認された．またCLIA法の測定結果によるHCVキャリア検出判定は現行のC型肝炎ウイルス検査による判定と一致する結果であったことからHCVキャリアの掘り起こしに向けた検診検査などに対してCLIA法適応の可能性が示唆された．今後追加検討による確認が望まれる．

以上総合すると，CLIA法はRNA定量法と同等の臨床的感度と良好な特異性をもつこと，IFNによる抗ウイルス療法の経過観察，効果判定，効果予測に対してRNA定量法と同様に適用可能であることなどから，臨床的有用であると考える．

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<Abstract>

Evaluation of a Highly Sensitive Chemiluminescent Immunoassay "ARCHITECT HCV Ag" for HCV core antigen.

by

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The aim of this study is to evaluate a chemiluminescent immunoassay "ARCHITECT HCV Ag" (CLIA ; Abbott Laboratories) for HCV core antigen in human serum or plasma using clinical specimens. This is done primarily to check for potential utility in management of HCV-infected patients with anti-HCV therapy by Interferon. The CLIA showed good reproducibility (Intra assay CV% : 3.9- 5.4, inter assay CV% : 3.1- 7.0) and dilution linearity with a wide dynamic range (3.00-20,000 fmol/L). The sensitivity of the CLIA with serially diluted HCV RNA positive specimens was 4- 8 times higher than the current HCV core antigen assays. The CLIA showed better sensitivity with seroconversion panels vs. the HCV core antigen assays and HCV RNA quantitative assay. The detection for RNA positive samples by the CLIA was statistically better than or equivalent to the HCV core antigen assays and HCV RNA quantitative assay respectively. The correlation between the CLIA and the HCV core antigen assays or HCV RNA quantitative assay was also good. Specificity of the CLIA was excellent (100%) with health check specimens (n=5003). In the monitoring of the antiviral therapy by IFN, the CLIA closely tracked HCV RNA dynamics and allowed the monitoring of the treatment. The CLIA indicated better positive predictive value and negative predictive value for SVR vs. RNA quantitative assay when viral load was categorized by 300 fmol/L for HCV core Ag or by 100 KIU/mL for RNA.

In conclusion, it is suggested that the CLIA can be applicable in viral load monitoring and predicting treatment outcome in patients receiving antiviral therapy. The CLIA could be an alternative to HCV RNA quantitative assay with faster laboratory turn around time to results and easier handling of the assay in the clinical practice of hepatitis C patients.

*　　*　　*

[監修] 山田 剛太郎(川崎医科大学附属川崎病院肝臓・消化器病センター長)

第4回

肝癌進展抑制を目的とした病診連携の重要性 ―高齢化する患者とその発癌率

八橋 弘
(国立病院機構長崎医療センター臨床研究センター 治療研究部長)

C型慢性肝炎の自然経過

C型慢性肝炎の自然経過，発癌リスクを，肝線維化stage(fibrosis stage:F0～F4)を指標に表現したものが図1である．すなわち，HCV持続感染者は，肝機能障害すなわち肝細胞の破壊と再生を繰り返しながら，F0からF1，F2，F3，F4(肝硬変)へと20年から30年以上かけてゆっくり階段を昇るように進展し，最終的には肝硬変を経由して肝癌が発生する．肝線維化stage別年間発癌率は，F0：0.5％，F1：1.5％，F2：3％，F3：5％，F4：7～8％であり，stage進展とともに肝発癌のリスクも上昇する．特に50歳前後に急速に肝線維化stageが進展し，60歳を過ぎて高率に癌化する例は珍しくない．肝線維化stageは，発癌の確率，発癌までの時間を推定する上で有用な指標であり，HCV持続感染者は可能な限り肝生検を行って発癌のリスクを把握することが望ましいが，肝線維化stageは，血小板数によってある程度の推定は可能である．

発癌抑止を目標としたインターフェロン治療

現在，C型慢性肝炎の治療は，週1回の投与でも抗ウイルス活性が持続するPEG-インターフェロン(IFN)と内服の抗ウイルス薬であるリバビリンを併用するPEG-IFN+リバビリン併用療法が治療の主流となっている．いわゆるIFN難治例とい

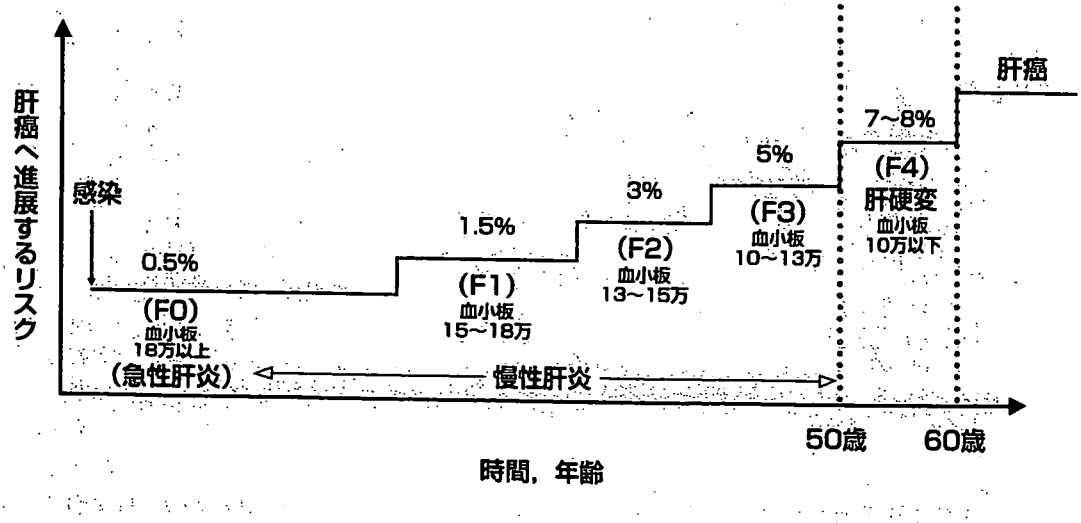

図1 C型慢性肝炎の自然経過，線維化stageと年間発癌率

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われるHCV 1型高ウイルス症例に対する治療成績は，従来型のIFNの24週間投与では10％に満たないウイルス駆除率（著効率）であったが，PEG-IFN+リバビリン併用療法48週間投与での著効率は50～60％にまで上昇した．

一方，日本のC型慢性肝炎患者の多くは60歳以上の高齢者である．上記に述べたように，ALT値異常を示す高齢のC型慢性肝炎患者の多くは，肝線維化stageは進展し，肝癌リスクも高いことから，高齢者での抗ウイルス療法の必要性，緊急性は若年者よりも高い．しかし，これら高齢者では，PEG-IFN+リバビリン併用療法の副作用の出現頻度，程度ともに高く，また著効率も低いといった問題が存在する．

筆者らは，高齢者，肝線維化進展例，PEG-IFN+リバビリン併用療法無効例などを対象に，IFN少量長期投与，PEG-IFNの2～4週に1回投与を数年前から実施している．本治療法はウイルス駆除を目的とせず，肝線維化進展抑止，発癌抑止を目的とした治療である．

その治療の理論背景は以下の2つに大別される．①IFNを抗炎症剤として用いて，ALT値の持続正常化，肝線維化進展抑止を目指す．②IFNには，細胞増殖抑制効果があり，肝癌細胞にも作用して，その増殖を抑制する，アポトーシスを誘導する，新生血管発育を抑制する，癌抑制遺伝子であるp53を誘導するといった作用がある．①を治療目標とする場合には，ALT値を指標とし，②の場合には，AFPなどの腫瘍マーカーを指標として，それぞれの治療法の効果を評価する．本治療法でALT値が低下，正常化しない例においても，AFP値は低下し正常化する場合が多く，IFNが前癌状態にある肝細胞に働きかけて肝癌の発生を抑えていることが想定される．

具体的には，従来型のIFNは，通常使用量の半分から1/4を，週2～3回投与する．PEG-IFNも通常使用量（180 μg）の半分から1/4量を2～4週間に1回投与する．投与量も少なく，投与間隔も長くなることから，IFNの副作用は軽微となる一方で，薬剤の安全性も高まり，また薬剤費用の負担も軽くなるという経済的なメリットも生まれる．ウイルス駆除を治療目的としないことから，患者が治療を続けることに疲れた場合や，1～2週間の旅行に出かける場合には，躊躇することなく治療を中止することが，この治療法のコツである．休薬によって体調が戻った場合や旅行から帰った場合に

