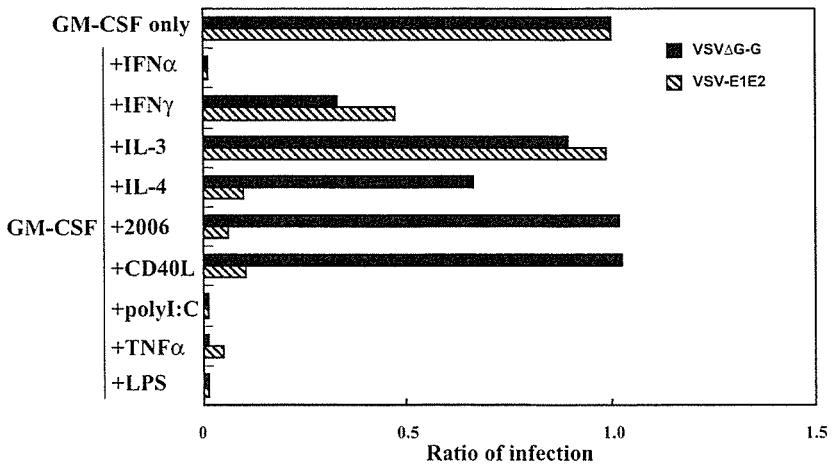

Fig. 4. IL-4, CpG ODN, or CD40L protects MDC from VSV-E1E2. Various immunomodulators were added to MDC cultured with GM-CSF, and the ratio of infection was determined between the cells treated with or without the reagents. IFN-α (100 U/ml), IFN-γ (100 U/ml), IL-3 (50 ng/ml), or IL-4 (10 ng/ml) was added on the day of MDC separation. CpG ODN 2006 (10 μM), CD40L (1 μg/ml), polyI:C (50 μg/ml), TNFα (20 ng/ml), or LPS (10 μg/ml) was added to MDC 24 hrs before the pseudovirus inoculation. Representative results are shown from three independent experiments.

ing that the molecules responsible for VSV-E1E2 entry differ between HepG2 and MDC. Furthermore, it also shows that mannan affects the molecules on MDC but not those on VSV-E1E2.

Discussion

Using the pseudotype VSV system, we have demonstrated that each DC subset has distinct susceptibility to HCV. First, VSV-E1E2 enters MDC but not PDC, which is in sharp contrast with PDC susceptibility to HIV (Patterson et al., 2001). Second, MDC cultured with GM-CSF are more susceptible to VSV-E1E2 than freshly prepared MDC or those cultured with GM-CSF and IL-4, showing that HCV targets immature MDC. Third, certain molecules containing the lectin domain on MDC are involved in the interaction with VSV-E1E2.

One of the suggested mechanisms of persistent HCV infection is the functional suppression of immunocompetent cells, including NK cells, T cells, and DC (Bain et al., 2001; Corado et al., 1997; Kanto et al., 1999; Wedemeyer et al., 2002). The possibility being raised for such immunological impairment is that HCV directly infects these blood cells. To elucidate this issue, investigators have used RT-PCR to examine whether the HCV genome is detectable or not in blood cells recovered from HCV-infected patients (Bain et al., 2001; Lerat et al., 1996, 1998). However, the existence of HCV-RNA does not enable to define whether HCV enters cells or only adheres to their surface. Instead of qualitative RT-PCR, we used the pseudotype VSV system to study the HCV E1E2-mediated virus entry to each DC subset. The pseudotype VSV system is a valid model for investigating the early steps of HCV infection, that is, viral attachment, receptor binding, and membrane fusion. Also, it enables us to estimate the efficiency of HCV E1E2-mediated virus entry to target cells. However, there are several limitations in this system. First, the positive results with pseudotype VSV do not indicate the replicative ability of HCV in the relevant cells. Because pseudotype VSV is constructed from VSV genome, their replication capacity is not exactly the same as HCV. Second, the evaluation of pseudotype VSV entry is possible only in cells that permit VSV replication. In other words, it cannot be used to determine the entry of VSV-E1E2 in the cells that suppress VSV replication. In this study, we could not evaluate the susceptibility of T, B, NK cells or fresh PDC to VSV-E1E2.

Alternatively, we performed an inoculation experiment with authentic HCV particle to confirm the reliability of the pseudotype VSV system. Quantitative RT-PCR assay showed that the highest titer of HCV-RNA was detected in MDC cultured with GM-CSF; however, low titer of HCV-RNA was detected in PDC. We hypothesized that the reason such discrepancy occurs between two assays is that RT-PCR amplified HCV genome from HCV attached to the surface of PDC. Strand-specific RT-PCR showed that negative strand of HCV-RNA, a surrogate marker of HCV replication, was detected in MDC cultured with GM-CSF but not in PDC. These results indicate that HCV enters and replicates in MDC but not in PDC, which are well correlated with those of pseudotype VSV entry.

