different. Probably, the hypovolemia, following the massive surgical blood loss, may affect on the difference of the CI levels between the two groups. The G/R ratio was significantly different between the two groups, being smaller in Group A than in Group B. The transaminase levels were significantly higher in Group A than in Group B. The AKBR at 24 hours after liver transplantation, representing the graft liver function, was lower in Group A than in Group B though the difference was not significantly different between the two groups. Probably, the small-for-size graft might lead to the delay of the recover of the graft liver function.

Based on these results, we believe that there are two primary factors, leading to severe renal dysfunction in the early postoperative period after LDLT. The first is hypovolemia. In fact, the CI levels were significantly lower in Group A than in Group B for approximately three days after surgery. Moreover, the surgical blood loss and the postoperative blood transfusion were clearly larger in Group A than Group B though neither factor was significantly different. The second likely contributing factor is small-for-size graft. As described previously, patients with liver failure have persistent hyperdynamic circulation. It has been hypothesized that this condition may be due to persistent collateral blood flow, and that it leads to renal dysfunction by affecting the balance between vasoconstricting and vasodilating factors. The portosystemic collateral pathways are generally thought to close spontaneously after cadaveric whole liver transplantation because portal hypertension can be relieved by implantation of a normal graft (19-21). However, in adult LDLT, portal hypertension can persist after transplantation because the graft is a partial liver (22,23). Therefore, renal dysfunction after adult LDLT may occur due to persistent portal hypertension and hyperdynamic state in patients with a small-for-size graft (5,24).

In conclusion, our study suggests that small-for-size graft or hypovolemia, resulting in the delay of the recover of graft liver function, may be an important cause of acute renal failure during early postoperative period in adult LDLT.

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Original Paper

Wall Shear Stress and Intrahepatic Leukocytes of Graft in Living Related Donor Liver Transplantation

Yoshinobu Sato[1], Hisami Watanabe[3], Takafumi Ichida[2], Satoshi Yamamoto[1]
Hideki Nakatsuka[1], Hiroshi Oya[1], Hiroshi Kameyama[1], Takaoki Watanabe[1]
Kazuhiko Shimamura[1], Toru Abo[3], Katsuyoshi Hatakeyama[1]
Division of [1]Digestive and General Surgery, [2]Gastroenterology and Hapatology, [3]Immunology
Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
Corresponding Author: Yoshinobu Sato, MD, PhD, First Department of Surgery, Niigata University School of Medicine, 1-757 Asahimachi-dori, Niigata 951-8510, Japan
Tel: +81 25 227 2228, Fax: +81 25 227 0779, E-mail: kanishok@med.niigata-u.ac.jp

ABSTRACT

Background/Aims: We investigated the influence of HTK solution against natural killer T cells and thymic T cells in liver graft before and after perfusion in adult living related donor liver transplantation.

Methodology: Graft samples were obtained before liver resection, after perfusion, and one hour after liver transplantation. Flowcytometry analysis was conducted using several human natural killer markers; CD16, CD56, CD57, and CD161.

Results: Natural killer T cells existed prominently in the liver leukocytes compared with their presence in peripheral blood lymphocytes, and the difference was significant. CD56+T and CD161+T cells, in comparison with CD16+T cells and CD57+T cells, were especially numerous in the liver. The proportion of CD56+T and CD161+T cells increased in the graft immediately after perfusion with HTK solution. However, CD16+T cells and CD57+T cells decreased in the graft immediately after perfusion and reperfusion of portal blood flow. Thymus-derived cells also decreased significantly after perfusion. The proportion of CD56+T cells among CD3+ cells showed a significant increase immediately after perfusion. All types of natural killer cells in the graft immediately increased after perfusion by HTK solution and reperfusion of portal blood flow. Compared with CD57+NKT cells, CD56+NKT cells showed a significant tendency to stay in the liver graft against the perfusion. CD57+NKT cells tended to wash out from the liver into the systemic circulation. Moreover, thymus-derived T cells showed the strongest tendency to wash out from the liver graft.