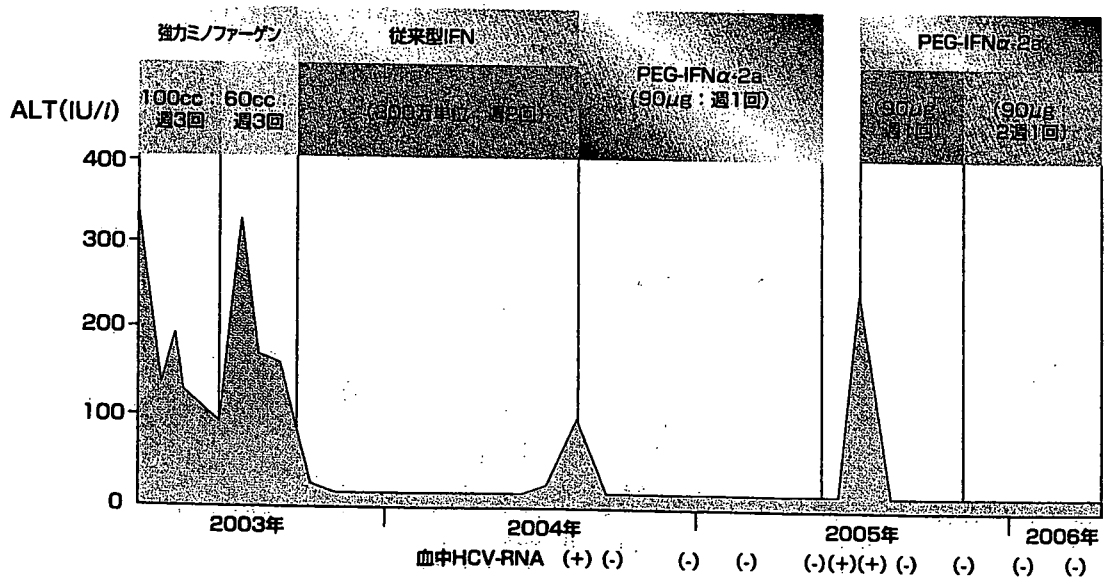

図2 70歳女性①
強力ミノファーゲンからIFN少量長期へ移行後に，PEG-IFNを導入した例．PEG-IFNを2週に1回の使用でもALT値は正常化し，血中HCV-RNA陰性化が持続している．

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は治療を再開始する．また，従来型のIFNでは，在宅自己注射が2005年4月に承認されている．年余に渡って治療を行うことで肝線維化の進展抑制，発癌抑止等の治療効果が期待できる(図2,3)．

これからの肝疾患診療体制 —かかりつけ医と肝疾患専門医療機関との医療連携

厚生労働省は，平成19年1月26日付けで「都道府県における肝炎検査後肝疾患診療体制に関するガイドライン」を公表した．そのガイドラインの中には，都道府県ごとに肝疾患診療ネットワーク(図4)を構築して肝疾患の医療連携を推進する，すなわち下記のようなことを実施するよう明記している．まず，各都道府県は，医師会，肝炎に関する専門医，関係市区町村や保健所等の関係者によって構成される肝炎診療協議会を設置する．同協議会では，各都道府県等の実情に応じて，①要診療者に対する保健指導，②かかりつけ医と専門医療機関の連携，③高度専門的ないし集学的な治療を提供可能な医療機関の確保，④受診状況や治療状況等の把握，⑤医療機関情報の収集と提供，⑥人材の育成，等について必要な検討を行うとともに，関係者との連絡・調整を図る．平成19年度内に，肝炎診療協議会は，肝疾患診療連携拠点病院を原則1箇所，また2次医療圏ごとに肝疾患専門医療機関を指定する．

一方，筆者が，日々の日常診療の中で実感している肝疾患診療の現状と問題点を下記に箇条書きにすると，①肝疾患は自覚症状がないにもかかわらず，最終的には肝硬変，肝癌を合併することがある，②肝疾患の診断法と治療法は毎年急速に進歩しているも専門医でないとその全容の理解は困難である，③わが国にはC型肝炎感染者200万人，B型肝炎感染者130万人，合わせて330万人存在する，④それに比して日本肝臓学会認定の肝臓

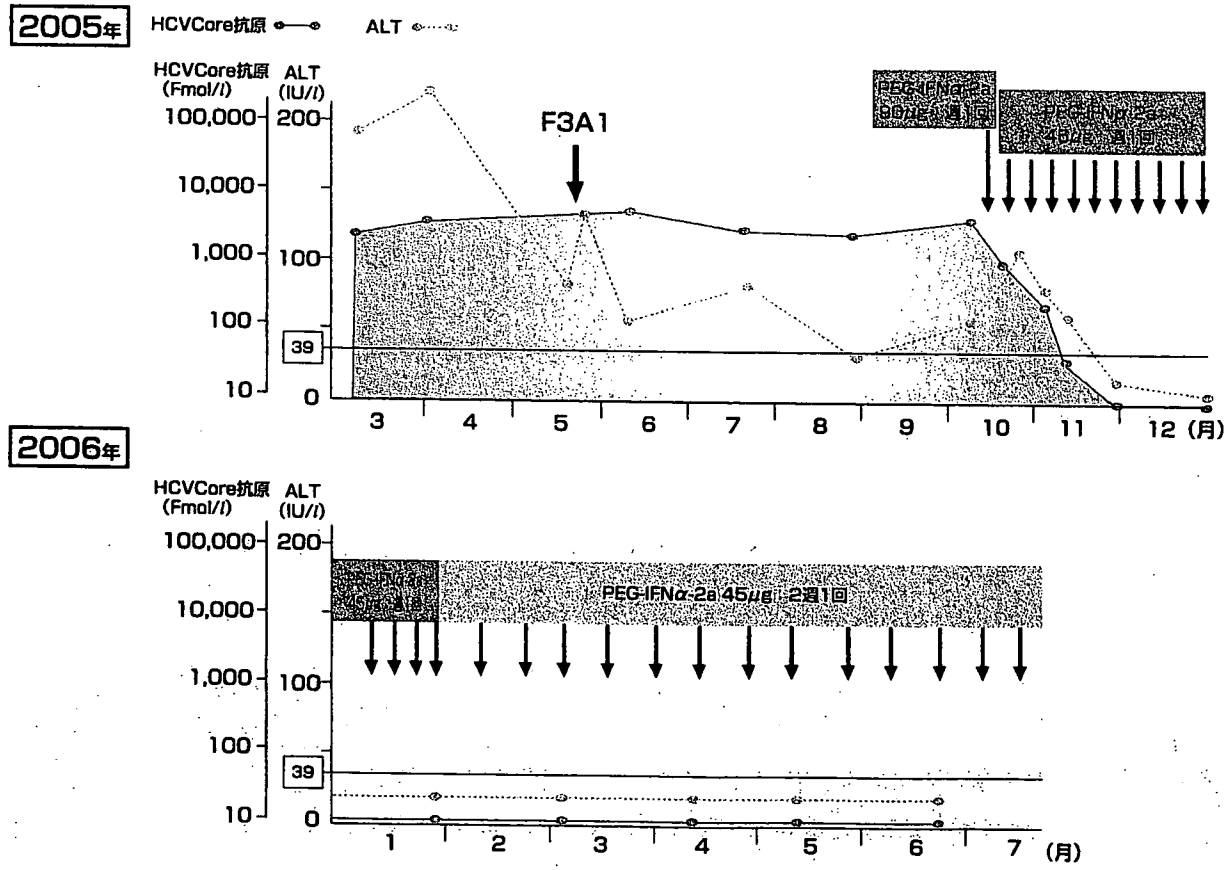

図3 70歳女性②

PEG-IFN90 μgを投与したが，ふらふら感を訴えたことから，翌週からは45 μgに減量した．副作用は軽減し継続投与可能となり，ALT値の正常化，HCV Core抗原の陰性化に至るも，体調が万全でないとの訴えがあることから45 μg 2週に1回投与と投与間隔を延長した．変更後，自覚症状はほぼ消失するも，ALT値の正常化，抗ウイルス効果は同様に持続している．

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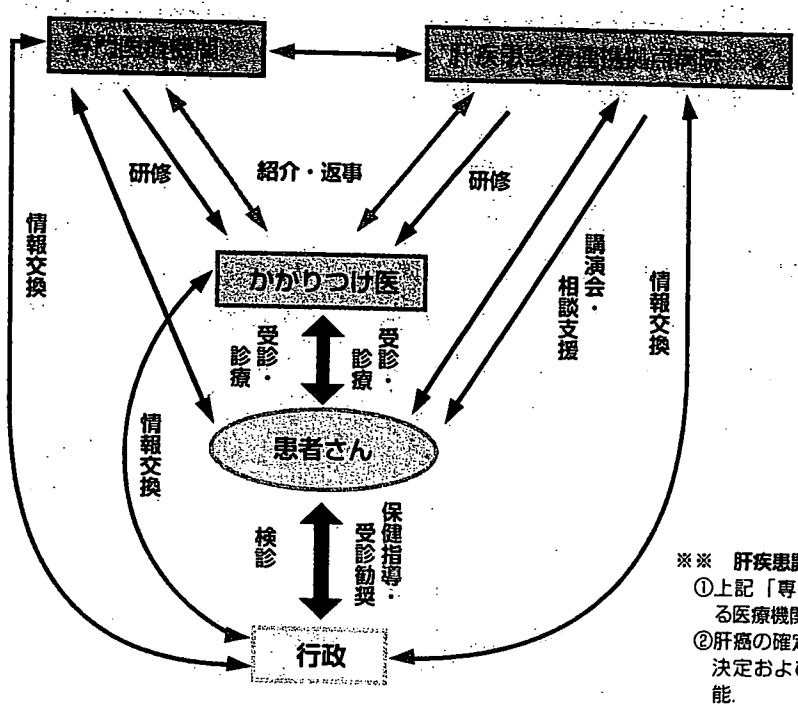

※ 専門医療機関
①専門的な知識をもつ医師による診断と治療方針の決定.
②IFNなどの抗ウイルス療法可能.
③超音波検査などによる肝癌の診断.