The Th1 response is thought to be needed to eradicate HCV from hosts (Gerlach et al., 1999). Myeloid DC potentially activate CD4+T cells to support Th1 differentiation (Liu, 2001). We found that MDC from HCV-infected patients are less able to induce the Th1 response than the normal counterpart (Kanto T., unpublished data). It has been reported that MoDC expressing HCV protein were impaired in the stimulation of allogeneic T cells and IL-12 production, indicating an inhibitory capacity of HCV

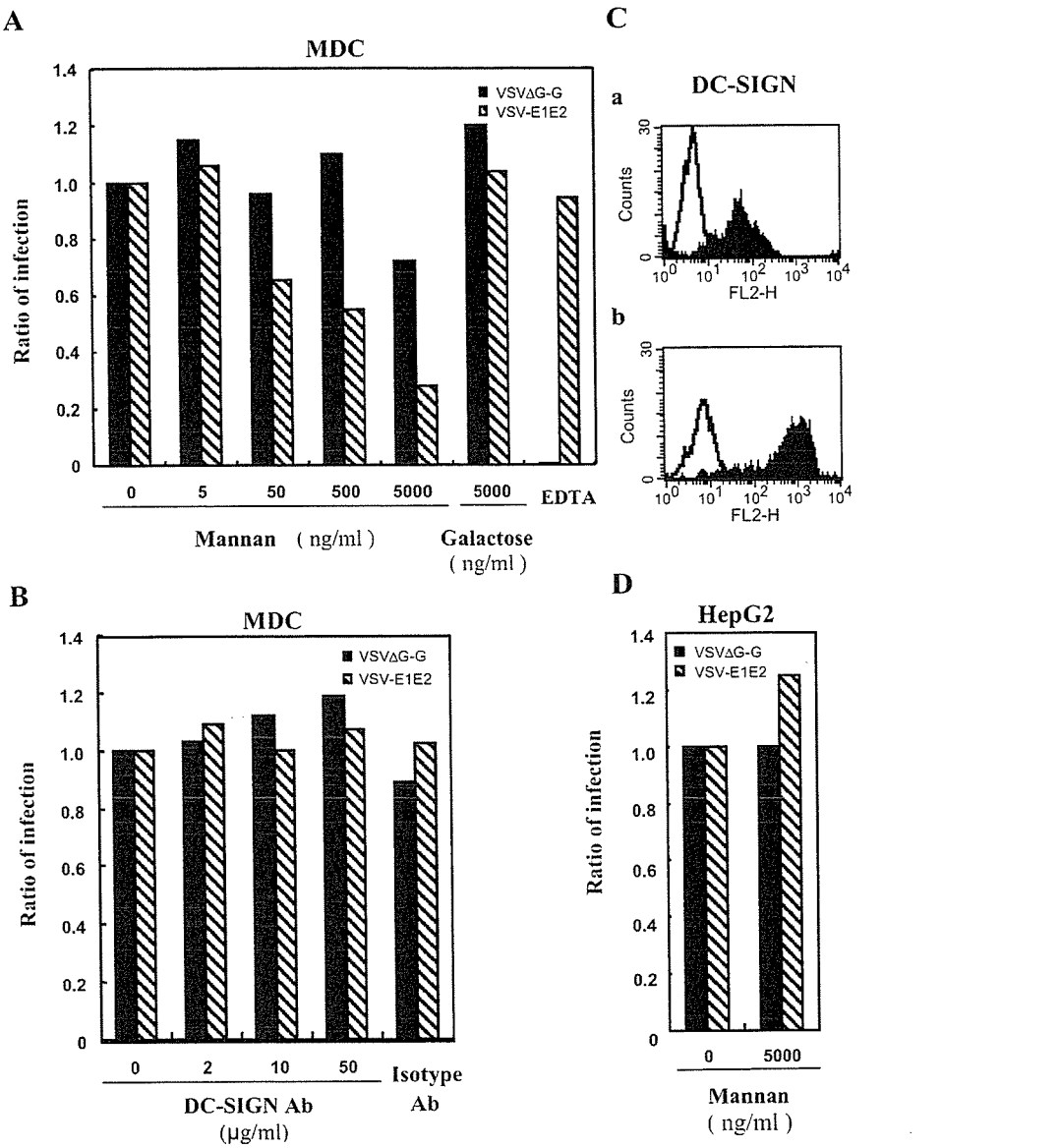

Fig. 5. Mannan inhibits VSV-E1E2 entry to MDC, but not to HepG2. Various concentrations of mannan, galactose, or 5 mM EDTA were added to day 4 MDC cultured with GM-CSF before the pseudotype VSV inoculation. Alternatively, day 4 MDC were treated with 2, 10, or 50 μg/ml antihuman DC-SIGN Ab or 10 μg/ml isotype IgG_{2B} for 30 min before the pseudotype VSV inoculation. HepG2 was treated with 5 μg/ml mannan before the addition of pseudotype VSV. The ratio of infection with pseudotype VSV in MDC (A, B) or HepG2 (D) was determined as described in Materials and method. Representative results are shown from three independent experiments. (C) Flow cytometric analyses were done for DC-SIGN expression on day 4 MDC cultured with GM-CSF (a) or with GM-CSF and IL-4 (b) generated from a healthy volunteer. Representative results from three subjects are shown. Open histograms represent the results with isotype Ab and filled ones represent those with anti-DC-SIGN Abs. Fluorescence intensity is shown in the *x*-axis and the number of cells is shown in the *y*-axis.

protein on DC function (Sarobe et al., 2002). These data suggest that direct HCV infection to myeloid DC suppress their function. Thus, the protection of DC from HCV infection is a rational approach to improve DC-mediated anti-HCV immune response. In the present study, we demonstrate that some of the maturation stimuli are capable of protecting DC from VSV-E1E2 entry. However, MoDC from HCV-infected patients are reported to be resistant to maturation stimuli, such as TNF-α (Auffermann-Gretzinger et al., 2001). Thus, further investigation is necessary to determine the effective modulation that allows MDC to mature in HCV infection. As shown in this study, CpG ODN or CD40L stimulated MDC to mature and become less susceptible to VSV-E1E2. The potent ability of CpG ODN to stimulate a DC-inducing Th1 response has been demonstrated in vivo tumor treatment models (Heckelsmiller et al., 2002). Therefore, CpG ODN are promising as a DC adjuvant in HCV-infected patients that potentially leads to MDC maturation as well as boosting Th1 response.

