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Journal of Viral Hepatitis, 2009, **16**, 506–512 doi:10.1111/j.1365-2893.2009.01102.x

Inhibition of intracellular hepatitis C virus replication by nelfinavir and synergistic effect with interferon-α

S. Toma,[1] T. Yamashiro,[2] S. Arakaki,[1] J. Shiroma,[1] T. Maeshiro,[1] K. Hibiya,[1] N. Sakamoto,[3] F. Kinjo,[4] M. Tateyama[1] and J. Fujita[1] [1]*First Department of Internal Medicine, School of Medicine, University of the Ryukyus, Okinawa, Japan;* [2]*Department of Blood Transfusion Medicine, Ryukyu University Hospital, Okinawa, Japan;* [3]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan; and* [4]*Department of Endoscopy, Ryukyu University Hospital, Okinawa, Japan*

Received September 2008; accepted for publication December 2008

SUMMARY. Liver diseases associated with hepatitis C virus (HCV) infection have become the major cause of mortality in patients with human immunodeficiency virus (HIV) infection since the introduction of highly active anti-retroviral therapy. HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients, but the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone. HIV protease inhibitors might have potential to down-regulate HCV load of HCV/HIV coinfected patients. In this study, we evaluated the effects of nelfinavir on intracellular HCV replication using the HCV replicon system. We constructed an HCV replicon expressing a neomycin-selectable chimeric firefly luciferase reporter protein. Cytotoxicity and apoptosis induced by nelfinavir were assessed and synergism between nelfinavir and interferon (IFN) was calculated using CalcuSyn analysis. Nelfinavir dose-dependently repressed HCV replication at low concentrations (IC_{50}, 9.88 μmol/L). Nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication. Clinical concentrations of nelfinavir (5 μmol/L) combined with IFN showed synergistic inhibition of HCV replication in our replicon model. Our results suggest that the direct effects of nelfinavir on the HCV subgenome and its synergism with IFN could improve clinical responses to IFN therapy in HCV/HIV coinfected patients.

Keywords: hepatitis C virus, human immunodeficiency virus, nelfinavir.

INTRODUCTION

Patients with human immunodeficiency virus (HIV) infection are frequently coinfected with hepatitis C virus (HCV), because these viruses have similar routes of transmission, including blood transfusion, intravenous drug use and sexual contact [1,2]. The optimal therapy for HIV infection is highly active anti-retroviral therapy (HAART), which combines HIV reverse transcriptase inhibitors, often with HIV protease inhibitors. Since the introduction of HAART, the morbidity and mortality associated with HIV infection have declined. This reduction in mortality due to opportunistic infections has made HCV-associated liver disease the leading causes of mortality [3].

Several studies have reported that HCV-related liver disease is more severe in HIV-infected patients than in non-HIV-infected patients [4,5]. The severity of liver disease increases as the immunodeficiency progresses and HIV seropositivity accelerates the progression of liver fibrosis related to chronic hepatitis C [6,7]. In addition, many studies have documented that HIV/HCV coinfected patients have higher HCV loads than do HCV monoinfected controls [8–10]. However, the standard therapies used to treat chronic hepatitis C in HCV/HIV coinfected patients are the same as those for patients infected with HCV alone [11].

HAART has been reported to reduce serum HCV RNA levels accompanied by immune improvement [12], but the decrease in HIV viral load was associated with a persistent and significant increase in HCV viral load [13]. There is no consistent evidence that HAART results in suppression of HCV viraemia, suggesting that multiple factors may be affecting viral load in coinfected patients [14,15]. However,

Abbreviations: CI, combination index; HAART, highly active anti-retroviral therapy; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; LDH, lactate dehydrogenase; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling.

Correspondence: Tsuyoshi Yamashiro, MD, PhD, Department of Blood Transfusion Medicine, Ryukyu University Hospital, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan.
E-mail: tsuyo-y@med.u-ryukyu.ac.jp

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Trimoulet *et al.* showed that patients treated with HAART that included protease inhibitors had significantly lower intrahepatic HCV loads than those treated with HAART without protease inhibitors [16].

Liver injury has been reported to be a potential side-effect of HAART [17] and potential hepatotoxicity of HIV protease inhibitors had been well realized before these drugs were licensed for the first time [18]. Concomitant hepatic damage prior to the start of HAART is an important risk factor, which can intensify hepatotoxic side-effects of HAART. The presence of chronic hepatitis C has been reported to increase the risk of HAART-associated hepatotoxicity (relative risk, 2.46; 95% confidence interval, 1.43–4.24) [19]. The mechanisms underlying the association of HCV and hepatotoxicity remain unclear, but in some patients liver enzyme elevations may be a manifestation of immune reconstitution that follows antiretroviral therapy. After immune recovery, CD4+ cell counts rise and the ability of T cells to identify and lyse HCV-infected hepatocytes may be increased [20]. The differences in the potential for hepatotoxicity have been reported to exist among the commercially available protease inhibitors and nelfinavir was associated with the low rate of severe hepatotoxicity among patients coinfected with hepatitis viruses [21].

HIV protease is a small, dimeric aspartyl protease that specifically cleaves the polyprotein precursors encoding the structural proteins and enzymes of the virus. This proteolytic activity is absolutely required for the production of mature, infectious virions. HIV protease inhibitors block HIV maturation and show remarkable antiviral potency [22]. It has recently been reported that HIV protease inhibitors also have nonviral effects on the host cells, beyond their effect of blocking HIV protease enzymatic activity [23]. NF-κB is central to the overall immune response through its ability to activate genes coding for regulators of apoptosis and cell proliferation [24]. The HIV protease inhibitor nelfinavir has been shown to regulate NF-κB activation [25].

In the present study, we investigated the action of nelfinavir alone, or in combination with interferon (IFN), on HCV replication using the replicon system.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line, Huh7, was maintained in Dulbecco's modified minimal essential medium (Sigma, St Louis, MO, USA) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 500 μg/mL G418 (Nakalai Tesque, Kyoto, Japan).

HCV replicon constructs and transfected cell lines

An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an infectious HCV clone, HCV-N, genotype Ib [26]. The replicon, pRep-N was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising firefly luciferase and neomycin phosphotransferase (pRep-Feo) [27–29]. RNA was synthesized from pRep-Feo using T7-polymerase (Promega, Madison, WI, USA) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established. We have previously reported that firefly luciferase activities of Feo replicon-expressing cells correlated well with HCV NS3, NS4A and NS5A protein expression levels and with replicon RNA expression levels [27].

Treatment with IFN and nelfinavir

Recombinant human IFN-α-2b (Schering-Plough, Kenilworth, NJ, USA) and purified nelfinavir (Japan Tobacco Inc., Tokyo, Japan) were used. IFN and nelfinavir treatment schedules were as described in the results.