Conclusions: CD56+NKT cells and natural killer cells are more involved in local immunity, whereas thymus-derived cells and CD57+NKT cells are involved in regulation of systemic immunity. Allo-immunity between local and systemic systems may be affected by the dynamic changes in hepatic circulation associated with living related donor liver transplantation.

KEY WORDS: Shear stress; Immunology; Intrahepatic leukocytes; NKT cell; Liver transplantation

ABBREVIATIONS: Living Related Donor Liver Transplantation (LRDLT); Mononuclear Cell (MNC); Peripheral Blood Lymphocytes (PBL); Sinusoidal Endothelial Cell (SEC); Natural Killer (NK); Auxiliary Orthotopic Partial Liver Transplantation (APOLT); Extra-Hepatic Portal Venous Obstruction (EHO)

INTRODUCTION

The systemic and local immune systems are strongly interrelated, and dynamic immunological changes in the remnant liver and extra-liver site are observed following partial hepatectomy (1-3). The wall shear stress, a simple hemodynamic force caused by venous flow directed against vessel walls (4,5), is the most important factor in the link between the systemic and intrahepatic immune systems following partial hepatectomy (3). We reported that there are two types of leukocytes in the liver: resident leukocytes, such as extrathymic T cells, which tend to stay in the liver against shear stress, and passenger leukocytes, such as thymic T cells, which are washed by the increased portal flow out of the liver and recruited into the systemic circulation (6,7). We confirmed this hypothesis in an experiment on perfused liver in mice (8). Intermediate TcR cells and NK1.1T cells tended to stay in the liver against perfused solution. Conversely, thymic T cells, compared with natural killer T (NKT) cells, increased in the irrigated solution. In nude mice, these phenomena were more prominent (8). These dynamic immunological changes may influence the allo-immune reaction in liver transplantation. Therefore, we investigated the changes in proportion of NKT cells and thymic T cells in the liver graft before and after perfusion by HTK solution in adult living related donor liver transplantation (LRDLT).

Hepato-Gastroenterology 2004; 51:329-333

METHODOLOGY

Patients

Adult patients (n=7, 17 to 55 years old) underwent LRDLT between January and December 2000. The primary diseases included two cases of liver cirrhosis, one related to hepatitis B and the other to hepatitis C, one case of primary biliary cirrhosis, one case of primary hepatic amyloidosis, one case of alcoholic liver cirrhosis, one case of neurological Wilson's disease, and