※※ 肝疾患診療連携拠点病院
①上記「専門的な医療が行える医療機関」の条件を満たす.
②肝癌の確定診断・治療方針の決定および集学的治療が可能.
③肝疾患に関する相談支援業務を行う.

図4 都道府県における肝疾患診療ネットワーク(2007年から)

〔全国C型肝炎診療懇談会報告書(厚生労働省, 2007)より改変〕

専門医数は，平成19年10月末の時点で3,695人と少ない(一専門医当たり1,000人の肝炎患者)，⑤現在，肝疾患の多くは適切な診断と治療によって完治ないしコントロールが可能な疾患となりつつある，などが挙げられる.

上記のような日本の医療状況の中で，肝疾患患者が理想的な医療を享受するためには，かかりつけ医と肝疾患専門医療機関の医療連携が重要である．肝疾患患者は，普段の診療，IFNを含む投薬等はかかりつけ医が行い，病状の正確な診断，治療方針決定などは専門医療機関で行う．病状が安定している場合でも1年に1回は専門医療機関に診察を依頼し，病態や治療方針を確認することが望ましい.

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BASIC STUDIES

NIM811, a nonimmunosuppressive cyclosporine analogue, suppresses collagen production and enhances collagenase activity in hepatic stellate cells

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Keywords
cyclosporine analogue – hepatic fibrosis – hepatic stellate cell – NIM811 – TGF-β

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Abstract

Background/Aims: A recent decrease in patient survival has been reported among hepatitis C virus (HCV)-infected liver transplant recipients and this may be attributable to progression of fibrosis. We reported previously that cyclosporine suppressed the proliferation of, and collagen production in, hepatic stellate cells (HSCs). Here, we investigated the effects of NIM811, a cyclosporine analogue, on cell growth, collagen production and collagenase activity in HSCs. *Methods:* Rat HSCs and human HSC-derived TWNT-4 cells were cultured for the study. The expression of collagen, matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) and collagenase activity was evaluated. Cell proliferation and apoptosis were measured. Phosphorylation of mitogen-activated protein kinases (MAPKs), Smad2 and Smad3 was evaluated. The expression of the tumour growth factor-β (TGF-β)-receptor and Smad7 genes was also evaluated. *Results:* NIM811, as well as cyclosporine, suppressed the transcription and synthesis of collagen and stimulated the production of MMP-1 with a concomitant enhancement of collagenase activity, although it did not change the expression of TIMP-1. NIM811 inhibited proliferation without induction of apoptosis. In the MAPKs and TGF-β signalling pathways, NIM811 enhanced the phosphorylation of JNK and p38, but not extracellular signal-regulated kinases 1 and 2, and suppressed the phosphorylation of Smad2 and Smad3, accompanied by increased Smad7 transcription and decreased TGF-β-receptor transcription. *Conclusion:* These findings demonstrate that NIM811 not only suppresses collagen production and proliferation but also increases collagenase activity. These effects are accompanied by inhibition of TGF-β signalling pathways.

In liver transplantation, a significant decline in patient survival, caused by rapid progression to decompensated graft cirrhosis, is an important clinical issue for hepatitis C virus (HCV)-positive patients, whereas there has been an improvement in the survival of HCV-negative patients (1–5). Progression to cirrhosis has been estimated to occur in up to 30% of patients at 5 years after transplantation, and the rate of disease progression has been greater in patients who underwent transplantation in recent years compared with those who underwent transplantation a longer period of time ago (1, 2, 4). Several variables, including donor age, degree of immunosuppression and viral load, are implicated in the recent increase in progression (2, 3). Indeed, Berenguer *et al.* (3, 5) reported that HCV-infected patients who received immunosuppressive therapy with cyclosporine were less likely to develop graft cirrhosis than those who received tacrolimus, indicating that the choice of calcineurin inhibitor may influence disease progression (6).

Hepatic stellate cells (HSCs) are nonparenchymal liver cells that have a characteristic stellate morphology

and reside in the perisinusoidal space of Disse (7). Following liver injury, HSCs undergo transdifferentiation to an activated myofibroblastic phenotype and express α-smooth muscle actin. Activated HSCs then proliferate and produce extracellular matrices (ECM) such as collagens (7, 8). Previously, we evaluated the effects of immunosuppressive drugs, such as cyclosporine and tacrolinus, on HSCs. Cell proliferation and collagen production were suppressed by cyclosporine but not by tacrolimus, indicating that cyclosporine potentially has an anti-fibrogenic effect (9).

Cyclosporine is an immunosuppressive cyclic undecapeptide, and binds with nanomolar affinity to cyclophilins. The complex of cyclosporine and cyclophilin A inhibits calcineurin, a calcium-dependent phosphatase that regulates the expression of various cytokine genes in activated T-lymphocytes (10–12). NIM811 is a four-substituted cyclosporine that does not bind to cyclophilin A and therefore lacks immunosuppressive activity; however, it retains the ability to bind other cyclophilins, such as cyclophilin B (13). Recently, NIM811, as well as cyclosporine, showed a suppressive effect on HCV at the RNA and protein levels in an HCV subgenomic replicon cell culture system (14). In this study, we investigated the effects of NIM811 on proliferation, collagen production and collagenase activity in HSCs *in vitro*. We demonstrated that NIM811 not only suppressed proliferation and collagen production but also enhanced collagenase activity in HSCs, indicating that NIM811 is a potential candidate for anti-fibrosis therapy.

Materials and methods

Cell culture

Hepatic stellate cells were isolated from the liver of male Wistar rats by sequential *in situ* perfusion with collagenase and digestion with pronase, followed by centrifugation in a double-layered (17%/11.5%) metrizamide solution (Sigma Chemical, St Louis, MO, USA), as described previously (15). HSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum (FCS). The experiments described in this study were performed on cells between the third and fourth serial passages. Because commercial kits for the measurement of mouse or rat matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were not available, we used TWNT-4 cells, a human cell line derived from HSCs (16), to evaluate the effects of NIM811 on MMP-1 and TIMP-1. TWNT-4 cells were cultured in DMEM with 10% FCS as reported previously (16). NIM811 was donated by Novartis Pharma AG (Basel, Switzerland). NIM811 was dissolved in DMEM and added to the cultures. The cell viability of HSCs was more than 90% under serum-free conditions for 24 h in the presence of 2 mM NIM811 under serum-free conditions.

Type I collagen assay

Cultured HSCs were incubated in a serum-free medium in the presence or absence of NIM811 for 24 h. Type I collagen was determined in culture media by ELISA as described previously (17). Anti-rat type I collagen antibody (LSL, Tokyo, Japan) was used as the primary antibody and peroxidase-conjugated goat-anti-rabbit IgG (Organon Teknika Corporation, Durham, NC, USA) was used as the secondary antibody. Rat tail tendon collagen type I (Advance Biofactures Corporation, Lymbrook, NY, USA) was used as the standard.

Matrix metalloproteinase 1, tissue inhibitor of metalloproteinase-1 and collagenase assay

Cultured TWNT-4 cells were incubated in a serum-free medium in the presence or absence of NIM811 for 24 h. MMP-1 and TIMP-1 productions were determined in culture media by ELISA using a Biotrak ELISA system for human MMP-1 (Amersham Biosciences, Piscataway, NJ, USA) and an hTIMP-1 kit (Daiichi Fine Chemical Co. Ltd, Toyama, Japan) respectively (18). Active MMP-1 and pro-MMP-1 in culture media were determined using an MMP-1 Biotrak Activity Assay System (Amersham) (18).

Analysis of gene expression using real-time reverse transcriptase-polymerase chain recation

Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from TWNT-4 cells that were maintained in either the presence or absence of NIM811 in 10% FCS for 24 h. cDNA was synthesized from 1.0 μg RNA with GeneAmp™ RNA PCR (Applied Biosystems, Branchburg, NJ, USA) using random hexamers. Real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green 1 (Roche, Tokyo, Japan) as described previously (19). The reaction mixture (20 μL) contained LightCycler-FastStart DNA Master SYBR Green 1, 4 mM $MgCl_2$, 0.5 μM of the upstream and downstream PCR primers and 2 μL of the first-strand cDNA as a template. To control for variations in the reactions, all PCRs were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The primers used were as follows: 5′-AGGGTGAGACAGGCGAACAG-3′ (forward primer) and 5′-CTCTTGAGGTGGCTGGGGCA-3′ (reverse primer) for human type I collagen α1 chain (GenBank™ accession number NM000088) (20);

5′-GATCATCGGGACAACTCTCCT-3′ (forward primer) and 5′-TCCGGGTAGAAGGGATTTGTG-3′ (reverse primer) for MMP-1 (GenBank™ accession number NM002421) (21); 5′-TTCTGCAATTCCG ACCTCGT-3′ (forward primer) and 5′-TCCGTCCAC AAGCAATGAGT-3′ (reverse primer) for TIMP-1 (Ref. 3; GenBank™ accession number NM003254) (22); 5′-GGATCTCAGGCATTCCTCGG-3′ (forward primer) and 5′-CAGTATGCCACCACGCACCA-3′ (reverse primer) for Smad7 (23); and 5′-GGCCGTTTGTATGTG CACCCTC-3′ (forward primer) and 5′-GGGCGAT CTAATGAAGGGTCC-3′ (reverse primer) for TGF-β-receptor I (TGF-β-RI) (24).