Luciferase assays

Luciferase activity was quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). Typically, 5×10^3 cells/well, plated onto 24-well plates and cultured for 48 h, were lysed with 100 μL 1× Glo luciferase Buffer (Promega), and the luciferase activity in 100 μL of the lysate was measured by adding an equal volume of Bright-Glo Luciferase Assay Reagent (Promega). Assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

Western blot analysis

Cells were lysed in buffer containing 62.5 μM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 6% 2-mercaptoethanol and 0.01% bromophenol blue. Equal amounts of protein (10 μg) were subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (Invitrogen, Carlsbad, CA, USA), followed by transfer to a polyvinylidene difluoride membrane (Roche, Basle, Switzerland) and sequential probing with a monoclonal anti-NS5A antibody (Virogen, Watertown, MA, USA) and β-actin antibody (Thermo Fisher Scientific, Fremont, CA, USA), respectively. The bands were visualized using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA).

Cytotoxicity assay

Lactate dehydrogenase (LDH) tests and 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium inner salt (MTS) reduction assays were performed to investigate cytotoxicity and cell viability. LDH levels were measured in the supernatants using the LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan), according

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to the manufacturer's instructions. The level of specific cytotoxicity was calculated using the following formula: % of specific LDH release = ([experimental LDH release − the mean of negative control release]/[the mean of positive control release − the mean of negative control release]) ×100. LDH release from cells treated with 0.2% Tween 20 was used as a positive control, while LDH release from nontreated cells was used as a negative control. Viable cell growth was determined by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer's instructions.

TUNEL method

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labelling (TUNEL) was used to detect DNA fragmentation of nuclei. Using 24-well plates, 5×10^3 cells/well were plated with 5.0 μM nelfinavir. After incubation for 2 days, the glass coverslips were harvested, fixed with 4% paraformaldehyde and washed with phosphate-buffered saline. The cells were permeabilized with 0.5% Tween 20 and treated with MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories Co., Nagoya, Japan). Cells were then treated with RNase and propidium iodide. The nick end-labelling was analysed using a confocal laser scanning microscope (Fluorview; Olympus, Tokyo, Japan).

Analysis of drug synergism

The effects of treatment of Huh7/Rep-Feo cells with nelfinavir and IFN, alone and in combination, were analysed using isobologram analysis. Dose-inhibition curves were drawn for IFN and nelfinavir, used alone or in combination. For each drug combination, the 50% inhibitory concentration (IC_{50}) values were plotted against the fractional concentration of IFN and nelfinavir on the x axis and y axis, respectively. IC_{50}, IC_{20} and IC_{80} values ware determined using the Calcusyn^{TM} software package (Biosoft, Cambridge, UK), which performs single and multiple drug dose-effect calculations and determines the presence of antagonism, additivity or synergism. Using the median effect equation, we used this program to plot dose-effect curves for each drug and combination of drugs. The x intercept of the median effect equation gives the ID_{50} for each drug. The median effect plot also gives information on the slope of the dose-effect curve. This information can then be used to calculate the combination index (CI). CI >1 denotes antagonism, CI = 1 denotes additivity, and CI < 1 denotes synergism.

Statistical analysis

Statistical analysis was performed using the Student's t-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of nelfinavir on HCV replication

To assess the effects of nelfinavir on intracellular replication of the HCV genome, Huh7/Rep-Feo cells were cultured with various concentrations of nelfinavir. The dose-effect correlation and time course of replicon expression were measured using the luciferase assay 48 h after treatment. Culture of Huh7/Rep-Feo cells with nelfinavir, at concentrations ranging from 0 to 10 μmol/L, showed dose-dependent repression of internal luciferase activity (Fig. 1a). The inhibition of HCV-RNA replication was detectable at concentrations of nelfinavir as low as 2.5 μmol/mL. Western blot hybridization also demonstrated a reduction of the replicon protein levels after nelfinavir treatment (Fig. 1b). To determine the cytotoxic effect of nelfinavir in Huh7/Rep-Feo cells, LDH levels in the supernatants were measured. No significant change in LDH levels was detectable after 48 h incubation (Fig. 1c). MTS assays of the cells cultured with nelfinavir indicated no significant effects on cell viability (Fig. 1d). Nuclear DNA fragmentation in Huh7/Rep-Feo cells, a possible mechanism of nelfinavir induced cytotoxicity, was evaluated by TUNEL staining. No fragmentation of nuclear DNA was observed in Huh7/Rep-Feo cells treated with 5.0 μM nelfinavir (Fig. 1e).

Inhibition of HCV RNA replication by IFN alone and in combination with nelfinavir

Huh7/Rep-Feo cells were cultured with various concentrations of IFN, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. IFN caused a marked dose-dependent inhibition of HCV RNA replication (Fig. 2a). The inhibition of HCV RNA replication was detectable at concentrations of IFN as low as 0.01 U/mL. In contrast, measurement of LDH levels and the results of the MTS assay suggested that IFN had little effect on cell viability and replication (data not shown). A dose-effect curve for the effects of nelfinavir and IFN on the replicon was generated by treating Huh7/Rep-Feo cells with various concentrations of IFN (1.0, 0.1, 0.01, 0.001 and 0 U/mL) and nelfinavir (5, 10 and 0 μmol/L). The luciferase activities were plotted against the drug concentrations after 48 h incubation. The inhibition curves were shifted to the left with increasing concentrations of nelfinavir (Fig. 2b), demonstrating synergy between the two drugs against the HCV replicon. There were no significant differences in MTS reduction values at the different drug concentrations (data not shown).