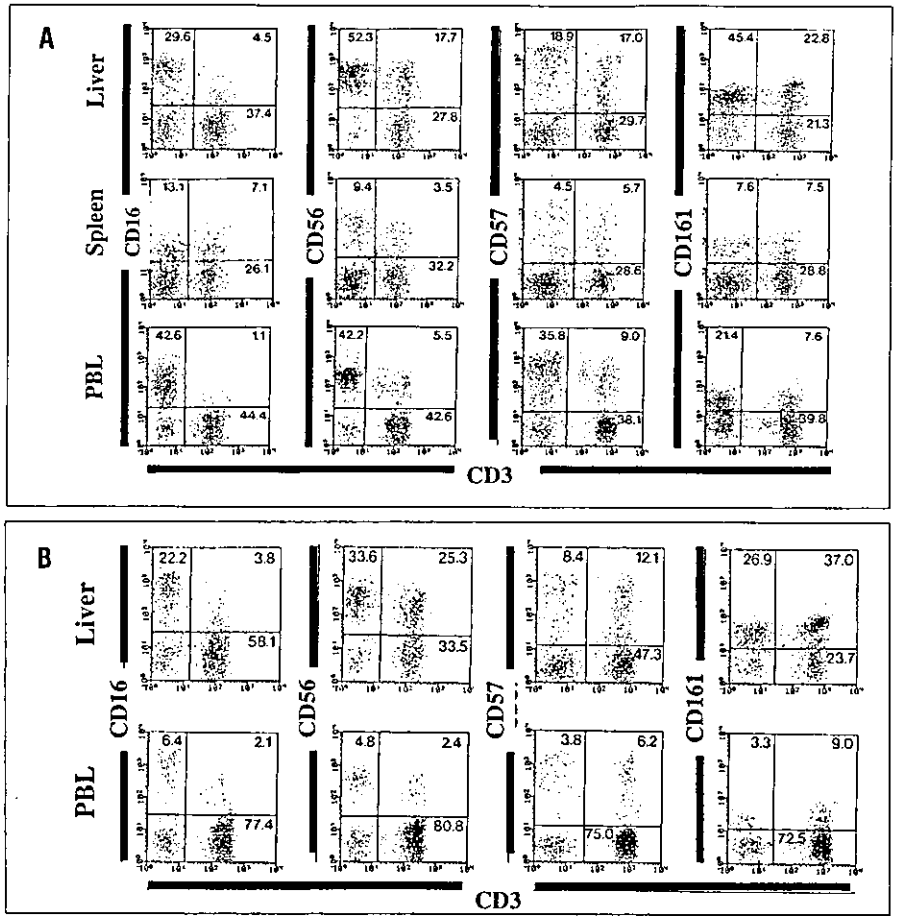

FIGURE 1
Phenotypic detection of NK and NKT cells. NKT cells existed prominently in the liver compared with those in the spleen and PBL of secondary extra-hepatic obliteration of only left gastric vein due to pancreatitis **(A)** and donor patient **(B)**. CD56+T cells and CD161+T cells were especially 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-

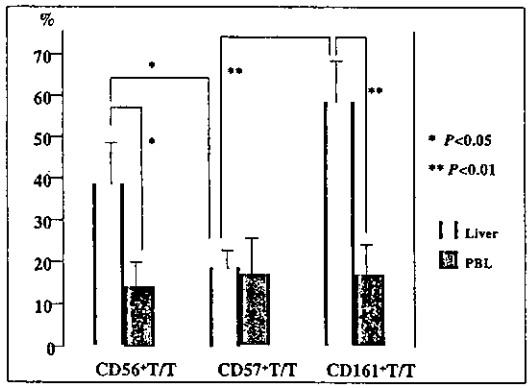

FIGURE 2 The proportion of NKT cells among CD3+T cells in donors of LRDLT (n=7).
CD56+T cells and CD161+T cells were abundant in the liver (39.3±11.2%, 58.3±6.0%) compared with those in PBL (13.4±6.5%, 18.7±10.4%). And also they were abundant compared with CD57+T cells in the liver.

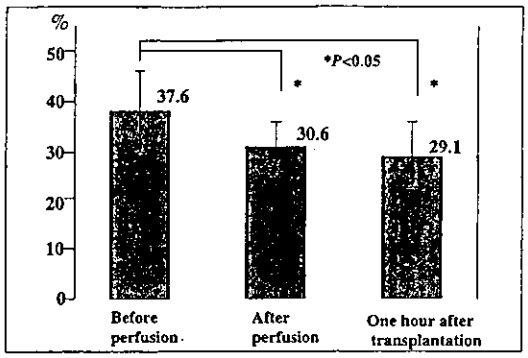

FIGURE 3 Changes of the proportion of thymus-derived cells in the graft liver by the perfusion of HTK solution in LRDLT. Thymus-derived cells of CD56⁻T cells in the graft before perfusion (37.6±10.3%) decreased immediately after perfusion by HTK solution (30.6±7.4%) and one hour after transplantation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

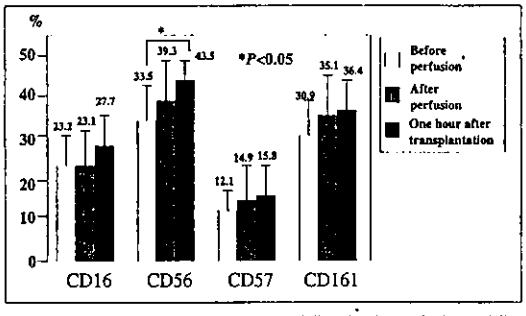

FIGURE 4 Changes of NK cells in the graft liver by the perfusion of HTK solution in LRDLT. All kinds of NK cells in the graft tended to increase immediately after perfusion by HTK solution and one hour after transplantation. Especially, CD56+T cells in the graft increased one hour after transplantation (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)**.