It is arguably necessary to identify molecules that are responsible for HCV entry to protect DC. Previously, tetraspanin CD81 has drawn much attention as a presumed HCV receptor due to its high affinity to HCV-E2 (Pileri et al., 1998). However, its involvement in VSV-E1E2 entry is unlikely because the CD81 is equally expressed on both MDC and PDC but is lacking on the VSV-E1E2-sensitive cell line HepG2 (Flint et al., 1999). In this study, we showed some of the characteristics of the molecules on DC involved in VSV-E1E2 entry. They are myeloid-lineage specific, inducible by GM-CSF, down-regulatable by IL-4 or other maturation stimuli. In addition, they possess some lectin domain, as evidenced by the inhibition of VSV-E1E2 as well as authentic HCV entry with mannan. These results raised the possibility that such molecules are categorized as members of C-type lectins, such as DC-SIGN, mannose receptor (MR), Langerin, DEC205, BDCA2, or asialoglycoprotein receptor. (Figdor et al., 2002) Recently, two independent studies have demonstrated that HCV E1 and E2 glycoproteins efficiently bind to DC-SIGN (Lozach et al., 2003; Pohlmann et al., 2003). However, the involvement of DC-SIGN in VSV-E1E2 entry is less likely because its expression on MDC did not parallel the susceptibility to VSV-E1E2. In addition, the treatment with anti-DC-SIGN Ab did not inhibit VSV-E1E2 entry. Furthermore, the treatment with EDTA failed to block VSV-E1E2 entry to MDC, showing that the VSV-E1E2 entry occurs in a Ca^{2+}-independent manner. It is still obscure in which step lectins are involved in VSV-E1E2 entry to MDC. From an analogy with the interaction of HIV with DC-SIGN (Geijtenbeek et al., 2000), it is conceivable that lectins are essential for HCV attachment to MDC. Nevertheless, the possibility remains that HCV entry receptors or co-receptors, which may be other than lectins, exist on MDC. With the aid of the pseudotype VSV system, exploration has been underway to identify the molecules on MDC that are critically involved in HCV infection.

Materials and method

Reagents

Recombinant human IL-4 and GM-CSF were purchased from PeproTech (London, UK). Recombinant human soluble CD40L, human TNF-α and IL-3, and mouse monoclonal antihuman DC-SIGN (CD209/DC-SIGN1) Ab (12507) were from R & D Systems (Minneapolis, MN). LPS, polyI:C, mannan, galactose, and methyl α-D-mannnopyranoside were from Sigma (St. Louis, MO). Recombinant human IFN-γ was from Strathman Biotech GmbH (Hamburg, Germany). Human lymphoblastoid IFN-α was provided by Sumitomo Pharmaceuticals (Osaka, Japan). Unmethylated CpG ODN 2006 (Krug et al., 2001) was synthesized at and purchased from Sigma Genosys (Hokkaido, Japan). Isotype IgG (mouse IgG_{2B}) for the blocking experiments was kindly provided from the JT laboratory (Osaka, Japan).

Separation of DC precursors and other cells from PBMC

After informed consent had been obtained from healthy volunteers, buffy coats were isolated from venous blood drawn from them at the Osaka Red Cross Blood Center (Osaka, Japan). PBMC were collected from buffy coats by Ficoll–Hypaque density-gradient centrifugation. B cells, MDC, and PDC were magnetically isolated by using CD19 microbeads, BDCA-1, or BDCA-4 DC isolation kits from Miltenyi Biotec (Bergish-Gladbach, Germany), respectively. BDCA-1^+ and BDCA-4^+ cells are phenotypically compatible with MDC and PDC, respectively (Dzionek et al., 2000). CD4, CD8 T cells, and NK cells were separated from PBMC by using the relevant Stem-Sep kits (Stem Cell Technologies Inc, Vancouver, BC). $CD34^+$ hematopoietic precursor cells were isolated from cord blood mononuclear cells by using CD34-microbeads from Miltenyi. The purity of all isolated cells was more than 90% as determined by FACS Caliber (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Culture of DC

Isolated MDC were cultured for 4 days in IMDM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 mM Hepes buffer, and 10 μM nonessential amino acid (Complete medium, CM) containing 50 ng/ml GM-CSF with or without 10 ng/ml IL-4. PDC were cultured for 4 days in CM in the presence of 50 ng/ml of IL-3.

Flow cytometry

The expression of surface molecules on DC was analyzed by FACS Caliber (Becton Dickinson). For the staining, DC were stored with specific Abs or isotype Abs for 30 min at 4 °C in PBS containing 2% of BSA and 0.1% of sodium azide. The following FITC-, PE-, PerCP-, or PC5-conjugated antihuman mAbs were used: CD1a (NA1/34; DAKO, Glostrup, Denmark), CD11c (KB90; DAKO), CD14 (M5E2; Becton Dickinson), CD40 (5C3; BD Pharmingen, San Diego, CA), CD80 (L307.4; BD Pharmingen), CD83 (HB15a; Immunotech, Marseille, France), CD86 (IT2.2; B70/B7-2, BD Pharmingen), CDw123 (7G3; IL-3 receptorα chain, BD Pharmingen), DC-SIGN (120507; R & D Systems), and HLA-DR (L243; Becton Dickinson).