Synergistic inhibitory effects of nelfinavir and IFN on the replicon

We investigated a possible synergistic anti-HCV effect between nelfinavir and IFN, using the isobologram method

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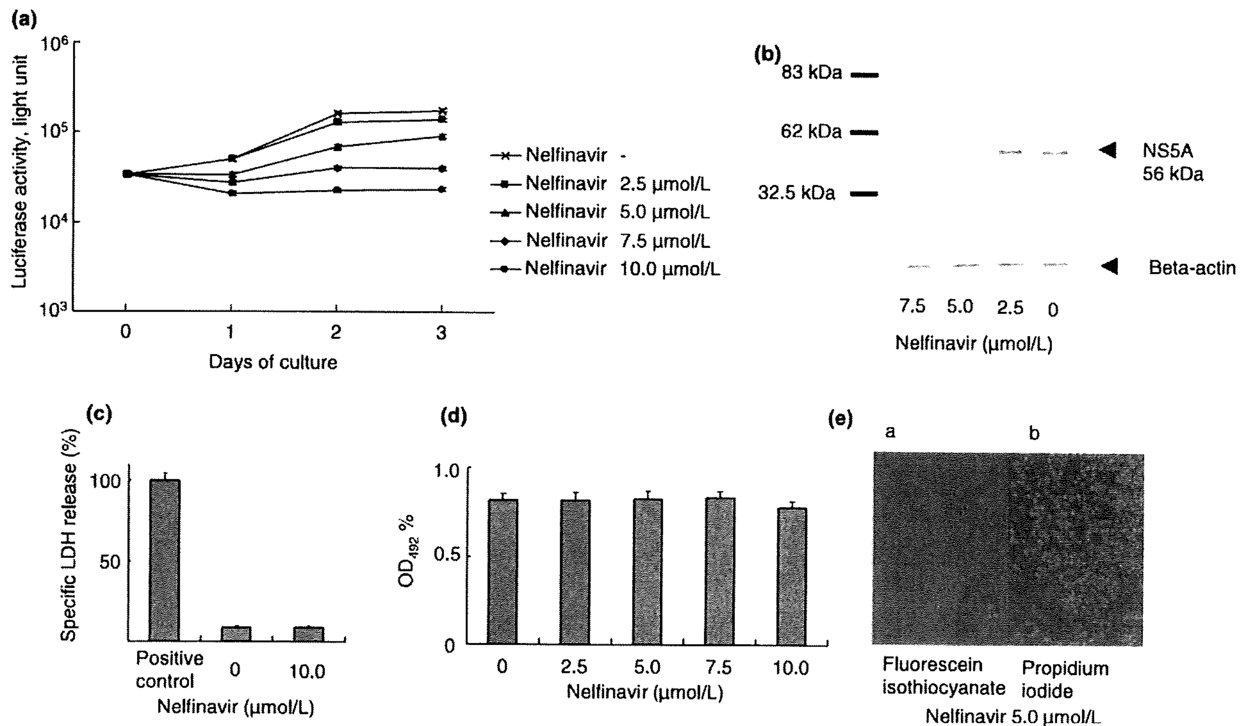

Fig. 1 Dose-dependent inhibition of hepatitis C virus (HCV) RNA replication by nelfinavir. (a) Huh7/Rep-Feo cells were cultured with concentrations of nelfinavir as indicated. (b) Western blotting. The cells were cultured in the presence of nelfinavir, as indicated and were harvested after 48 h exposure. (c) Cytotoxicity assay. Lactate dehydrogenase (LDH) assay of Huh7/Rep-Feo cells cultured with the concentrations of nelfinavir indicated. (d) 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) assay of Huh7/Rep-Feo cells cultured with the concentrations of nelfinavir indicated. (e) Nuclear DNA fragmentation in Huh7/Rep-Feo cells detected by the TUNEL method. Cells were observed using a confocal laser scanning microscopy (all 200×). Nuclear DNA fragmentation is shown in green (a: fluorescein isothiocyanate staining), and Huh7/Rep-Feo cell nuclei in red (b: propidium iodide staining).

and Calcusyn software, as described in Material and methods. A log dose-effect curve and median effect plot were made for both drugs. Both drugs showed linear regression of effect on the logarithms of doses (R^2 = 0.94 for nelfinavir; R^2 = 0.99 for IFN). The IC_{50} values were 9.88 ± 0.43 μmol/L for nelfinavir and 0.099 ± 0.14 U/mL for IFN (Fig. 3a,b).

In order to determine if nelfinavir and IFN had a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were treated with combinations of IFN and nelfinavir at various concentrations. Isobolograms were generated based on the two drugs administered in combination at fixed ratios, adjusted for the IC_{50} of each drug (FIC ratio): 1:0, 4:1, 1:1, 1:4 and 0:1. Using the Calcusyn software, each 90% inhibition of HCV replication (Fa = 0.90), 75% inhibition of HCV replication (Fa = 0.75), and 50% inhibition of HCV replication were plotted on the x and y axes (Fig. 3c). The ED_{90}, ED_{75} and ED_{50} plots for each drug ratio fell below the line representing additivity, indicating synergistic effects of the drug combination on intracellular HCV-RNA replication. The CI at an Fa value of 0.5 was 0.58, generated from Fig. 3c using CalcuSyn. There was no significant difference in MTS reduction at different drug concentrations (data not shown), suggesting that the synergistic action of nelfinavir and IFN on HCV replication was through their pharmacological effects, and not due to augmentation of cytotoxicity.

DISCUSSION

The results of this study suggest that nelfinavir inhibits HCV replication at concentrations that show no cytotoxicity, and that nelfinavir and IFN act synergistically against HCV.

Nelfinavir inhibited HCV replication in a concentration-dependent manner and its effects could be observed at concentrations as low as <3.0 μmol/L. In clinical use, the plasma concentration of nelfinavir ranges from 3.3 to 6.0 μmol/L. These results support those of Trimoulet *et al.*, who found a reduction in HCV loads in patients treated with HAART including nelfinavir [16]. García-Samaniego *et al.* reported that indinavir, another HIV protease inhibitor, failed to reduce HCV viral titres [4]. In a preliminary study using the replicon system, we tested the ability of several unpurified HIV protease inhibitors to inhibit HCV replication: nelfinavir, ritonavir and saquinavir reduced HCV-replication, but indinavir and fosamprenavir had no effect (data not

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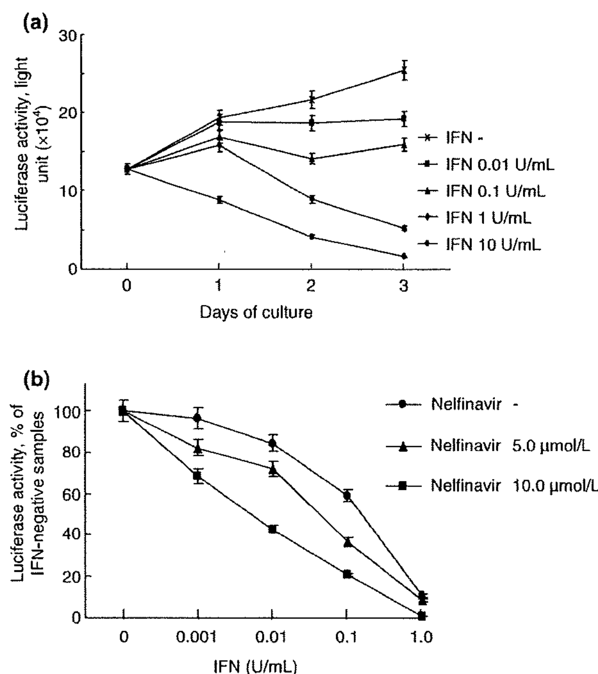

Fig. 2 Dose-dependent inhibition of hepatitis C virus (HCV) RNA replication by IFN. (a) Huh7/Rep-Feo cells were cultured with concentrations of IFN as indicated. (b) Dose-inhibition curves for IFN combined with the concentrations of nelfinavir indicated. Luciferase activities are displayed as percentages of the IFN-negative samples.