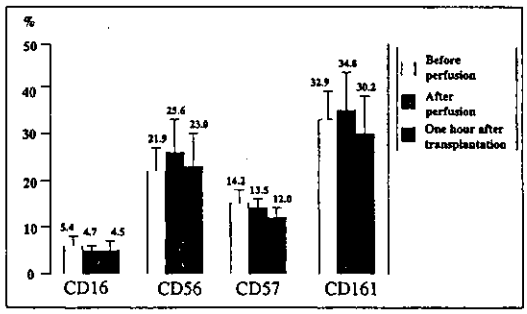

FIGURE 5 Changes of NKT cells in the graft liver by the perfusion of HTK solution in LRDLT. CD56+T and CD161+T cells in the graft liver tended to increase immediately after perfusion by HTK solution without statistical significance. However, CD16+T cells and CD57+T cells in the graft liver tended to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

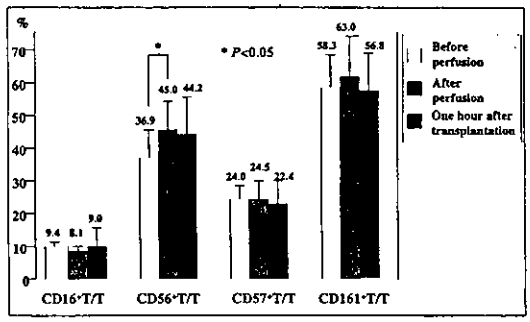

FIGURE 6 Changes of NKT cells among CD3+T cells in the graft liver by the perfusion of HTK solution in LRDLT. CD56+T cells and CD161+T cells among CD3+T cells in the graft liver tended to increase immediately after perfusion and decrease one hour after transplantation. Especially, CD56+T cells among 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

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 12671147 and 13557099) from the Ministry of Education, Science, Sports and Culture of Japan.

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ELSEVIER

Rapid Progressive Hepatitis C After Liver Transplantation: A Case Report

T. Takeishi, Y. Sato, T. Ichida, S. Yamamoto, K. Hirano, T. Kobayashi, T. Watanabe, and K. Hatakeyama

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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From the Division of Digestive and General Surgery (T.T., Y.S., S.Y., K.H., T.K., T.W., K.H.) Gastroenterology and Hepatology (T.I.) Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan.

Address reprint requests to Toshiyuki Takeishi, MD, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata 951-8510, Japan. E-mail: tochiogou@yahoo.co.jp

0041-1345/04/$–see front matter
doi:10.1016/j.transproceed.2004.07.009

Reevaluation of Prognostic Factors for Survival after Liver Resection in Patients with Hepatocellular Carcinoma in a Japanese Nationwide Survey

Iwao Ikai, M.D.[1]
Shigeki Arii, M.D.[2]
Masamichi Kojiro, M.D.[3]
Takafumi Ichida, M.D.[4]
Masatoshi Makuuchi, M.D.[5]
Yutaka Matsuyama, Ph.D.[6]
Yasuni Nakanuma, M.D.[7]
Kiwamu Okita, M.D.[8]
Masao Omata, M.D.[9]
Kenichi Takayasu, M.D.[10]
Yoshio Yamaoka, M.D.[1]
for The Liver Cancer Study Group of Japan

[1] Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan.

[2] Department of Hepato-Biliary-Pancreatic Surgery, Tokyo Medical and Dental University, Graduate School of Medicine, Tokyo, Japan.

[3] Department of Pathology, Kurume University School of Medicine, Kurume, Japan.

[4] Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan.

[5] Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

[6] Department of Biostatistics, School of Health Sciences and Nursing, University of Tokyo, Tokyo, Japan.

[7] Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan.