Analysis of bromodeoxyuridine incorporation

Hepatic stellate cell incorporation of bromodeoxyuridine (BrdU) was measured using a cell proliferation ELISA (Roche Diagnostics GmbH, Mannheim, Germany) as described previously (25). Briefly, subconfluent HSCs were serum starved for 24 h. They were then washed with DMEM and incubated for 24 h with BrdU in DMEM with 10% FCS in the presence or absence of NIM811. After labelling the cells with BrdU, cellular DNA was digested and incubated with the anti-BrdU antibody conjugated with peroxidase. BrdU incorporation was estimated by measuring the fluorescence intensity of the supernatant at 450 nm (excitation) and 690 nm (emission).

Analysis of apoptosis

Hepatic stellate cells were maintained in either the presence or absence of NIM811 under serum-free conditions for 24 h. Cells were fixed for 30 min in 4% paraformaldehyde/PBS at room temperature, and permeabilized for 5 min in PBS containing 0.2% Triton X-100 at 4 °C. Cells were then stained with Hoechst 33 342 and analysed by the terminal deoxynucleotidyl Transferase Biotin-dUTP nick end labeling method using an *in situ* Cell Death Detection Kit (Roche) according to the manufacturer's instructions. The samples were visualized with an LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany). At least 100 cells from three independent experiments and from three different cell preparations were counted for each condition.

Western blot analysis for phospho- and nonphospho-mitogen-activated protein kinases

Western blot analysis was basically performed essentially as described previously (26). After starving HSCs for 24 h, they were treated with or without NIM811 for 2 h or mock treated. Whole-cell lysates containing 1×10^7 TWNT-4 cells were prepared in 100 mL sodium dodecyl sulphate-polyacrylamide gel electrophoresis sample buffer. Protein lysates were subjected to 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and probed with the primary antibodies for extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinase (MAPK), phospho-ERK1/2 MAPK (Thr202/Tyr204), Jun N-terminal kinase (JNK), phospho-JNK (Thr183/Tyr185), p38 MAPK or phospho-p38 MAPK (Thr180/Tyr182) (New England Biolabs, Beverly, MA, USA). Antibody binding was detected using peroxidase-linked anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as the secondary antibody. The blots were developed using ECL-plus (Amersham Pharmacia Biotech) to visualize the antibodies. The levels of ERK1/2 MAPK, phosphorylated-ERK1/2 MAPK, JNK, phosphorylated-JNK, p38 MAPK and phosphorylated-p38 MAPK were quantified by densitometry using an optical scanner system. For comparison, the ratios of phosphorylated ERK1/2, JNK and p38 MAPK to nonphosphorylated ERK1/2, JNK and p38 MAPK, respectively, were calculated from the densitometric data.

Western blot analysis for phospho- and nonphospho-Smad2 and Smad3

Western blot analysis was performed as described above, probed with the primary antibody for Smad2, phospho-Smad2 (Thr/Tyr), Smad3 or phospho-Smad3 (Thr/Tyr) (Cell Signaling Technology, Danvers, MA, USA). For comparison, the ratios of phosphorylated Smad2 and Smad3 to nonphosphorylated Smad2 and Smad3, respectively, were calculated from the densitometric data.

Statistical analysis

All results are shown as the mean ± SEM. Comparisons were made using one-way ANOVA, followed by Scheffe's test or the Mann–Whitney test.

Results

Effects of NIM811 on type I collagen accumulation, matrix metalloproteinase 1 and tissue inhibitor of metalloproteinase-1 production and collagenase activity

To assess the effect of NIM811 on ECM production by HSCs, we determined type I collagen concentrations in culture media after adjusting the number of rat HSCs. Treatment of the cells with increasing concentrations

of NIM811, as well as cyclosporine, led to a concentration-dependent suppression of collagen accumulation; 0.5 mM NIM811 reduced collagen accumulation by approximately 50% (Fig. 1). As reported previously (9), cyclopsorine at the clinically relevant concentration of 0.125 mM (150 ng/mL) reduced collagen production by approximately 50%, whereas tacrolimus at the clinically relevant concentration of 12.5 nM (10 ng/mL) did not reduce collagen production significantly (Fig. 1).

Because collagenase activity affects accumulation of type I collagen, we evaluated the effects of NIM811 on collagenase activity in TWNT-4 cells. NIM811 led to a concentration-dependent increase in collagenase activity (active MMP-1); in the presence of 0.5 mM NIM811, collagenase activity increased roughly two-fold (Fig. 2A). Because collagenase activity is regulated by the balance between MMP-1 and TIMP-1, we also evaluated TIMP-1 production in TWNT-4 cells. NIM811 tended to reduce TIMP-1 production in a concentration-dependent manner; however, NIM811 did not reduce TIMP-1 production significantly, even at a concentration of 2.0 mM (Fig. 2B).

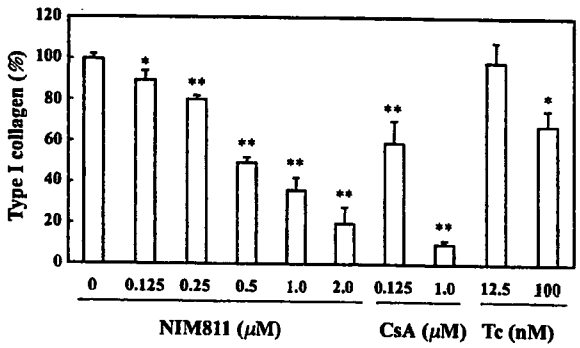

Fig. 1. Effects of NIM811, cyclosporine (CsA) and tacrolimus (Tc) on the accumulation of type I collagen in rat hepatic stellate cells (HSCs). NIM811, as well as cyclosporine, caused a concentration-dependent inhibition of type I collagen production. In contrast, tacrolimus did not show these suppressive effects at a clinically relevant concentration (i.e. 12.5 nM, equivalent to 10 ng/mL). The data represent mean ± SEM from five independent experiments. *and **Statistically significant differences ($P < 0.05$ and < 0.01 respectively) compared with HSCs in the absence of NIM811, CsA and Tc.

Effects of NIM811 on gene expression of type I collagen, matrix metalloproteinase 1 and tissue inhibitor of metalloproteinase-1

We used RT-PCR to evaluate the effects of NIM811 or cyclosporine on the mRNA levels of type I collagen, MMP-1 and TIMP-1. The expression of type I collagen was reduced by roughly 30% in the presence of 0.5 mM NIM811 (Fig. 3A). In contrast, 0.5 mM NIM811 increased the expression of MMP-1 nearly two-fold (Fig. 3B) but did not affect that of TIMP-1 (Fig. 3C). These results indicated that the effects of NIM811 on gene expression were similar to its effect on protein production.

Effect of NIM811 on cell proliferation and apoptosis

Bromodeoxyuridine incorporation was measured to investigate the effect of NIM811 on cell proliferation. Quantitative analysis showed that 2.0 mM NIM811 treatment decreased new DNA synthesis by nearly 30%, although treatment with lower concentrations had a reduced effect (Fig. 4). Next, we evaluated the effects of NIM811 on apoptosis; even in the presence of 2 mM NIM811, little apoptosis was observed (data not shown).

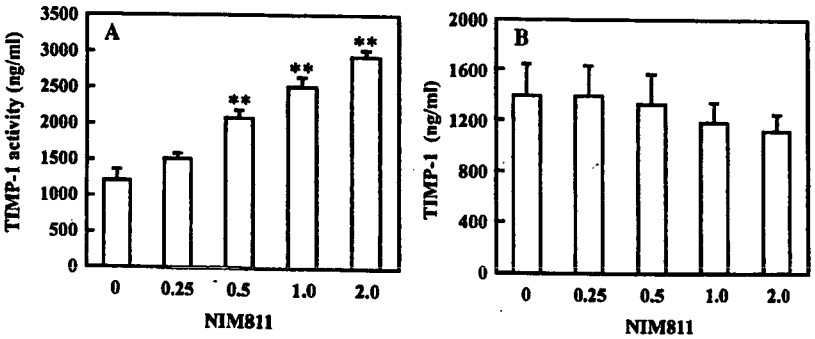

Fig. 2. Effects of NIM811 on matrix metalloproteinase 1 (MMP-1) activity (collagenase activity) and tissue inhibitor of metalloproteinase-1 (TIMP-1) production in TWNT-4 cells. NIM811 caused a concentration-dependent enhancement of MMP-1 activity (collagenase activity) (A). NIM811 also tended to reduce TIMP-1 production in a concentration-dependent manner (B). The data represent mean ± SEM from five independent experiments. *and **Statistically significant differences ($P < 0.05$ and < 0.01 respectively) compared with hepatic stellate cells (HSCs) in the absence of NIM811.