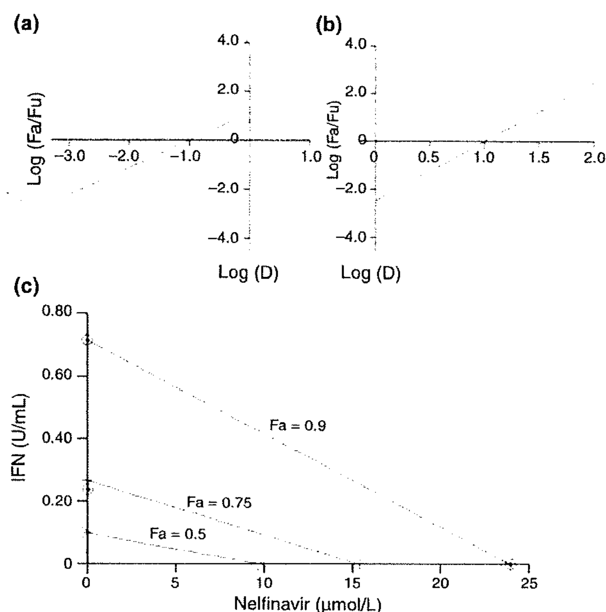

Fig. 3 CalcuSyn analysis of the interferon (IFN)/nelfinavir combination effects on intracellular hepatitis C virus (HCV) replication. Huh7/Rep-Feo cells were cultured with various concentrations of nelfinavir and IFN. Luciferase activities of the cell lysates were measured after 48 h exposure. The CalcuSyn median-effect plot was generated from three separate experiments in triplicate with SD <20% (Fa: affected fraction, Fu: unaffected fraction, D: concentration of drug used). (a) Log dose-effect curve and median effect plot for nelfinavir. Median effect plot has the form of a straight line, $y = 2.47x + 0.43$. (b) Log dose-effect curve and median effect plot for IFN. Median effect plot has the form of a straight line, $y = 1.12x - 0.14$. (c) Isobologram analysis of the combination of IFN and nelfinavir in Huh7/Rep-Feo cells. The individual doses of IFN and nelfinavir required to achieve 90% inhibition of HCV-replication (Fa = 0.90), 75% inhibition of HCV-replication (Fa = 0.75), 50% inhibition of HCV-replication were plotted on the x and y axes. Combination index (CI) values calculated using the Calcusyn software are represented by points above (indicate antagonism between drugs) or below the lines (indicate synergy). (X symbol) ED50, (plus sign) ED75 and (open dotted circle) ED90.

shown). These discrepancies in the effects of different protease inhibitors could explain the different results found in clinical studies.

Combination therapy using ribavirin and IFN is a standard therapy for patients with chronic HCV infection, including HCV/HIV coinfected patients [11]. We previously reported that the use of a clinically achievable concentration of ribavirin, in combination with IFN, showed strong synergistic inhibitory effects on HCV replication using the replicon system [29]. In this study, nelfinavir showed similar strong synergy with IFN. These results suggest that nelfinavir could improve the antiviral effects of IFN in HCV/HIV coinfected patients.

HIV protease inhibitors have a strong affinity for the active site of the HIV viral aspartyl protease, and irreversibly inhibit the catalytic activity of the enzyme. However, HIV protease inhibitors are thought not to inhibit HCV viral serine protease. The above-mentioned findings which demonstrate that different HIV protease inhibitors have different effects on HCV replication support this idea because if HIV protease inhibitors inhibited HCV serine protease, then all HIV protease inhibitors should inhibit HCV replication. The mechanism by which nelfinavir inhibits HCV replication is uncertain: Several studies have shown that HCV infection alters NF-κB promoter activity, possibly contributing to the persistence of HCV infection [30–32]. Equils *et al.* reported that nelfinavir blocked TLR2-, TLR4- and TNF-α-induced NF-κB activation [25]. Nelfinavir may play an important role in the regulation of the cellular inflammatory and immune responses through NF-κB, but further studies are needed to investigate the role of NF-κB promoter activity in nelfinavir-induced HCV replication inhibition.

The decreased clearance of antiretoroviral drugs is suspected to be a possible cause of increased susceptibility for HAART-associated liver toxicity in HIV/HCV coinfected patients, because the metabolism of the HIV protease inhibitors depends on the amount of functional cytochrome

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P450, which is reduced in severe liver disease [33]. Indeed, increased toxic drug concentrations have been reported in patients with hepatic disease, receiving standard dose of nelfinavir [34]. Meanwhile, Bruno *et al.* have reported, in a clinical study, that nelfinavir was associated with the lowest rates of severe hepatotoxicity in patients coinfected with hepatitis viruses among the available HIV protease inhibitors [21]. In our study, nelfinavir failed to induce cytotoxicity or apoptosis at concentrations that inhibited HCV replication.

The implications of our results for understanding the effect of nelfinavir on HCV replication are limited because this study used an *in vitro* HCV subgenomic replicon system, which only expresses viral nonstructural, and not structural, proteins. To elucidate the effects of nelfinavir on full-length, infectious hepatitis C virions, further studies using full genomic replicons are needed. In addition, the influence of immune reconstitution induced by HAART, including nelfinavir, on HCV replication needs to be investigated before clinical application of our data to therapy for HCV/HIV coinfected patients.

Because end-stage chronic liver disease resulting from co-infection with HCV is now the major cause of death in individuals infected with HIV, our results suggest a potentially promising approach for improving the standard therapies for chronic hepatitis C in HCV/HIV coinfected patients.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Clinical Utility of Serum β-D-Glucan and KL-6 Levels in *Pneumocystis jirovecii* Pneumonia

Hideta Nakamura[1], Masao Tateyama[1], Daisuke Tasato[1], Syusaku Haranaga[1], Satomi Yara[1], Futoshi Higa[1], Yuji Ohtsuki[2] and Jiro Fujita[1]

Abstract

Objective New serum markers (1→3) β-D-glucan (β-D-glucan) and KL-6 are reported to be useful for the clinical diagnosis of *Pneumocystis jirovecii* pneumonia (PCP). However, the utility of these markers in PCP with HIV infection (HIV PCP) and without HIV (non-HIV PCP) is unknown. This study was aimed to evaluate the utility of β-D-glucan and KL-6 for the diagnosis of PCP in patients with HIV infection (HIV PCP) and non-HIV PCP.