[8] Department of Gastroenterology and Hepatology, Yamaguchi University School of Medicine, Ube, Japan.

[9] Department of Gastroenterology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

[10] Department of Diagnostic Radiology, National Cancer Center Hospital, Tokyo, Japan.

Address for reprints: Iwao Ikai, M.D., The Liver Cancer Study Group of Japan, 403 Bear House, 40, Sanno-cho, Shogoin, Sakyo-ku, Kyoto, 606-8392, Japan; Fax: (011) 81 757525411; E-mail: ikai@kuhp.kyoto-u.ac.jp

This study was performed as a group project by the Liver Cancer Study Group of Japan.

Received February 9, 2004; revision received April 22, 2004; accepted May 10, 2004.

BACKGROUND. Advances in the diagnosis and surgical treatment of hepatocellular carcinoma (HCC) have improved the prognosis for patients with HCC who undergo liver resection. The objective of this study was to evaluate prognostic predictors for patients with HCC who underwent liver resection in a Japanese nationwide data base.

METHODS. In this study, the authors analyzed 12,118 patients with HCC in a Japanese nationwide data base who underwent liver resection between 1990 and 1999 and compared them with a previous analysis of patients between 1982 and 1989. All patients were evaluated for prognostic factors.

RESULTS. During the last decade, the increases in patients who were without hepatitis B virus surface antigen, who had small tumors, and who had portal vein invasion were noted. The 5-year overall survival rates for patients with HCC improved to 50.5%, compared with < 40% in the previous analysis. A multivariate analysis using a stratified Cox proportional hazards model according to associated liver disease indicated that age, degree of liver damage, α-fetoprotein level, maximal tumor dimension, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis, portal vein invasion, hepatic vein invasion, surgical curability, and free surgical margins were independent prognostic predictors for patients with HCC. Operative mortality decreased from 2.3% in 1990–1991 to 0.6% in 1998–1999.

CONCLUSIONS. Outcomes and operative mortality rates in patients with HCC improved during the last decade. Age, degree of liver damage, α-fetoprotein level, maximal tumor dimension, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis, portal vein invasion, hepatic vein invasion, surgical curability, and free surgical margins were prognostic factors for patients with HCC who underwent liver resection. *Cancer* 2004;101:796–802.

KEYWORDS: hepatocellular carcinoma, prognostic factor, liver resection, nationwide survey data base.

DOI 10.1002/cncr.20426
Published online 30 June 2004 in Wiley InterScience (www.interscience.wiley.com).

Hepatocellular carcinoma (HCC) is a common malignancy in Japan. Recent progress in the diagnosis and treatment of HCC has improved patient outcomes. With earlier diagnosis and tumors detected at an early stage, there is substantially increased survival, and curative surgical resection possible.[1–3] Advances in surgical techniques and perioperative management have led to a decline in the operative morbidity and mortality of patients who have HCC with chronic liver disease. However, long-term survival remains unsatisfactory.

The Liver Cancer Study Group of Japan (LCSGJ) has been conducting a nationwide survey of patients with primary liver carcinoma since 1965 to evaluate epidemiologic and clinical characteristics, histopathologic features, diagnosis, treatment modalities, and outcomes. In 1994, the LCSGJ reported on predictive factors for long-term prognosis after liver resection for patients with HCC between 1982 and 1989 in Japan.[2] Since that study was published, new imaging methods and laboratory tools have been introduced for the diagnosis of HCC.[4,5] In addition, it is recognized now that hepatitis C viral infection confers a high risk for HCC.[6] In the 1990s, a screening program for HCC was initiated, and several new therapeutic modalities for HCC, such as thermal ablation therapy and locoregional infusion chemotherapy through the hepatic artery, have been applied. Liver transplantation also has become an effective treatment for patients with HCC who have severe liver dysfunction.[7] However, new strategies for the treatment of patients with HCC still need to be established.