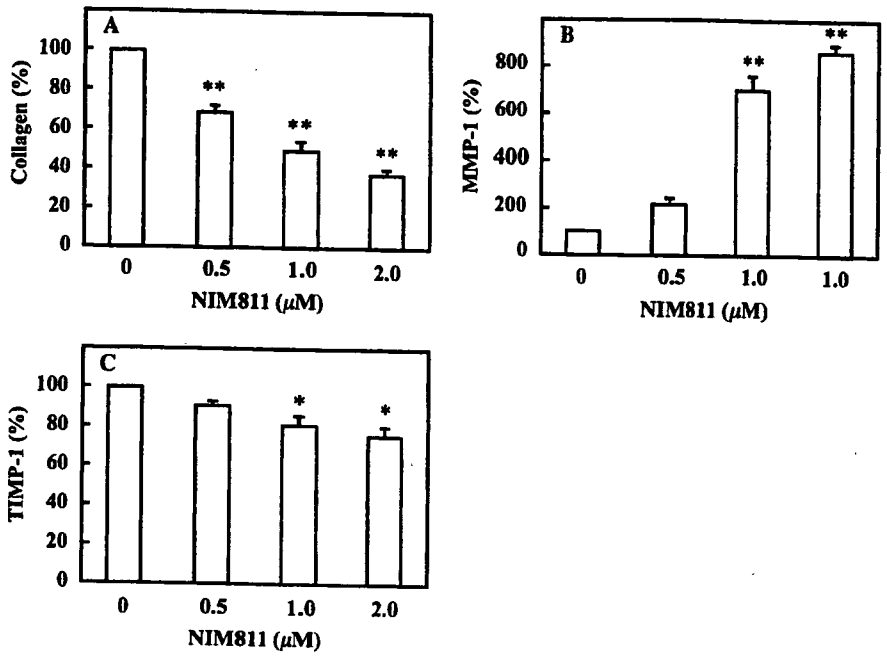

Fig. 3. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of type I collagen, matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) mRNA expression. Total RNA was extracted from hepatic stellate cells (HSCs) treated with NIM811 (0.5, 1 or 2 mM) or mock treated. Samples were analysed by real-time RT-PCR and all PCR reactions were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. NIM811 reduced the expression of type I collagen (A) and TIMP-1 (C) but it enhanced the expression of MMP-1 (B). The ratio of expression in the absence of NIM811 was used as a control (100%). The data represent mean ± SEM from three independent experiments. *and **Statistically significant differences ($P < 0.05$ and $P < 0.01$ respectively) compared with HSCs in the absence of NIM811.

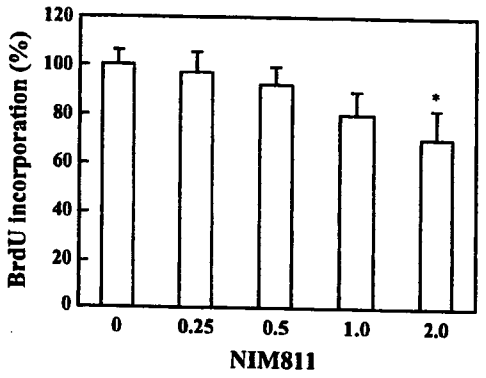

Fig. 4. Effects of NIM811 on BrdU incorporation into hepatic stellate cells (HSCs). NIM811 (2.0 mM) decreased BrdU incorporation in cells by nearly 30% compared with untreated cells, although treatment with a lower concentration did not. The data represent mean ± SEM from five independent experiments. *Statistically significant difference ($P < 0.05$) compared with HSCs in the absence of NIM811.

Effects of NIM811 on MAPKs signalling pathways

Because cell growth and the expression of genes involved in the process of cell growth are widely regulated through MAPK signal cascades, we assessed the effects of NIM811 on MAPK activity, including ERK1/2, JNK and p38. Treatment with NIM811 significantly enhanced the phosphorylation of JNK and p38 MAPK in a concentration-dependent manner, but did not enhance the phosphorylation of ERK1/2 (Fig. 5A–C). NIM811 at a concentration of 0.5 mM enhanced the phosphorylation of JNK and p38 MAPK by nearly 3.6- and 2.3-fold respectively (Fig. 5B and C).

Effects of NIM811 on tumour growth factor-β signalling pathways

Because TGF-β signal cascades through Smad2 and Smad3 strongly regulate the expression of the type I collagen gene (27), we evaluated the effects of NIM811 on the phosphorylation of Smad2 and Smad3. Treatment with NIM811 significantly suppressed the phosphorylation of Smad2 and Smad3 in a concentration-dependent manner; 0.5 mM NIM811 suppressed the phosphorylation of Smad2 and Smad3 by nearly 70 and 60% respectively (Fig. 6A and B). Next, we evaluated the expression of Smad7, which negatively regulates TGF-β signalling pathways by inhibition of TGF-β-RI phosphorylation (28). 0.5 mM NIM811

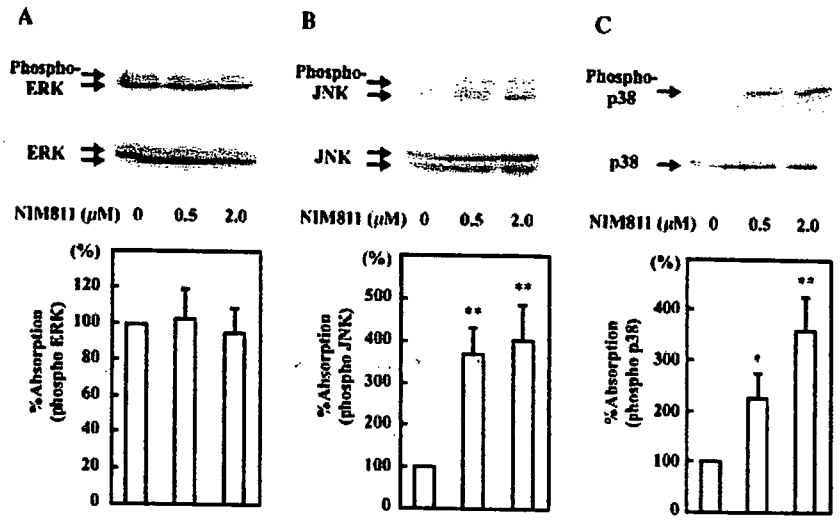

Fig. 5. Western blot analysis of phosphorylated and nonphosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2) (A), JNK (B) and p38 (C). NIM811 enhanced the phosphorylation of JNK and p38, but not ERK1/2. Each figure is representative of three independent experiments. Band intensities were measured to determine the ratios of phosphorylated to nonphosphorylated ERK1/2, JNK and p38. The ratio of phosphorylation in the absence of NIM811 was used as a control (100%). The values shown are the mean ± SEM of triplicate determination. *and **Statistically significant differences ($P < 0.05$ and < 0.01 respectively) compared with hepatic stellate cells (HSCs) in the absence of NIM811.

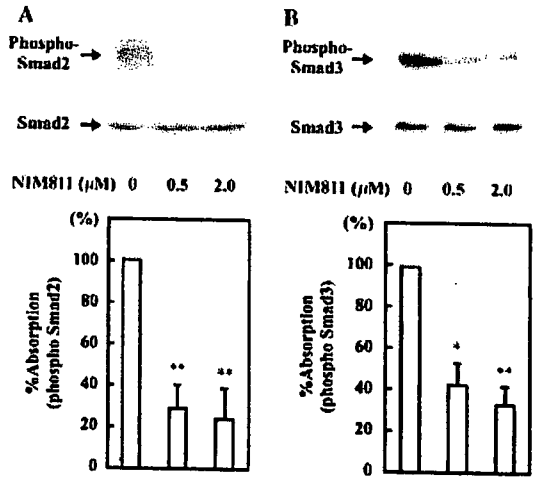

Fig. 6. Western blot analysis of phosphorylated and nonphosphorylated Smad2 and Smad3. NIM811 strongly suppressed the phosphorylation of Smad2 (A) and Smad3 (B). Each figure is representative of three independent experiments. Band intensities were measured to determine the ratios of phosphorylated to nonphosphorylated Smad2 and Smad3. The ratio of phosphorylation in the absence of NIM811 was used as a control (100%). The values shown are the mean ± SEM of triplicate determination. *and **Statistically significant differences ($P < 0.05$ and < 0.01 respectively) compared with hepatic stellate cells (HSCs) in the absence of NIM811.

enhanced the expression of Smad7 nearly two-fold (Fig. 7A) and it suppressed that of TGF-β-RI by nearly 50% (Fig. 7B).

Discussion

A recent study by Berenguer *et al.* (3) demonstrated that HCV-infected patients receiving immunosuppressive therapy with cyclosporine were less likely to develop graft cirrhosis than those treated with tacrolimus. We reported previously that cyclosporine at the clinically relevant concentration of 0.125 mM (150 ng/mL) significantly reduced collagen production in HSCs, while tacrolimus at the clinical concentration of 12.5 nM (10 ng/mL) did not (9). In this study, we demonstrated that NIM811 (0.125 mM), as well as cyclosporine, produced a concentration-dependent suppression of collagen accumulation (Fig. 1). We also found that this suppression was regulated at least as far upstream as the transcriptional level because treatment with NIM811 suppressed collagen gene expression. Collagen accumulation, in addition to being determined by the rate of collagen production, is also regulated by collagenase activity, specifically, by the balance between MMP-1 and TIMP-1. We found that treatment of the cells with NIM811 increased collagenase activity, accompanied by stimulation of the transcription and synthesis of MMP-1 and weak suppression of the expression of TIMP-1 (Figs 2 and 3).