Methods Retrospective study

Patients We reviewed the medical records of consecutive 35 patients. The serum levels of β-D-glucan and KL-6 in HIV PCP and non-HIV PCP were comparatively evaluated. We evaluated these markers in survivors and non survivors.

Results The detection rates of serum β-D-glucan and KL-6 levels in non-HIV PCP were lower than those in HIV PCP (88% vs. 100%, 66% vs. 88%, respectively). The false positive rates of these markers in both groups were similar (12%, 37%, respectively). Oxygenation index, serum albumin, and mechanical ventilation were the variables which were significantly associated with poor outcome in the univariate analysis.

Conclusion In conclusion, β-D-glucan was a reliable diagnostic marker for PCP. However, the detection rate of β-D-glucan and KL-6 in non-HIV PCP was lower than in HIV PCP. Neither β-D-glucan nor KL-6 was associated with the outcome of PCP.

Key words: (1→3) β-D-glucan, human immunodeficiency virus, KL-6, *Pneumocystis jirovecii* pneumonia

(Inter Med 48: 195-202, 2009)
(DOI: 10.2169/internalmedicine.48.1680)

Introduction

Pneumocystis jirovecii pneumonia (PCP) is a major cause of morbidity and mortality in patients with human immunodeficiency virus (HIV) infection (HIV-related PCP) and other conditions associated with immunosuppressive therapy (1). Invasive diagnostic techniques such as bronchoalveolar lavage (BAL) or transbronchial lung biopsy (TBLB) and specific staining of cyst and/or trophozoites are the gold standard for PCP diagnosis. New techniques such as the identification of *P. jirovecii* DNA by polymerase chain reaction (PCR) from expectorated and induced sputa showed greater sensitivity for PCP diagnosis than conventional histochemical staining techniques (2).

Yasuoka et al reported that (1→3) β-D-glucan (β-D-glucan), one of the major components of the cyst wall of *P. jirovecii*, can be a serological marker for the diagnosis and monitoring of PCP (3); some reports have also supported this evidence (4-6). On the other hand, it has been reported that KL-6, a mucin-like glycoprotein, which is expressed on type II pneumocytes, is another possible diagnostic marker of PCP (7). More recently, Tasaka et al have reported that β-D-glucan is more reliable as a serum diagnostic marker of PCP than KL-6 or lactate dehydrogenase (LDH), especially when BAL could not be performed because of severe respiratory failure (8).

The clinical characteristics of HIV-related PCP and non-

[1]Department of Medicine and Prevention and Control of Infectious Diseases, Faculty of Medicine, University of the Ryukyus, Okinawa and [2]Division of Pathology, Matsuyama-Shimin Hospital, Matsuyama
Received for publication September 10, 2008; Accepted for publication October 8, 2008
Correspondence to Dr. Hideta Nakamura, hnhideta@gmail.com

Inter Med 48: 195-202, 2009 DOI: 10.2169/internalmedicine.48.1680

HIV PCP are different. However, the previous reports failed to show the differences in these serum indicators between HIV-related PCP and non-HIV PCP. Therefore, we compared serum β-D-glucan and KL-6 as well as clinical characteristics in patients with different clinical backgrounds; HIV-related PCP and non-HIV PCP. In addition, the influence of these serum markers on the outcome of PCP was investigated.

Material and Methods

Patients

For this study, consecutive 35 patients diagnosed with PCP, 19 with HIV-related PCP and 16 with non-HIV PCP, in our institution between 1989 and 2006 were retrospectively evaluated. This study was approved by the institutional review board for clinical research in the University of the Ryukyus.

PCP diagnosis and BAL evaluation

BAL evaluation

BAL and/or TBLB were/was performed by fiberoptic bronchoscopy after obtaining written informed consent from the patients. BAL was performed by positioning the bronchoscope in the distal airway of the relevant bronchus (middle or lingual lobe in patients with diffuse pulmonary infiltrates) and administering 50 mL sterile normal saline solution per lavage. Conventionally, 3 lavages were performed. The fluid was immediately divided into aliquots and dispatched to different laboratories for examinations and culturing. Routine evaluation for *P. jirovecii* included 3 stains (Diff Quik®, toluidine blue O, and Grocott stain) and polymerase chain reaction (PCR). PCR was performed according to the well-established method of Wakefield et al (9). The diagnosis of PCP was defined by positive results of staining and/or PCR. BAL fluid was also evaluated for other pathogens, including opportunistic bacteria, viruses, fungi, protozoa, and acid-fast bacteria.

Data collection

We reviewed the medical records of all patients and collected the following data: (i) epidemiological data (age, gender, predisposing factors for PCP, and duration of symptoms prior to diagnosis), (ii) laboratory data (serum albumin, C-reactive protein (CRP), Lactate dehydrogenase (LDH), oxygenation index, β-D-glucan, and KL-6), and (iii) BAL data. β-D-Glucan was measured with a G test (Seikagaku Corporation, Tokyo, Japan), and KL-6 was measured by an enzyme-linked immunosorbent assay (ELISA) by using a KL-6 antibody kit (ED046; Eisai Co., Tokyo, Japan). The conventional cut-off points of these markers were defined as 20 pg/mL (10) and 520 U/mL (11), respectively. The oxygenation index was determined from the arterial oxygen tension (PaO_2) and fraction of inspired oxygen (FiO_2) values. We enrolled patients with other infectious lung diseases, asymptomatic HIV-1 infection, and healthy volunteers so that the levels of β-D-glucan and KL-6 could be compared. We examined a total of 24 patients with other infectious lung diseases, including 6 patients with bacterial pneumonia, 6 with lung tuberculosis, and 15 with *Legionella* pneumonia. Bacterial pneumonia diagnosis was confirmed by the isolation of a bacterial pathogen from sputum. We diagnosed *Legionella* pneumonia on the basis of specific urine antigen detection (12). Lung tuberculosis was diagnosed on the basis of isolation of *Mycobacterium tuberculosis* from sputum culture. The CD4 cell count of all asymptomatic HIV carriers was above 300 cells/mm^3. Healthy volunteers were recruited from among the medical staff in the hospital.

Immunohistochemical staining

Pulmonary tissues were obtained by TBLB from the patients who were examined; the tissue slices were fixed in 10% formalin and embedded in paraffin using the standard procedure. The tissue sections (4-μm thick) were dewaxed and stained according to the following method. The tissue sections of each patient were examined after Hematoxylin and Eosin staining, Grocott staining, and periodic acid Schiff (PAS) reaction. Monoclonal antibody to KL-6 (kindly provided by Eisai, Tokyo, Japan) was used at a dilution of 1:5,120 without pretreatment, as previously described (13). The general staining method followed the instructions in the kit manual for LSAB2 kit/horseradish peroxidase (HRP) (DakoCytomation, Kyoto, Japan) with diaminobenzidine as the substrate for HRP.