Assessment of pseudotype VSV entry into cells

To find which blood cells are susceptible to HCV infection, we used pseudotype VSV possessing chimeric HCV E1 and E2 protein which was generated as described

previously (Matsuura et al., 2001). The pseudotype VSV consists of recombinant VSV in which glycoprotein (G) gene is replaced with a reporter gene encoding GFP.

As an envelope, it possesses chimeric HCV E1 and E2 proteins (VSV-E1E2). The viruses were purified by centrifugation at 25000 rpm for 2 h at 4 °C in SW28 rotor (Beckman Coulter Inc., Fullerton, CA) through 20% (v/w) and 60% (v/w) discontinuous sucrose gradient and were stored at −80 °C. To determine RNA copy numbers in the viral samples, TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA) was used. We used forward and reverse primers (5′-cattattatcattaaaaggctc-3′ and 5′-gatacaaggtcaaatattccg-3′) that amplify a 323-bp segment of the pseudotyped VSV RNA and also used a dual fluorophore-labeled probe 5′-(6-carboxy-fluorescein)-atccagtggaatacccggcagattac-(6-carboxy-tetramethyl-rhodamine)-3′. The sequence detector (ABI Prism 7000, PE Applied Biosystems) allows measurement of the amplified products in indirect proportion to the increase in fluorescence emission continuously during the PCR amplification. The copy numbers in samples were determined based on the standard curve drawn by a known amount of in vitro synthesized pseudotyped VSV RNA. Because VSV efficiently replicates in a wide range of mammalian cells, we are able to determine the cells exhibiting susceptibility to pseudotype VSV by the expression of GFP. We used VSV ΔG which has no envelope protein as a negative control. Similarly, VSV ΔG-G was used as a positive control which is complemented with the VSV G protein. Various separated blood cells were prepared in CM at 5×10^4 cells/well on 96-well culture plates. Next, they were inoculated with the pseudotype viruses, VSV-E1E2 (1×10^{12} RNA copies/well), VSV ΔG (1×10^{12} RNA copies/well), or VSV ΔG-G (1×10^{11} RNA copies/well) and incubated for 16 h at 37 °C. The infected cells (GFP^+ cells) were observed under fluorescence microscopy, and their positive percentages were determined by FACS analysis. The net percentage of infected cells was expressed as % infection = (% of GFP^+ cells with VSV-E1E2 or VSV ΔG-G) − (% of GFP^+ cells with VSV ΔG).

To find the substances which potentially protect DC from HCV infection, we examined IFNα, IFNγ, IL-3, IL-4, CpG ODN 2006, CD40L, polyI:C, TNFα, or LPS for this purpose. The appropriate concentrations of these reagents were determined in a separate series of experiments. IFNα, IFNγ, IL-3, or IL-4 was added to DC on the day of separation. CpG ODN 2006, CD40L, polyI:C, TNFα, or LPS was added to DC 24 h before the inoculation of pseudovirus. To compare the inhibitory effect of reagents in VSV-E1E2 entry into cells, we determined the ratio of infection of cells with and without treatment.

DC express various molecules containing the lectin domain, some of which are reported to be essential for the attachment to virus (Figdor et al., 2002). To examine whether lectin-containing molecules on DC are involved in HCV infection, we tested mannan, methyl α-D-mannopyranoside, and galactose for the inhibition of VSV-E1E2 entry. Day 4 MDC cultured with GM-CSF were preincubated with various concentrations of mannan, methyl α-D-mannopyranoside, or galactose at 37 °C for 180 min and inoculated with the pseudotype VSV. We also treated DC with EDTA (5 mM), monoclonal antihuman DC-SIGN Ab (50, 10, or 2 μg/ml), or isotype IgG_{2B} before the pseudotype VSV inoculation. To compare DC with hepatoblastoma cell line, HepG2, we treated HepG2 with mannan before the inoculation.

Quantitative analysis of HCV RNA in cells inoculated with HCV particles from patient serum

To test the susceptibility of each DC subset to authentic HCV, we quantified HCV RNA in cells that had been inoculated with patient serum by means of real-time PCR. We used the commercial HCV seroconversion panel as an inoculum, which contains high HCV RNA titer (1×10^5 copies/μl) and no anti-HCV antibody (BioClinical Partners, Inc, USA). We added 3 μl/well of inoculum to DC on 96-well plates and incubated them at 37 °C for 24 h. DC were harvested and washed three times with IMDM supplemented with 1% FCS and then total RNA was extracted from DC using RNeasy Mini Kit (QIAGEN, Germany). To measure HCV RNA, TaqMan EZ RT-PCR kit (PE Applied Biosystems) was used. We used forward and reverse primers [5′-cgggagagccatagtgg-3′ (positions 130–146) and 5′-agtaccacaaggcctttcg-3′ (positions to 272 to 290)] that amplify a 161-bp segment of the 5′ noncoding region of HCV RNA and also used a dual fluorophore-labeled probe [5′-(6-carboxy-fluorescein)-ctgcggaaccggtgagtacac (positions 148–168)- (6-carboxy-tetramethyl-rhodamine) -3′]. The sequence detector (ABI Prism 7000) allows measurement of the amplified products in indirect proportion to the increase in fluorescence emission continuously during the PCR amplification. The copy number in the samples was determined based on the standard curve drawn by a known amount of in vitro synthesized HCV RNA.