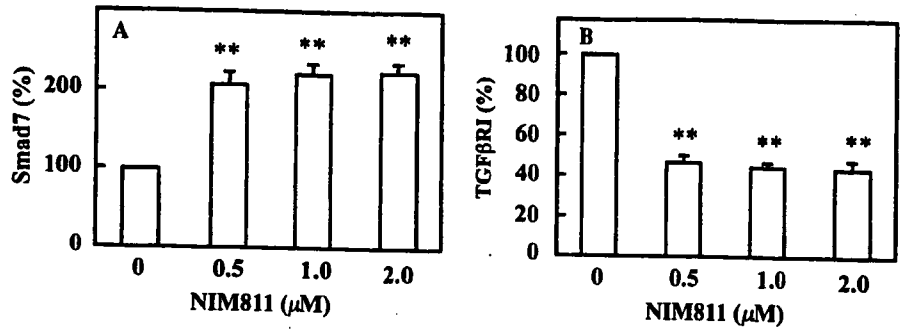

Fig. 7. Real-time reverse transciptase-polymerase chain reaction (RT-PCR) analysis of Smad7 and tumour growth factor-β (TGF-β)-RI mRNA expression. Total RNA was extracted from hepatic stellate cells (HSCs) treated with NIM811 (0.5, 1, or 2 mM) or mock treated. Samples were analysed by real-time RT-PCR, and all PCR reactions were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. NIM811 enhanced the expression of Smad7 (A), and it reduced the expression of TGF-β-RI (B). The ratio of expression in the absence of NIM811 was used as a control (100%). The data represent mean ± SEM from three independent experiments. **Statistically significant differences ($P < 0.01$) compared with HSCs in the absence of NIM811.

Previous work has demonstrated that, in addition to stimulating collagen production, activated HSCs inhibit the degradation of interstitial collagens by collagenases such as MMP-1, indicating that matrix degradation is inhibited during the progression of fibrosis (29–31). TIMP-1 has been reported to regulate cell growth and apoptosis independent of the inhibition of matrix degradation (32). We demonstrated that NIM811 suppressed the growth of HSCs in a concentration-dependent manner without apoptosis (Fig. 4). Taken together, these results indicate that NIM811 has therapeutic potential for liver fibrosis through suppression of collagen production and enhancement of collagenase activity.

To explore the mechanism by which NIM811 suppresses collagen production and cell proliferation, and enhances collagenase activity, we examined the effects of NIM811 on intra-cellular signalling cascades, such as MAPK cascades, which play important roles in collagen production and cell proliferation in HSCs (33). It is intriguing that NIM811 enhanced the activation of JNK and p38 but not ERK1/2 (Fig. 5). In contrast, cyclosporine suppressed the activation of JNK and p38, as we reported previously (9). It was shown that cyclosporine exerts its immunosuppressive effects through both the calcineurin-dependent nuclear factor of activated T cells (NFAT) pathway and the calcineurin-independent activation pathway for JNK and p38 (34). NIM811, an analogue of cyclosporine, does not activate the NFAT pathway because it cannot bind to cyclophilin A (13). The different effects of NIM811 and cyclosporine on JNK and p38 might be attributable to the absence of an effect of NIM811 on the NFAT pathway.

In addition to MAPKs, TGF-β signalling cascades strongly stimulate collagen production by HSCs (28). TGF-β binds to TGF-β-RII on the cell membrane, and then TGF-β-RII phosphorylates TGF-β-RI at the serine and threonine residues located in its glycine-/serine-rich domain (35). The phosphorylated TGF-β-RI phosphorylates Smad2 and Smad3 at a C-terminal SSXS motif and these form a complex with their common partner Smad4. These Smad proteins translocate to the nucleus and activate the transcription of target genes such as collagen (35). In this study, we demonstrated that Smad2 and Smad3 were constitutively phosphorylated in activated HSCs, as reported previously (36), and that NIM811 suppressed the phosphorylation of Smad2 and Smad3 (Fig. 6). These results suggest that NIM811 may inhibit the kinase activity of TGF-β-RII and/or TGF-β-RI. Several molecules such as Smad7 (28, 37), immunophilin FK506-binding protein (FKBP) 12 (38) and Smad anchor for receptor activation (SARA) (39) are associated with TGF-β-R and regulate TGF-β signalling. We found that NIM811 enhanced the expression of Smad7, and suppressed that of TGF-β-RI, indicating that NIM811 inhibits the TGF-β signalling pathways, at least partially through blockade at the receptor level. We also found that cyclosporine had similar effects on Smad2, Smad3, Smad7 and TGF-β-RI (unpublished data). As mentioned above, NIM811 had effects opposite to those of cyclosporine on JNK and p38, although both showed similar effects on collagen production and cell proliferation, suggesting that NIM811 and cyclosporine exhibit antifibrogenic effects mainly by blockade of TGF-β signalling pathways.

Because NIM811 lacks the ability to bind to cyclophilin A (13), NIM811 exerts its pharmacological effects by binding to other cyclophilins, such as cyclophilin B or D. Cyclophilins are a family of PPIases that catalyse the cis-trans interconversion of peptide-bound amino-terminal proline residues, facilitating

changes in protein conformation (40). There are more than 10 subtypes of cyclophilin and they are involved in numerous cellular processes, including transcriptional regulation, immune response, protein secretion and mitochondrial function (40, 41). Watashi *et al.* (42) recently reported that NIM811 suppressed the replication of an HCV replicon *in vitro*, whereas tacrolimus did not show this effect. Notably, NIM811 exerts its antiviral effects via binding cyclophilin B, which is a functional regulator of HCV RNA polymerase (43). NIM811 has also been reported to have cytoprotective properties depending on interference with the interaction with cyclophilin D, which regulates the mitochondrial permeability transition (13). Kon *et al.* (44) reported that NIM811 prevented acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. In order to explain the detailed working mechanism of NIM811, cyclophilins interacting with NIM811 are important factors; however, we have not determined which cyclophilin is utilized by NIM811 to exert its anti-fibrogenic and anti-proliferating activity. We are now in the process of identifying the target cyclophilin.

In conclusion, we demonstrated that NIM811, as well as cyclosporine, had anti-fibrogenic effects. NIM811 has no immunosuppressive activity and, in consideration of the toxicity data, seems more favourable for clinical use because of the absence of significant changes in kidney-specific parameters following 10 days of 50 mg/kg of oral NIM811, whereas the same dose regimen of cyclosporine produced signs of renal dysfunction (45). NIM811 would be a plausible candidate for prevention of the progression of HCV-related graft-cirrhosis after liver transplantation because of its anti-viral and anti-fibrogenic effects *in vitro*. Further studies *in vivo* will be required to determine whether NIM811 is effective for the treatment of hepatic fibrosis.

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BASIC STUDIES

Methylprednisolone injection via the portal vein suppresses inflammation in acute liver failure induced in rats by lipopolysaccharide and D-galactosamine

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Keywords
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Abstract

Background: We have reported that hepatic arterial steroid injection is an effective therapy to rescue patients from fulminant or severe acute hepatic failure. We speculate that a high concentration of steroid suppresses inflammatory processes in the liver directly by restraining activated inflammatory cells, including macrophages. To analyse the detailed mechanism, steroid injection via the portal vein was performed in an experimental model of liver damage. *Methods:* Rats subjected to lipopolysaccharide and D-galactosamine injection were treated with a methylprednisolone injection via the tail vein or the portal vein. The survival rate, serum levels of inflammatory cytokines and apoptotic cell counts in the liver were analysed. *Results:* The survival rate was significantly improved by steroid injection, especially via the portal vein. Serum values of alanine aminotransferase, tumor necrosis factor-α and interferon-γ were reduced in the treated groups, especially the group given portal venous injections. Apoptotic cell counts in the liver were significantly lower in the group injected with steroid via the portal vein. *Conclusion:* In the model rats, high concentrations of steroid in the liver acted on inflammatory cells and suppressed inflammatory cytokines and liver cell death. The mechanism is suggested to be the same for arterial steroid injection therapy in patients with acute hepatic failure.

Acute liver damage occasionally progresses to severe liver failure with extremely high mortality, even today (1–3). Plasma exchange and haemodiafiltration have been used as artificial liver support systems for affected patients but are only partially effective (4, 5). Although liver transplantation is valuable for the rescue of patients and improving prognosis (6, 7), it is accompanied by an extraordinary financial burden, and the possibility of life-threatening complications to living donors cannot be eliminated completely.

Recently, we reported that transcatheter arterial steroid injection therapy (TASIT), by which methylprednisolone is administered directly into the liver through a catheter located in the hepatic artery, dramatically suppressed liver injury and reduced the mortality of patients with severe acute hepatic failure (8). More than 75% of the patients treated with TASIT were cured without liver transplantation; however, although 24% of the patients without TASIT recovered, 35% underwent liver transplantation and 41% died. Because corticosteroid can suppress the effects of activated macrophages and other inflammatory cells (9, 10), it has been used for the treatment of severe acute hepatic failure by oral or intravenous administration; however, this therapy has not shown a satisfactory clinical effect and has not been definitively established (11, 12). We speculate that a sufficient concentration of corticosteroid cannot be achieved in the liver when corticosteroid is administered through a peripheral vein because of the injured circulation that accompanies severe hepatic damage. Therefore, it is possible that TASIT can supply a sufficient

concentration of steroid to the liver, even in such a poor condition.