Statistical methods

Statistical analysis was performed using the statistical software (SPSS for Windows, version 15; Chicago, IL). Data were expressed as the median with the interquartile range in parentheses. Comparisons between patients were performed using the chi-square test for categorical variables and the Mann-Whitney U test followed by the Kruskal-Wallis test for continuous variables. The relationships between variables were analyzed by the Spearman rank-over correlation test. Statistical significance was defined as $p<0.05$.

Results

Patient characteristics

During the study period, 35 episodes of PCP were confirmed in 34 individuals. Nineteen patients presented as HIV-related PCP patients and 16 had other conditions associated with immunosuppression. Of the 19 HIV-infected patients, 13 presented with PCP as the first manifestation of acquired immunodeficiency syndrome (AIDS). One patient had been previously diagnosed with PCP. The underlying diseases in patients with non-HIV PCP were described in Table 1. All patients with non-HIV PCP had been adminis-

Inter Med 48: 195-202, 2009 DOI: 10.2169/internalmedicine.48.1680

Table 1. Comparison of Patient Characteristics between HIV-related PCP and Non-HIV PCP

	HIV related PCP (n = 19)	Non-HIV PCP (n = 16)	p values
Age, yr	39.0 (29–58)	57.5 (43–75)	0.001
Male/Female	17/2	9/7	0.05
Diagnostic methods			
BAL/TBLB	17(89.4)	11(68.7)	
PCR assay	19(100)	16(100)	
Predisposing factors			
HIV infection	19 (100)	0	
Hematological malignancy	0	9 (56)	
Solid tumor	0	3 (18)	
Miscellaneous	0	4 (25)	
Immunosuppressive agents	1	8	
Previous PCP*	1 (5)	0	
Duration**, days	42 (1–112)	5.5 (2–28)	0.001
Laboratory findings			
PaO_2/FiO_2	281 (117–461)	153.5 (150–260)	0.07
LDH, U/mL	398 (245–1043)	594.5 (368–912)	NS
Serum albumin, mg/dL	2.75 (2.4–3.8)	3.15 (2.4–3.8)	NS
CRP, mg/dL	5.78 (1.15–12.2)	6.08 (1.83–26.0)	NS
β-D-glucan, pg/mL	300(32-4822)	85.4(7.2-429)	0.027
KL-6, U/mL	1120(331-4330)	644(49.6-1860)	0.045
CD4, cells/μL	39.0 (1.14–308)	ND	
HIV-RNA, $\times10^5$ copy/mL	1.1 (0.004–5.6)	-	

Unless otherwise indicated, data are presented as number (%) or median (range)
NS: No significant, ND: No data
*Previous PCP: Patient who had a PCP in the past
**Duration of symptoms prior to diagnosis

tered immunosuppressive agents, except those with adult T-cell leukemia. PCP was diagnosed by microscopic methods in 17 of the 19 patients with HIV-related PCP and 11 of 16 patients with non-HIV PCP. All of them also had positive results in the PCR assay. Seven patients (2 with HIV-related PCP and 5 with non-HIV PCP) with negative results in the microscopic analyses were diagnosed based on the PCR assay of BAL fluid.

Serum β-D-glucan and KL-6 values on admission

The serum levels of β-D-glucan were significantly higher in patients with HIV and non-HIV PCP than in patients with other infectious lung diseases and the controls ($p<0.05$) (Fig. 1a). Serum β-D-glucan levels were significantly higher in patients with HIV PCP than in those with non-HIV PCP. Serum KL-6 levels were significantly higher in patients with HIV-related PCP than in those with non-HIV PCP and other pulmonary infections (Fig. 1b). Although the levels of serum KL-6 were significantly higher in non-HIV PCP patients than in the controls, these levels were not significantly different from those of patients with other infectious lung diseases. Considering the conventional cut-off point (10, 11), the detection rate and false positive rate of β-D-glucan were 100% and 12%, respectively, in the HIV-related PCP patients and 88% and 12% in the non-HIV PCP patients, respectively. The detection rate and false positive rate of KL-6 were 88% and 37%, respectively, in the HIV-related PCP patients, and 66% and 37% in the non-HIV PCP patients, respectively. In cases in whom both β-D-glucan and KL-6 were positive, the detection rate in HIV-related PCP and

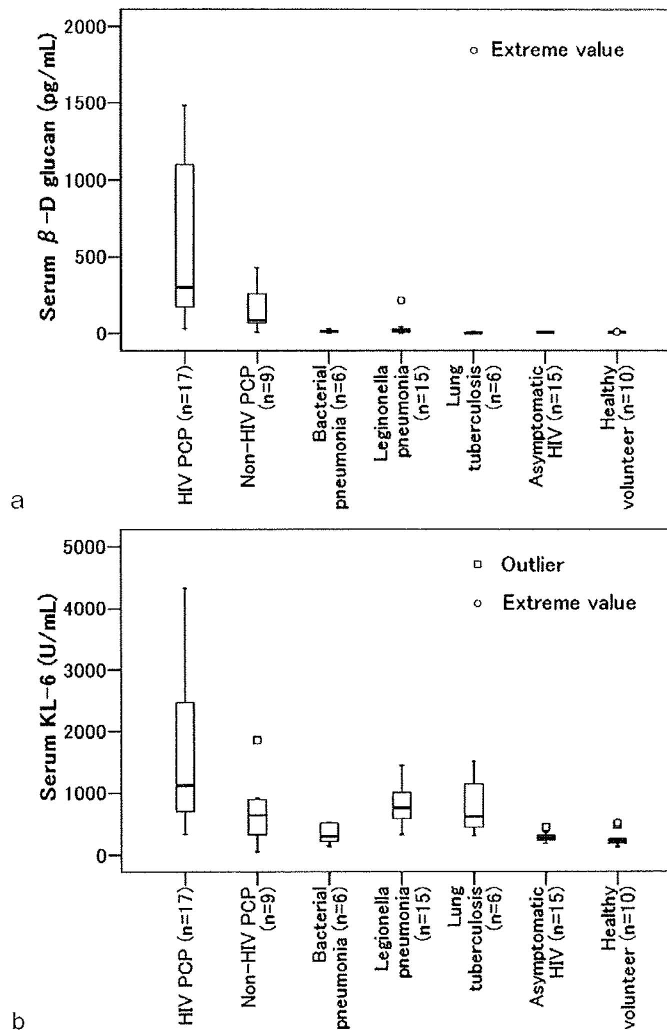

Figure 1. Serum β-D-glucan (a) and KL-6 (b) levels in patients with HIV-related PCP, non-HIV PCP, and other respiratory infectious disease and controls. The box and whisker plots show the 25th and 75th percentiles, the median (horizontal line within the box), and the 10th and 90th percentiles (whiskers). Data pointing away from the median by 1.5–3 times the IQR are termed as outliers and those away from the median by 3 times the IQR are extreme values.

non-HIV PCP were 88.2% and 55.5%, respectively. The false positive rate in this setting, was 9.6% in both groups.