The strand-specific RT-PCR assay for HCV-RNA in cells inoculated with authentic HCV particles

To detect negative-strand HCV-RNA that is indicative of RNA replication, we performed the strand-specific RT-PCR assay referring to the methods described by Navas et al. (2002) with some modifications. We used the same batch of HCV seroconversion panel as an inoculum as described in the above section. We added 3 μl/well of inoculum to DC on 96-well plates and incubated them at 37 °C for 24 h. After the washing of DC for three times, total RNA was extracted from DC as the same way as we did in quantitative RT-PCR. As a control for the detection of HCV-RNA, 9 μl of inoculum was used. We used sense and anti-sense primers [5′-cactcccctgtgaggaactactgtc-3′ (positions 38–62) and 5′-atggtgcacggtctacgagacctcc-3′ (positions 319–343) that amplify a 306-bp segment of the 5′ noncoding region of HCV

genome. Ten microliters of purified RNA was used for reverse transcription (RT) with 10 μl of RT reaction mixture containing the thermostable recombinant *Thermus thermophilus* (rTth) enzyme (PE Applied Biosystems). Synthesis of cDNA was carried out with strand-specific primers, sense primer was used to obtain negative-strand RNA, and antisense primer was used to obtain positive-strand RNA, respectively. Reverse transcription was carried out at 70 °C for 15 min. Subsequently, the same primers were used in reverse order in the PCR rounds. Thirty-five cycles of PCR (94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s) followed by 7 min of extension at 72 °C were carried out on GeneAmp PCR System (PE Applied Biosystems). One-fifth of the PCR products was subjected to electrophoresis on 2% agarose gel and stained with ethidium bromide for observation under UV light. The expected molecular size of PCR products derived from target HCV RNA was 306 bp.

Statistical analysis

The paired *t* test was used to test the significance of the pseudotype VSV entry to MDC. Statistical analyses were performed with the Statview version 4.5 software (Abacus Concepts, Berkeley, CA). A *P* value of less than 0.05 was considered statistically significant.

Acknowledgment

We are grateful to Dr. Keiji Ueda (Osaka University) for providing cord blood cells.

References

Alter, H.J., Purcell, R.H., Shih, J.W., Melpolder, J.C., Houghton, M., Choo, Q.L., Kuo, G., 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. 321 (22), 1494–1500.

Auffermann-Gretzinger, S., Keeffe, E.B., Levy, S., 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 97 (10), 3171–3176.

Bain, C., Fatmi, A., Zoulim, F., Zarski, J.P., Trepo, C., Inchauspe, G., 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 120 (2), 512–524.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392 (6673), 245–252.

Corado, J., Toro, F., Rivera, H., Bianco, N.E., Deibis, L., De Sanctis, J.B., 1997. Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. Clin. Exp. Immunol. 109 (3), 451–457.

Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D.W., Schmitz, J., 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J. Immunol. 165 (11), 6037–6046.

Farci, P., Alter, H.J., Govindarajan, S., Wong, D.C., Engle, R., Lesniewski, R.R., Mushahwar, I.K., Desai, S.M., Miller, R.H., Ogata, N., et al., 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science 258 (5079), 135–140.

Figdor, C.G., van Kooyk, Y., Adema, G.J., 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev., Immunol. 2 (2), 77–84.

Flint, M., Thomas, J.M., Maidens, C.M., Shotton, C., Levy, S., Barclay, W.S., McKeating, J.A., 1999. Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. J. Virol. 73 (8), 6782–6790.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., Figdor, C.G., van Kooyk, Y., 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100 (5), 587–597.

Gerlach, J.T., Diepolder, H.M., Jung, M.C., Gruener, N.H., Schraut, W.W., Zachoval, R., Hoffmann, R., Schirren, C.A., Santantonio, T., Pape, G.R., 1999. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. Gastroenterology 117 (4), 933–941.

Grosjean, I., Caux, C., Bella, C., Berger, I., Wild, F., Banchereau, J., Kaiserlian, D., 1997. Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells. J. Exp. Med. 186 (6), 801–812.

Hart, D.N., 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90 (9), 3245–3287.

Heckelsmiller, K., Beck, S., Rall, K., Sipos, B., Schlamp, A., Tuma, E., Rothenfusser, S., Endres, S., Hartmann, G., 2002. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. Eur. J. Immunol. 32 (11), 3235–3245.

Kaku, H., Goldstein, I.J., Oscarson, S., 1991. Interactions of five D-mannose-specific lectins with a series of synthetic branched trisaccharides. Carbohydr. Res. 213, 109–116.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A., Hori, M., 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J. Immunol. 162 (9), 5584–5591.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S., Krieg, A.M., Hartmann, G., 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur. J. Immunol. 31 (7), 2154–2163.

Lerat, H., Berby, F., Trabaud, M.A., Vidalin, O., Major, M., Trepo, C., Inchauspe, G., 1996. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J. Clin. Invest. 97 (3), 845–851.

Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M.A., Trepo, C., Inchauspe, G., 1998. In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. Blood 91 (10), 3841–3849.

Liu, Y.J., 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 106 (3), 259–262.

Lozach, P.Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J.L., Arenzana-Seisdedos, F., Altmeyer, R., 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. 278 (22), 20358–20366.

Matsuura, Y., Tani, H., Suzuki, K., Kimura-Someya, T., Suzuki, R., Aizaki, H., Ishii, K., Moriishi, K., Robison, C.S., Whitt, M.A., Miyamura, T., 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology 286 (2), 263–275.

Miller, R.H., Purcell, R.H., 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. U.S.A. 87 (6), 2057–2061.

Navas, M.C., Fuchs, A., Schvoerer, E., Bohbot, A., Aubertin, A.M., Stoll-Keller, F., 2002. Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J. Med. Virol. 67 (2), 152–161.