We reevaluated prognostic predictors for patients with HCC in a large-scale data base, taking into consideration recent advances in the diagnosis and treatment of HCC. In this study, we analyzed a Japanese nationwide data base between 1990 and 1999 to reevaluate prognostic factors for patients with HCC who underwent liver resection and compared the findings with data from our previous report.[2]

MATERIALS AND METHODS

In the nationwide follow-up survey of primary hepatic carcinoma conducted by the LCSGJ, patients with primary malignant liver tumors who were diagnosed with imaging studies, preoperative clinical data, and/or histopathologic studies at approximately 800 institutions in Japan were registered every 2 years, and registered patients were followed prospectively. In this data base, there were 50,267 patients who were diagnosed with HCC between 1990 and 1999. Among them, we enrolled 12,118 patients with HCC who underwent liver resection between January, 1990 and December, 1999. Follow-up ended on December 31, 1999. The median follow-up was 21.5 months (range, 0.03–119.7 months), the mean patient age was 62.5 years, and the male:female ratio was 3.73:1.0.

TABLE 1
Degree of Liver Damage

Item	Degree of liver damage* A	B	C
Ascites	None	Controllable	Uncontrollable
Serum bilirubin (mg/dL)	< 2.0	2.0–3.0	> 3.0
Serum albumin (g/dL)	> 3.5	3.0–3.5	> 3.0
$ICGR_{15}$ (%)	< 15	15–40	> 40
Prothrombin activity (%)	> 80	50–80	< 50

$ICGR_{15}$ indocyanine green retention rate at 15 minutes.
* The severity of each finding is evaluated separately. Degree of liver damage is recorded as A, B, or C, based on the highest grade that contained at least two findings.

In the data base, 21 clinicopathologic and biologic variables were selected from LCSGJ questionnaires that were used to survey patients with HCC. Patients were then stratified by gender, age, history of blood transfusion, hepatitis B virus surface antigen (HBs-Ag) status, hepatitis C virus antibody (HCV-Ab) status, degree of liver damage, preoperative serum α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) levels, maximal tumor dimension, number of tumors, intrahepatic extent of tumor, extrahepatic metastasis (including lymph node metastasis indicated in the preoperative imaging studies or operative findings), growth appearance (expansive growth or infiltrative growth), capsular formation, septum formation, portal vein invasion, hepatic vein invasion, bile duct invasion, surgical curability of liver resection, free surgical margins, and histologic-associated liver disease (normal liver, chronic hepatitis, and cirrhosis). The degree of liver damage was classified as A, B, and C (also called clinical Stages I, II, and III, respectively in the *Classification of Primary Liver Cancer* by the LCSGJ[8]) and was defined by preoperative measurements of ascites, serum bilirubin level, serum albumin level, indocyanine green retention rate at 15 minutes, and prothrombin activity (Table 1). Intrahepatic extent of tumor also was defined in the *Classification of Primary Liver Cancer* by the LCSGJ.[8] We defined a free surgical margin as a distance of 1 cm between the cut surface and the tumor edge in the resected specimen. Surgical curability was defined by the LCSGJ as follow: Absolute curative resection included live resection with 1 cm of free surgical margin in patients with Stage I disease; relative curative resection included live resection without 1 cm of free surgical margin but with the excised tumor tissue in patients with Stage I disease or liver resection with 1

cm of free surgical margin in patients with Stage II or III disease (in either instance, no tumor thrombi may remain in the portal vein, hepatic vein, or bile duct in images of the remnant liver); relative noncurative resection, in which all macroscopic tumor tissue is removed; and absolute noncurative resection, which is liver resection with part of the macroscopic tumor tissue remaining. Capsular infiltration was excluded, because this factor was determined only in patients who had HCC with capsular formation; and, in 25% of our patients, capsular formation was absent.