Correlations between these markers in the sera and laboratory findings were evaluated in all cases (Fig. 2). There were significant correlations between LDH levels and PaO_2/FiO_2 (p=0.016, R^2 0.18); the proportion of BAL fluid neutrophils and PaO_2/FiO_2 (p=0.001, R^2 0.36). There was a significant correlation between the KL-6 levels and the duration of symptoms prior to PCP diagnosis (p=0.003, R^2 0.32). There was no significant correlation between the levels of β-D-glucan and KL-6.

Histopathological and immunohistochemical analysis of the TBLB tissues of an HIV case

Histopathological findings revealed eosinophilic foamy exudates in the intra-alveolar spaces, which were characteristic of *P. jirovecii* infection (Fig. 3A, arrow); these exudates also tested positive with the PAS reaction (Fig. 3B, arrow). Grocott staining clearly revealed black round or crescent *P. jirovecii* cysts in the identical part with Fig. 3B (Fig. 3C, arrow). Strong positive immune reaction with anti-KL-6 antibody was detected not only on the surface of proliferating type II alveolar epithelial cells (Fig. 3D, double arrow) but also within the exudates in a granular or fine vesicular pattern (Fig. 3D, arrow).

Clinical findings on admission and outcome

To clarify the prognostic factors of PCP at diagnosis, the clinical findings on admission of the survivors and non-survivors were compared (Table 2). The mortality was higher in the non-HIV PCP group than in the HIV-related PCP group. On admission, the following parameters were

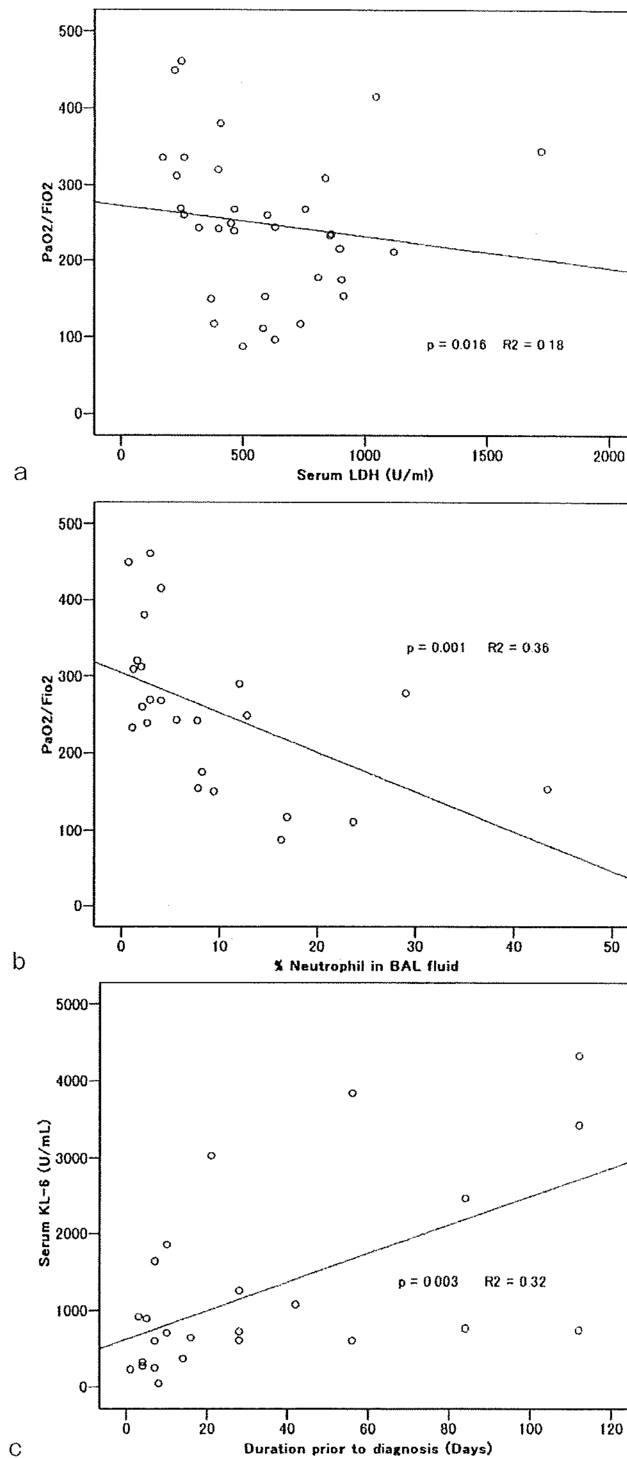

Figure 2. Correlations between several parameters that were evaluated. a: Serum levels of LDH and PaO_2/FiO_2 are inversely correlated ($p=0.016$, R^2 0.18). b: The proportion of neutrophils in BAL fluid is inversely correlated with that of PaO_2/FiO_2 ($p=0.001$ R^2 0.36). c: Duration prior to the diagnosis of PCP and serum KL-6 levels are positively correlated ($p=0.003$, R^2 0.32).

significantly different: age, serum albumin, PaO_2/FiO_2, the proportion of neutrophils in the BAL fluid, and the requirement of mechanical ventilation. The values of both serum KL-6 and β-D-glucan were not significantly different between the survivors and non-survivors. In addition, the serum LDH levels did not significantly influence the progno-