Patterson, S., Rae, A., Hockey, N., Gilmour, J., Gotch, F., 2001. Plasmacytoid dendritic cells are highly susceptible to human immunodeficiency virus type 1 infection and release infectious virus. J. Virol. 75 (14), 6710–6713.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R.,

Weiner, A.J., Houghton, M., Rosa, D., Grandi, G., Abrignani, S., 1998. Binding of hepatitis C virus to CD81. Science 282 (5390), 938–941.

Pohlmann, S., Zhang, J., Baribaud, F., Chen, Z., Leslie, G.J., Lin, G., Granelli-Piperno, A., Doms, R.W., Rice, C.M., McKeating, J.A., 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. 77 (7), 4070–4080.

Sarobe, P., Lasarte, J.J., Casares, N., Lopez-Diaz de Cerio, A., Baixeras, E., Labarga, P., Garcia, N., Borras-Cuesta, F., Prieto, J., 2002. Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. J. Virol. 76 (10), 5062–5070.

Schnorr, J.J., Xanthakos, S., Keikavoussi, P., Kampgen, E., ter Meulen, V., Schneider-Schaulies, S., 1997. Induction of maturation of human blood dendritic cell precursors by measles virus is associated with immunosuppression. Proc. Natl. Acad. Sci. U.S.A. 94 (10), 5326–5331.

Tailleux, L., Schwartz, O., Herrmann, J.L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L.P., Gluckman, J.C., Lagrange, P.H., Gicquel, B., Neyrolles, O., 2003. DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. J. Exp. Med. 197 (1), 121–127.

Wedemeyer, H., He, X.S., Nascimbeni, M., Davis, A.R., Greenberg, H.B., Hoofnagle, J.H., Liang, T.J., Alter, H., Rehermann, B., 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J. Immunol. 169 (6), 3447–3458.

Weiner, A., Erickson, A.L., Kansopon, J., Crawford, K., Muchmore, E., Hughes, A.L., Houghton, M., Walker, C.M., 1995. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc. Natl. Acad. Sci. U.S.A. 92 (7), 2755–2759.

Wu, L., Martin, T.D., Vazeux, R., Unutmaz, D., KewalRamani, V.N., 2002. Functional evaluation of DC-SIGN monoclonal antibodies reveals DC-SIGN interactions with ICAM-3 do not promote human immunodeficiency virus type 1 transmission. J. Virol. 76 (12), 5905–5914.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Hepatology Research 29 (2004) 142–147
Hepatology Research
www.elsevier.com/locate/ihepcom

The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan

Naoki Hiramatsu[a], Akinori Kasahara[b], Fumihiko Nakanishi[c], Takashi Toyama[c], Masahiko Tsujii[c], Shingo Tsuji[c], Tatsuya Kanto[a], Tetsuo Takehara[a], Michio Kato[d], Harumasa Yoshihara[e], Masafumi Naito[f], Kazuhiro Katayama[f], Taizo Hijioka[g], Hideki Hagiwara[h], Shinji Kubota[i], Masahide Oshita[j], Haruya Meren[j], Manabu Masuzawa[j], Yoshimichi Haruna[k], Eiji Mita[l], Kunio Suzuki[l], Norio Hayashi[a,*]

[a] *Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita City, Osaka 565-0871, Japan*
[b] *Department of General Medicine, Osaka University Graduate School of Medicine, Osaka, Japan*
[c] *Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Osaka, Japan*
[d] *Osaka National Hospital, Osaka, Japan*
[e] *Osaka Rousai Hospital, Osaka, Japan*
[f] *Osaka Kouseinenkin Hospital, Osaka, Japan*
[g] *National Osaka South Hospital, Osaka, Japan*
[h] *Higashiosaka City Central Hospital, Osaka, Japan*
[i] *Kansai Rousai Hospital, Hyoga, Japan*
[j] *Osaka Police Hospital, Osaka, Japan*
[k] *Osaka Prefectural Hospital, Osaka, Japan*
[l] *Saiseikai senri Hospital, Osaka, Japan*

Received 26 December 2003; received in revised form 16 March 2004; accepted 25 March 2004

Abstract

One hundred seventy-one patients with chronic hepatitis C were included in this study (genotype1 and high viral loads (1H), $n = 130$; non-1H, $n = 37$; N.D., $n = 4$). The combination therapy of interferon and ribavirin for 24 weeks with an additional 24 weeks of interferon monotherapy (48-week treatment) was undergone by 42 1H patients and 5 non-1H patients. The combination therapy of interferon and ribavirin was administered for 24 weeks in 67 1H patients and 22 non-1H patients. Among the 1H patients, the HCV relapse rate was significantly higher in those receiving 24-week combination treatment than in those receiving 48-week treatment (78% versus 42%, $P = 0.003$). Among the non-1H patients, no significant difference was found between them. Sustained virological response (SVR) rates were observed to decrease as the timing of HCV RNA disappearance was delayed. In spite of the small rate (16%), SVR was obtained from the patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment. In 1H patients, 24-week combination treatment followed by interferon monotherapy for 24 weeks was concluded to be the treatment offering the most hope among those that the medical insurance can be applied in Japan.

Keywords: Chronic hepatitis C; Interferon and ribavirin combination therapy; Combination therapy followed by IFN monotherapy

1. Introduction

Interferon is the only available treatment for patients with chronic hepatitis C since HCV was discovered in 1989 [1–4]. Thirty percent of patients with chronic hepatitis C achieved SVR by interferon therapy but the efficacy was not satisfactory. Furthermore, in the patients considered to be the most treatment-resistant, that is, the 1H patients, only 5–8% showed SVR. In Japan, 40–50% of the patients with chronic hepatitis C belong to the 1H group. Therefore, finding how to eradicate the HCV RNA

* Corresponding author. Tel.: +81-6-6879-3441; fax: +81-6-6879-3449.
E-mail address: hayashin@moltex.med.osaka-u.ac.jp (N. Hayashi).