To estimate the effect of exposure of the injured liver to high concentrations of steroid, we carried out experiments in a rat model of acute liver failure induced by lipopolysaccharide (LPS) and D-galactosamine (GalN) (13). It is well known that the liver damage in this model is caused by the activation of lymphocytes and macrophages and that cytokines released from these inflammatory cells accelerate the progression of liver cell destruction, including apoptosis of hepatocytes (14, 15). Although, in the case of TASIT, steroid is injected through a catheter located in the hepatic artery, via a puncture of the femoral artery, we decided to inject steroid via the portal vein in this study because of the technical difficulty of the arterial approach in rats. Because the blood supply to the liver tissue from the portal vein is twice in volume that from the hepatic artery, a sufficient or a greater drug supply to the liver tissue should be achieved using the portal vein approach (16). In this study, using the rat model of liver damage, the survival period and serum values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were compared among three groups: rats without steroid treatment, with steroid injection via the tail vein and with injection via the portal vein. Liver damage was examined histologically, and apoptotic cell counts were estimated. Our results showed that steroid injection therapy via the portal vein was superior in suppressing hepatic inflammation and improving the survival rate in this animal model.

Materials and methods

Animal treatment

Eight- to 10-week-old male Wister rats weighing 175–225 g were maintained under controlled conditions with free access to standard chow and water. During all the experimental procedures, the animals were given human care in compliance with institutional guidelines according to the '*Guide for the Care and Use of Laboratory Animals*' published by the National Institutes of Health. One-hundred microgram per rat of LPS and 500 mg/kg of GalN (Sigma, St Louis, MO, USA), each of which was dissolved in 500 μL of phosphate buffered saline (PBS), were injected intraperitoneally into the rats. Two hours after the injection of LPS and GalN, animals were anaesthetized with pentobarbital sodium, and then 10 mg of methylprednisolone sodium succinate (mPSL) (Pfizer, New York, NY, USA) dissolved in 100 μL of PBS was injected into the portal vein or the tail vein. Control animals were injected with the same amount of PBS into the portal vein or the tail vein. Seven rats were examined in each group.

Histological examination

Liver tissue samples were isolated 10 h after the injection of mPSL or PBS, fixed in 10% formalin and embedded in paraffin. Serial sections (5 μm thick) were cut from each block. Liver injury was evaluated morphologically in hepatic sections stained with haematoxylin and eosin. Apoptotic cells were detected using an apoptosis detection kit (Wako, Osaka, Japan). The TdT-mediated dUTP nick end labelling (TUNEL) method was performed using deparaffinized liver sections (17). The number of positive cells in 10 high-performance fields (HPF) was counted on each section by three independent examiners who did not have information about the sections, and the average value of positive cell counts from 10 HPF from three examiners was used for statistical analysis.

Transaminase and cytokine assays

Blood samples were taken from the tail veins at 10 h after the injection of mPSL or PBS. The serum levels of AST and ALT were measured by a colorimetric assay (Fuji Film, Tokyo, Japan). TNF-α and IFN-γ were measured using ELISA kits (Endogen, Rockford, IL, USA).

Statistical analysis

The results are expressed as mean ± SD. Significant difference between two groups was assessed using unpaired two-tailed *t*-tests. A value of $P < 0.05$ was considered to be significant. Survival curves were compared using the log-rank test.

Results

Effect of methylprednisolone sodium succinate injection on the survival rate of animals with acute liver failure

The time course of our experimental procedure is summarized in Figure 1. We set the time of mPSL administration at 2 h after LPS and GalN challenges, when the inflammatory processes were thought to have started but not to have progressed fully and irreversibly to fatal liver failure. In our experimental model of acute liver damage induced by LPS and GalN, the spontaneous survival rate was 20% at 17 h of observation. The control animals without steroid treatment began to die 4–5 h after the chemical

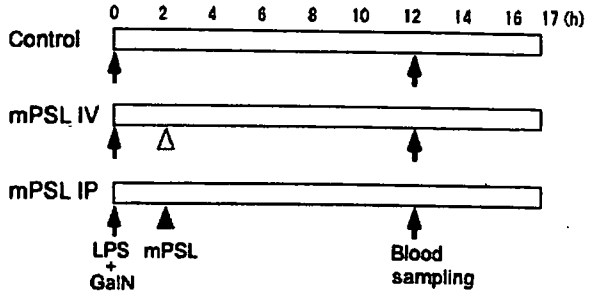

Fig. 1. Time course of experiments. Two hours after the intraperitoneal injection of 100 μg of lipopolysaccharide (LPS) and 500 mg/kg of D-galactosamine, 10 mg of methylprednisolone (mPSL) was administered into the tail vein (mPSL IV) or portal vein (mPSL IP). Blood for transaminase and cytokine measurements was taken at 10 h after mPSL injection and the survival rate of animals was estimated at 17 h from the beginning of experiments. Open triangles, mPSL injection via tail vein; closed triangles, mPSL injection via portal vein.

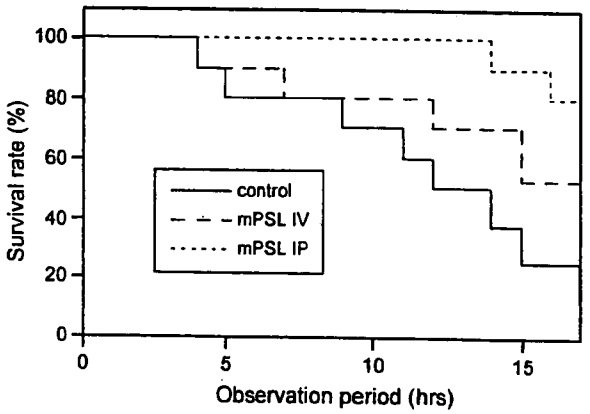

Fig. 2. Survival rate of animals with acute liver damage treated with or without methylprednisolone (mPSL). Two hours after the injection of D-galactosamine and lipopolysaccharide, mPSL was administered into the tail vein (mPSL IV) or portal vein (mPSL IP). Animals with mPSL injection via the tail vein showed lower mortality than the controls (50% at 17 h) and animals injected with mPSL via the portal vein showed the highest survival rate (80% at 17 h). The difference among the groups was significant (log-rank test: $P = 0.0083$). ($n = 10$ in each group).

injection and the number of dead animals increased with the passage of time (Fig. 2). The rats with mPSL injection via the tail vein showed a similar tendency, but the rate of death was lower than the control animals, and 50% of the animals survived throughout the observation period. However, the mortality of animals with an mPSL injection via the portal vein was significantly lower than that of the other groups. All the animals in this group survived for 12 h after the chemical injection. The final survival rate of this group was 80% after the complete 17-h observation period and surviving animals showed an almost normal appearance, in contrast to the surviving animals in the other groups, which looked ill.

Effect of methylprednisolone sodium succinate injection on serum levels of aspartate aminotransferase, alanine aminotransferase, tumor necrosis factor-α and interferon-γ

Liver damage was estimated by the serum levels of transaminases. AST and ALT levels in the control animals were extremely high (Fig. 3) but were reduced by mPSL treatment, both the mPSL-treated groups having significantly lower transaminase levels than the controls (decrease of about 50%). However, no difference in the levels was evident between the treated groups (injection via the tail vein and the portal vein). Next, cytokine levels were determined in the animals (Fig. 4). Serum TNF-α and IFN-γ levels in the groups injected with mPSL were much lower than those in the control animals, and the levels were lower in the group treated with mPSL via the portal vein than that via the tail vein. TNF-α and IFN-γ levels in the group injected with mPSL via the portal vein were about 30 and 25% of those in the control group respectively, and the difference between the groups was significant.

Effect of methylprednisolone sodium succinate injection on histological improvement

As mentioned above, the effect of mPSL on the levels of inflammatory cytokines depends on the injection route. It is possible that the considerable reduction of these cytokines results in the suppression of hepatic inflammation and improvement of survival rate. Therefore, liver damage in the animals was evaluated histologically. In control animals without mPSL treatment, zonal necroses were observed (Fig. 5A). These pathological changes were attenuated in the group injected with mPSL via the tail vein and extensive necroses were not found but confluent necroses were scattered (Fig. 5B). Further histological improvement was seen in the group injected with mPSL via the portal vein (Fig. 5C). A few spotty necroses with disarranged hepatic cords were observed.

In both the experimental liver damage model and human patients with fulminant hepatitis, it is known that a substantial increase in apoptotic cells may be observed in the injured liver and this may be evidence of progression of liver cell death. We analysed the number of apoptotic cells in each group of model animals. TUNEL-positive cells were counted in 10 HPF in each liver section from each group of animals.