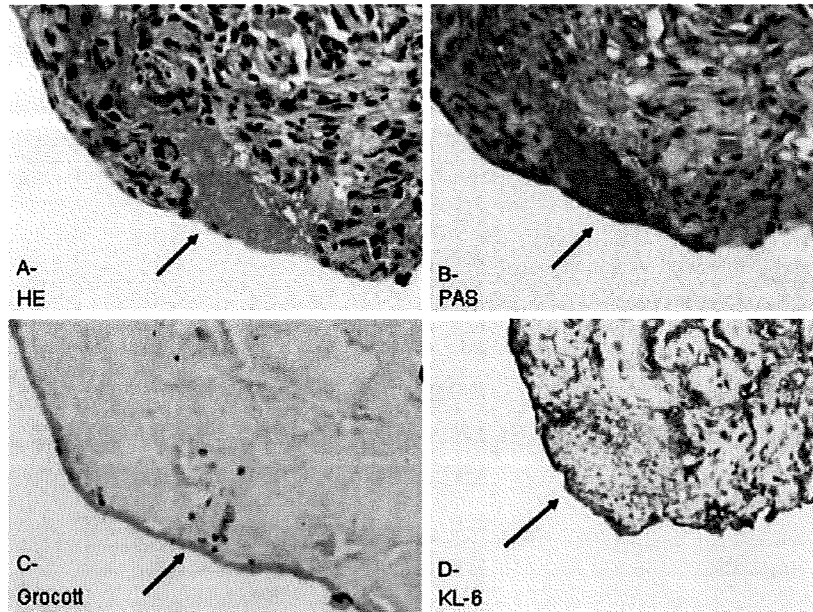

Figure 3. Histopathological and immunohistochemical staining of PCP tissues. Eosinophilic foamy exudates were observed by Hematoxylin and Eosin staining (A, arrow), and these showed strong positive reaction with PAS (B, arrow). Grocott stain revealed cysts of *P. jirovecii* (C, arrow), and these exudates were KL-6 immunopositive (D, arrow). KL-6 was also expressed on the surface of type II alveolar epithelial cells (D, double arrows). A–D: ×400

sis.

Discussion

In the present study, we evaluated the laboratory and BAL findings to compare the clinical features of HIV-related PCP and non-HIV PCP. The main finding of our study is that β-D-glucan is the most reliable adjunctive diagnostic marker for detecting PCP, particularly in HIV-related PCP patients, but the detection rate of β-D-glucan in the diagnosis of non-HIV PCP is inferior to that of HIV PCP. The levels of β-D-glucan on admission have no influence on the prognosis of this disease. Serum KL-6 was a less useful marker because of its low detection rate and high false positive rate in both groups. The LDH level and the proportion of neutrophils in BAL fluid were correlated with oxygenation impairment.

Our data confirmed the observation of previous studies which reported that mortality is influenced by 3 factors present on admission; these factors are severe hypoxia, low serum albumin level, and BAL neutrophilia as well as the need for mechanical ventilation during therapy (14-16). Although LDH and pneumothorax are described as prognostic factors in previous reports (17), in the present study, these factors were not significantly different between surviors and non survivors. The discrepancy might have resulted from the fact that this study included PCP with different clinical backgrounds.

β-D-Glucan is one of the major components of the yeast cell wall and has been used for the presumptive diagnosis of invasive fungal infections (18). The utility of serum β-D-glucan in the adjunct diagnosis of PCP has been proved in several studies (3-6). However, these studies have been conducted on a small number of patients. Tasaka et al have reported its usefulness in a large number of patients (8). The present result is consistent with those of previous studies, and we observed that the detection rate of β-D-glucan was higher in patients with HIV-related PCP than in those with non-HIV PCP. This result can be associated with the fact that the number of *P. jirovecii* in the lungs of HIV-related PCP patients is significantly increased as compared with that in non-HIV PCP patients (19). This marker was not a predictor of outcome. Serum S-adenosylmethionine is reported to be another marker for PCP (20), but the role in the outcome of the marker is unknown, which needs further study.

The serum KL-6 level is known to be a sensitive indicator of various interstitial lung diseases and acute lung injuries (11, 21). Furthermore, the serum KL-6 level is elevated in some infectious lung diseases such as *Legionella* pneumonia (22) and severe lung tuberculosis (23). There has been a report describing elevated serum KL-6 levels in a small number of PCP patients (7). A new finding in the present study is that the serum KL-6 levels seem to correlate with the duration of symptoms prior to the diagnosis; therefore, the KL-6 level was significantly higher in patients with HIV-related PCP than in those with non-HIV PCP because the clinical progression is more rapid in patients with non-HIV PCP than in those with HIV-related PCP.

The positive results of β-D-glucan and KL-6 contributed

Table 2. Comparison of Clinical Findings on Admission between Survivors and Non Survivors

	Survivors (n = 26)	Non survivors (n = 9)	p values
Age, yr	48 (29–70)	66 (34–70)	0.042
Male/Female	21/5	5/4	NS
HIV related PCP	17	2	
Non-HIV PCP	9	7	
PaO_2/FiO_2	251.5 (150–461)	117 (87.5–154)	0.001
LDH, U/mL	398 (226–1043)	499 (381–912)	NS
Serum albumin, g/dL	3.0 (2.4–3.8)	2.5 (1.9–3.3)	0.028
CRP, mg/dL	5.78 (1.15–26)	7.06 (6.67–10.3)	NS
β-D-glucan, pg/mL	217 (7.2–4822)	278 (85.4–429)	NS
KL-6, U/mL	767 (275–3840)	780 (49.6–4330)	NS
BAL fluid			
Cellularity, $\times 10^5$	1.8 (0.67–8.1)	3.04 (0.3–22.4)	NS
Lymphocytes, %	37.5 (4.9–82)	19.0 (7–67.4)	NS
Neutrophils, %	2.9 (0.7–43.4)	16.6 (7.8–23.7)	0.028
Eosinophils, %	0.5 (0–5.3)	0.95 (0–2.8)	NS
Macrophages, %	51.9 (3.3–90.1)	63.9 (15.1–75.4)	NS
Pneumothorax	3 (11.5)	2 (22.2)	NS
Mechanical ventilation	1 (3.8)	6 (66.6)	0.001

Unless otherwise indicated, data are presented as number (%) or median (range)
NS: No significance

only a slight improvement in the false positive rate, but a decline of the detection rate in the diagnosis of PCP.

A limitation of this study was the small number of patients. Another limitation might be that we included cases of PCP that were diagnosed on the basis of not only microscopic findings but also on the results of PCR assays. A recent study showed that PCR of BAL fluid can detect asymptomatic colonization of *P. jirovecii*, particularly in patients receiving corticosteroid therapy or in immunocompromised patients with lung disease (24). However, all PCR positive cases in this study were symptomatic and successfully treated with Trimethoprim-sulfamethoxazole, and we believe that PCR tests in those cases were not false positive.

In summary, β-D-glucan is a reliable marker for adjunctive diagnosis of PCP, but physicians should be aware of its lower detection rate in non-HIV PCP. Factors influencing outcome are the underlying disease, oxygenation index, serum albumin, and the association of mechanical ventilation. It is important to note that neither β-D-glucan nor KL-6 was associated with the prognosis of PCP.

Acknowledgement
This work was supported by the Takeda Science Foundation.

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