1386-6346/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.hepres.2004.03.008

of 1H patients is most important for the treatment of chronic hepatitis C.

Recently, ribavirin, a nucleic acid analogue, exhibiting in vitro activity against various kinds of DNA and RNA viruses has been developed. The combination therapy of ribavirin and interferon has been shown to be very useful in the eradication of HCV in patients with chronic hepatitis C [5–7], although the mechanism of action of ribavirin remains speculative and ribavirin monotherapy led to no significant decrease of the amount of HCV RNA in the patients with chronic hepatitis C [8]. Most recent studies, performed with large numbers of naïve patients, have shown that the combination therapy of interferon and ribavirin can increase the SVR rate two-fold compared with interferon monotherapy for patients with chronic hepatitis C [9–12]. Especially, in the 1H patients, the combination therapy of interferon and ribavirin was more useful than in the other patients. Furthermore, Poynard et al. [10] showed that 1H patients treated by combination therapy for 48 weeks had a higher SVR rate than those treated for 24 weeks (28% versus 8%). Therefore, the combination therapy of interferon and ribavirin for 48 weeks is recommended as the standard therapy for 1H patients in Europe and the United States [13,14].

In Japan, the combination therapy of interferon and ribavirin was approved in 2001. However, the duration of the combination therapy is limited to 24 weeks in the medical insurance. As mentioned above, the SVR rate in 1H patients treated by the combination therapy for 24 weeks was clearly lower than those treated for 48 weeks. Furthermore, prolonged interferon monotherapy was reported to suppress relapse after cessation of therapy and to achieve a higher SVR rate in patients with chronic hepatitis C [15]. This study assessed the efficacy of the combination therapy of interferon and ribavirin for 24 weeks with an additional 24 weeks of interferon monotherapy compared with that of the combination therapy for 24 weeks.

2. Patients and methods

2.1. Patients

The current study was conducted at Osaka University Hospital and the institutions of the Osaka Liver Disease Study Group. The 171 patients included in this study had HCV RNA detectable in serum by the polymerase chain reaction (PCR) method, had elevated ALT (above the upper limit of the normal) and had been histologically proven to have chronic hepatitis. No patients were positive for hepatitis B surface antigen and anti-human immunodeficiency virus antibody or had other forms of liver disease (such as alcoholic liver disease and autoimmune liver disease). This study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

2.2. Determination of HCV RNA levels and HCV genotype

Serum HCV RNA levels were quantified using branched DNA (bDNA) probe assay (version 2; Chiron, Dai-ichi Kagaku, Tokyo) [16,17] or combined PCR assay (Amplicor-HCV monitor assay) [18]. In this study, a high viral load, as described previously [16,18,19], was designated as the condition of a serum HCV RNA level of more than 10^6 equivalents/ml by bDNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay. HCV genome typing was classified by serological genotyping assay [20].

2.3. Treatment schedule

Of the 171 patients with chronic hepatitis C enrolled in this study, 130 had HCV RNA with genotype 1 and high viral loads (1H group), which were difficult to eradicate by anti-viral therapy. Of the remaining 41 patients, 37 had HCV RNA with genotype 2 or low viral loads (non-1H group); genotype or viral levels could not be determined for four. One hundred thirty-six patients in whom treatment had been done without the discontinuation of interferon till the end of the scheduled duration were studied (1H, $n = 109$; non-1H, $n = 27$).

The combination therapy of interferon-α-2b and ribavirin was administered for 24 weeks in 67 patients of the 1H group and 22 patients of the non-1H group. In this protocol, interferon-α-2b was given intramuscularly every day for the first 2 weeks and then three times a week for the following 22 weeks in combination with ribavirin at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥ 60 kg, respectively). The combination therapy of interferon-α-2b and ribavirin for 24 weeks, followed by interferon-α-2b monotherapy three times a week for a further 24 weeks, was administered to 42 patients of the 1H group and 5 patients of the non-1H group. The pretreatment characteristics of the patients were similar (Table 1).

The starting doses of interferon-α-2b were 10 MU per day for 38, 6 MU per day for 127, and 3 MU per day for 6 patients. With ribavirin, 800 mg per day was started in 92, 600 mg per day in 77, and 400 mg per day in 2 patients. Among the 171 patients, the interferon dose was decreased in six patients during the treatment, and the interferon was stopped along with ribavirin in 33 patients (19%) due to side effects. The ribavirin dose was decreased in 43 patients (25%) during the treatment, and stopped without discontinuance of interferon in six patients. Eighty-seven patients (51%) completed treatment without discontinuance or dosage decrease of both drugs.

After the sufficient informed consent at the end of the combination therapy of interferon and ribavirin, the patients themselves decided whether to be treated for 24 or 48 weeks. The information included the results of clinical trials of the combination therapy for 24 and 48 weeks in other countries, such as the SVR rate, HCV relapse rate.