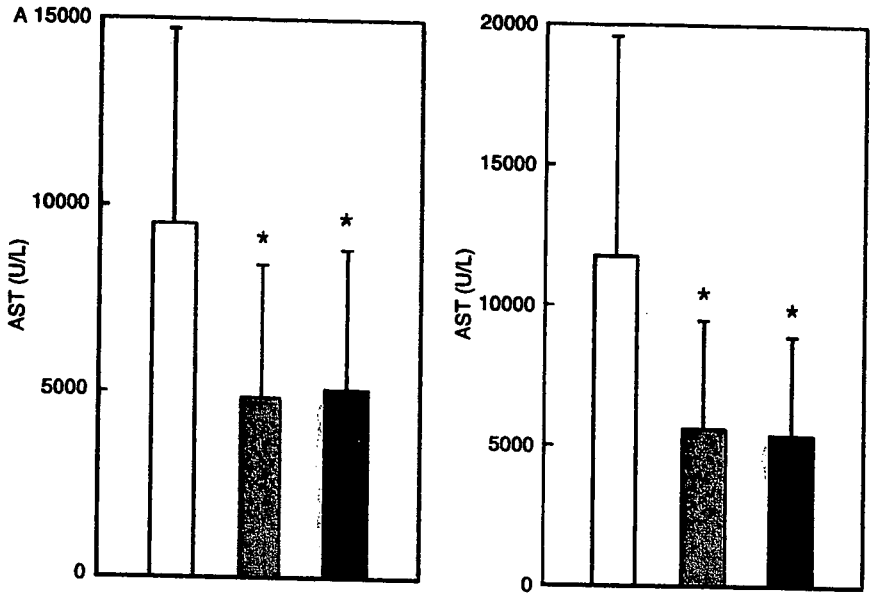

Fig. 3. Effect of methylprednisolone (mPSL) on serum transaminase levels. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in mPSL treated groups were significantly lower than those of the control group but no difference was observed between the group injected with mPSL via the tail vain and via the portal vein. Open squares, control; shaded squares, mPSL injection via the tail vein; closed squares, mPSL injection via the portal vein. (A) AST. (B) ALT. Values are expressed as mean ± SD. ($n = 7$ in each group; *significantly different from the control; $P < 0.05$).

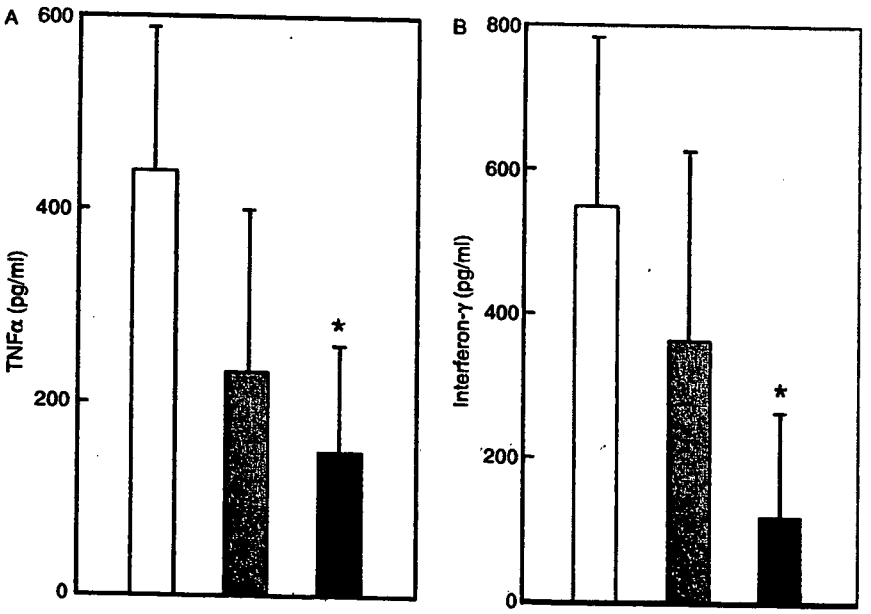

Fig. 4. Effect of methylprednisolone (mPSL) on serum tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) levels. mPSL treatments using two different routes reduced serum concentrations of TNF-α and IFN-γ and the effects of steroid were more pronounced in the group injected with mPSL via the portal vein than via the tail vein. Open squares, control; shaded squares, mPSL injection via the tail vein; closed squares, mPSL injection via the portal vein. (A) TNF-α. (B) IFN-γ. Values are expressed as mean ± SD ($n = 7$ in each group; *significantly different from the control; $P < 0.05$).

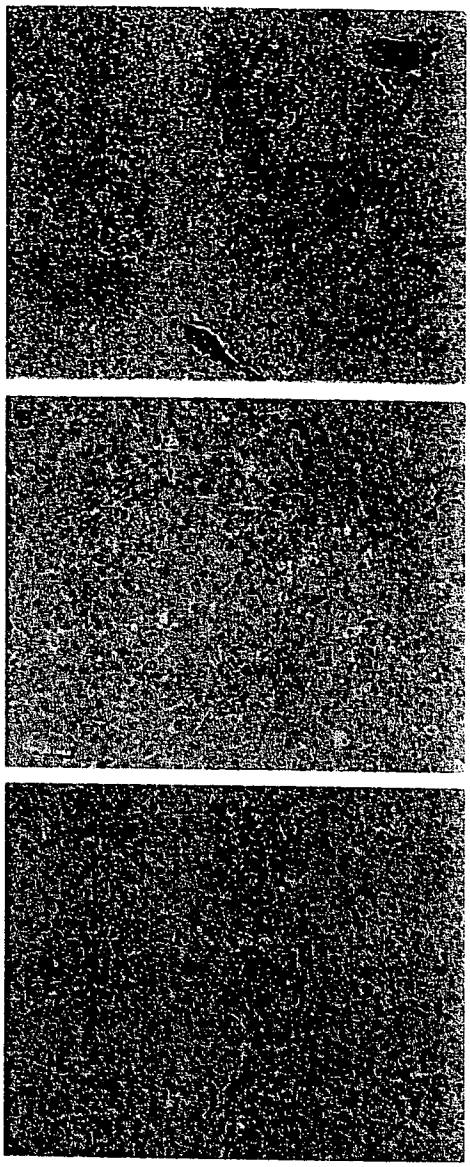

Fig. 5. Histological features of liver sections. A representative example from each group is shown (magnification: A, × 100; B, × 200; C, × 200). The control animals with injection of D-galactosamine and lipopolysaccharide without steroid treatment showed extensive eosinophilic areas, suggestive of zonal necroses (A). Extensive necroses were not found but confluent necroses were scattered in the animals injected with methylprednisolone (mPSL) via the tail vein (B). A few spotty necroses with disarranged hepatic cords were seen in the animals injected with mPSL via the portal vein (C).

As shown in Figure 6, numerous apoptotic cells were observed in the liver sections of animals treated without steroid. The number of apoptotic cells decreased substantially in both groups of mPSL-treated animals. In the group treated with mPSL via the tail vein, the

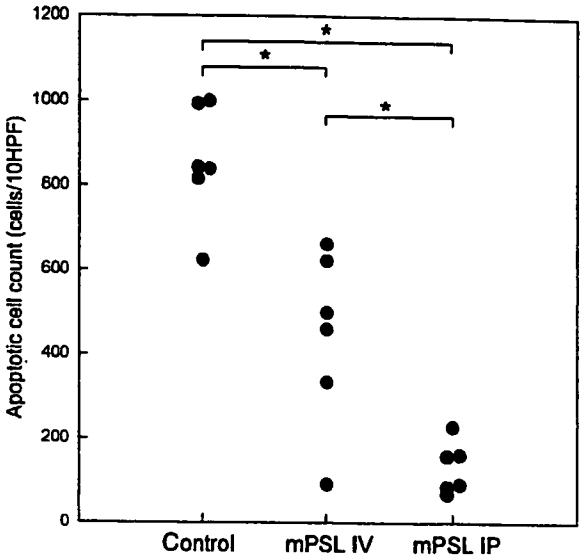

Fig. 6. Effect of methylprednisolone (mPSL) treatment on the number of apoptotic cells in liver. TdT-mediated dUTP nick end labelling (TUNEL) staining was performed to detect apoptotic cells in liver sections. TUNEL-positive cells were counted in 10 high-performance fields (HPF) for every group. Significantly fewer apoptotic cells were observed in the group treated with mPSL via the tail vein (mPSL IV) than the controls and the reduction was more pronounced in the group treated with mPSL via the portal vein (mPSL IP) ($n = 6$ in each group; *significantly different; $P < 0.05$).

number of apoptotic cells decreased significantly and was around half that of the control group. This reduction was more pronounced in the group treated with mPSL via the portal vein; the number of apoptotic cells was less than a quarter that of the control group and the difference was significant compared with the controls and the animals injected with mPSL via the tail vein.

Discussion

In this study, we showed that steroid injection via the portal vein led to an improvement of the survival rate, serum levels of transaminases and inflammatory cytokines and histological findings in model animals of acute liver failure induced by LPS and GalN. Steroid injection via the tail vein could also reduce serum transaminase levels; however, the effect of this treatment on other parameters was inferior to that of steroid injection via the portal vein.

The effects of steroid on suppression of transaminase elevation and improvement of survival have been reported in rodent models of acute liver damage (18–22); for instance, dexamethasone administered