Overall, cumulative survival rates were obtained using the Kaplan–Meier method. The differences in survival between the groups were compared using the log-rank test. The starting point for calculating survival was the date of surgery, and the endpoint was the date of death. All deaths, including operative deaths, were considered the endpoint. Patients who remained alive on December 31, 1999, were censored. After the univariate analysis of the factors affecting survival, only significant variables except PIVKA-II were used in the multivariate analysis using the stratified Cox proportional hazards model, because data records of PIVKA-II were not complete. In the Cox model, baseline hazards were stratified by underlying liver disease, because the underlying liver disease affected survival in the univariate analysis and crossed the Kaplan–Meier curves. It was expected that this stratified Cox model would be more powerful than the subgroup analysis by underlying liver disease. The data derived from 7056 patients with complete records were used in the multivariate analysis. P values < 0.05 were considered statistically significant. Statistical analysis was carried out using SAS software (version 8.02; SAS Inc., Cary, NC).

RESULTS

The 1-year, 3-year, and 5-year overall survival rates for patients with HCC were 85.3%, 67.0%, and 50.5%, respectively (Fig. 1). The 5-year survival rate was 10% greater than the rate in our previous report, in which the 5-year survival rate was < 40%.[2] Categorization of variables, patient ratios, and the 3-year and 5-year survival rates are shown in Table 2. Compared with our previous report, the proportion of patients age > 60 years increased from 46% to 66%. The percentage of patients with positive HBs-Ag status decreased from 25% to 20%; and the percentage of patients with positive HCV-Ab status, which was not examined in the previous study, was > 60%. The development of diagnostic imaging studies made it possible to detect small tumors, so that patients with tumor s that measured ≤ 2 cm in greatest dimension increased from 16% to 21%, and their 5-year survival rate improved from 53% to 66%. This early detection of small HCC tumors led to an increase in patients with AFP levels < 20 ng/mL from 32% to 43%. The percentage of patients who had solitary tumors was similar to the percentage in our previous report; however, their 5-year survival rate increased from 41% to 56%. Improved surgical techniques have made it possible to resect advanced hepatic tumors. The proportion of patients with portal vein invasion who underwent liver resection increased from 15% to 24%. The indications for surgical treatment of advanced HCC have been expanding.

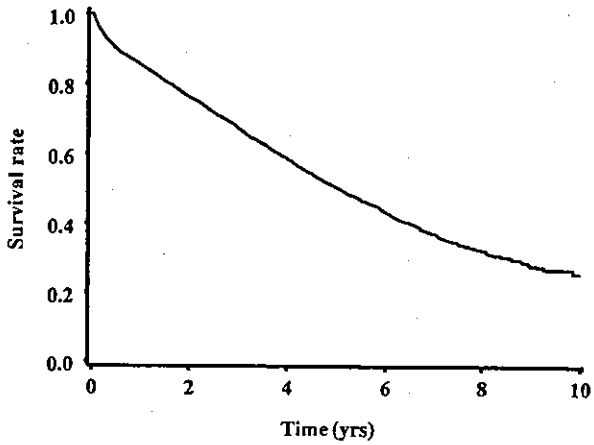

FIGURE 1. Survival curve for patients with hepatocellular carcinoma (HCC) who underwent liver resection between 1990 and 1999 (n = 12,118 patients). The 1-year, 3-year, and 5-year overall survival rates for patients with HCC patients were 85.3%, 67.0%, and 50.5%, respectively.

In the univariate analysis, there were significant differences in survival among the groups stratified by age, degree of liver damage, serum level of AFP and PIVKA-II levels, maximal tumor dimension, number of tumors, intrahepatic extent of tumors, extrahepatic metastasis, growth appearance, capsular formation, septum formation, portal vein invasion, hepatic vein invasion, bile duct invasion, surgical curability, free surgical margin, and associated liver disease (Table 2). Compared with our previous report, the survival of patients with negative HBs-Ag status, including patients with HCV-Ab, was no better than the survival of patients with negative HBs-Ag status. There also were no significant differences in survival between patients with negative and positive HCV-Ab status.

The multivariate analysis using a Cox proportional hazards model stratified by associated liver disease indicated that age, degree of liver damage, AFP level, maximal tumor dimension, number of tumors, intrahepatic extent of tumors, extrahepatic metastasis, portal vein invasion, hepatic vein invasion, surgical curability, and free surgical margin were independent