Table 1
Baseline characteristics of patients according to therapeutic protocol

	24-week treatment		48-week treatment
	1H group	Non-1H group	1H group
	67	22	42
Age (yo)	55.8 ± 10.9	55.7 ± 12.8	54.0 ± 11.7
M/F	40/27	15/7	28/14
ALT (IU/L)	107 ± 71	102 ± 45	103 ± 58
Fibrosis	1.9 ± 0.9	1.9 ± 1.2	1.8 ± 1.1
History of IFN treatment			
Naïve	34	11	17
Relapser	21	7	17
Non-responder	11	4	8
Unknown	1	0	0

Note: All comparisons are not significant. Twenty-four-week treatment, interferon plus ribavirin treatment for 24 weeks; 48-week treatment, interferon plus ribavirin treatment for 24 weeks followed by interferon monotherapy for 24 weeks. 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than 1H group. Fibrosis, Knodell's histological score (category 4).

Also, side effects were presented and the combination therapy of interferon-α-2b and ribavirin for 48 weeks was explained as not being covered by medical insurance in Japan. In the 47 patients who agreed to receive the additional 24 weeks of interferon monotherapy, the starting doses of interferon-α-2b were 10 MU per day for 10, 6 MU per day for 35, and 3 MU per day for 2 patients. All patients completed the additional treatment although interferon was decreased only in one patient from 10 to 6 MU per day.

2.4. Statistical analysis

Age, histological scores before interferon therapy, and serum ALT levels are expressed as mean ± S.D. The chi-squared test was used for statistical analysis of the comparison between group frequencies. When appropriate, the clinical and laboratory features of the two groups were compared by Student's *t*-test. Histological evaluation was substituted as a variable for Knodell's histological scores [21].

3. Results

3.1. Results of interferon and ribavirin combination therapy

Seventy-five percent of all of the patients of 1H group (82/109), including not only patients who received 24-week treatment but also those who received 48-week treatment, had no detectable HCV RNA at 24 weeks after the beginning of combination therapy of interferon and ribavirin. This was also the case for 100% of the non-1H patients (27/27). In patients given 24-week treatment of combined interferon and ribavirin, 45 out of 67 of the 1H group were negative for HCV RNA at the end of therapy, but only 22% of the patients (10/45) showed no detectable HCV RNA at 24 weeks after cessation of therapy. On the other hand, HCV RNA was negative in all non-1H patients at the end of the 24-week treatment, and the SVR rate was 86% (19/22) (Fig. 1). In patients with 48-week treatment (24-week combination treatment, followed by 24-week interferon monotherapy), HCV RNA reappeared during interferon monotherapy (break through) in 11 out of 37 patients (30%) who were negative for HCV RNA at the end of 24-week combination therapy: SVR was finally reached in 15 out of 26 patients who continued to be sero-negative for HCV RNA at the end of 48-week treatment. On the other hand, HCV RNA was not cleared even by 48-week treatment in all five patients who were positive for HCV RNA at the end of 24-week treatment (Fig. 2). In the non-1H patients who received 48-week treatment, HCV RNA was negative in all five patients at the end of the 24-week treatment, and SVR was attained by 80% (4/5).

The HCV RNA relapse rate after treatment was compared according to the duration of treatment. In all patients, 57% of those receiving 24-week treatment (38/67) had HCV RNA relapse, as compared with 39% of those receiving 48-week treatment (12/31). Among the 1H patients, a significant dif-

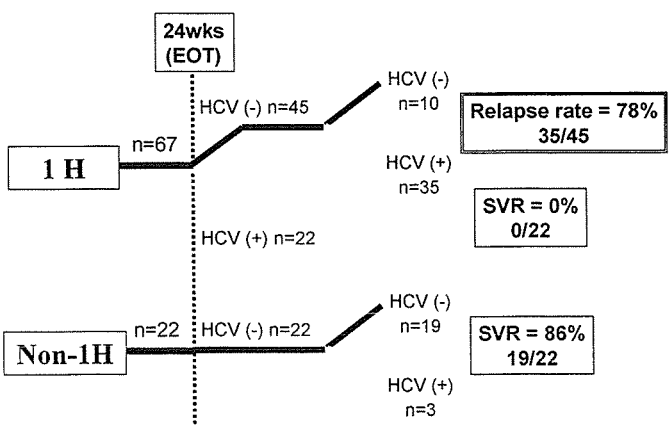

Fig. 1. Efficacy of the combination therapy (24-week treatment). 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than those of the 1H group. EOT, end of treatment. HCV, serum HCV RNA positivity by polymerase chain reaction.

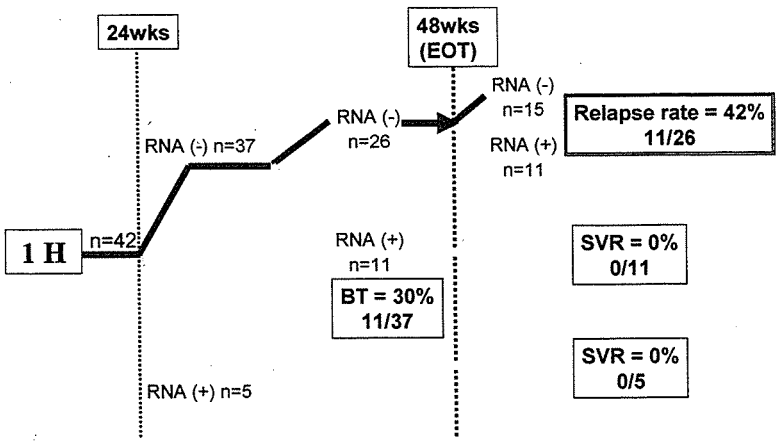

Fig. 2. Efficacy of the combination therapy followed by interferon monotherapy (48-week treatment). 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than those of the 1H group. EOT, end of treatment. HCV, serum HCV RNA positivity by polymerase chain reaction. BT, break through.

ference was found in HCV relapse rate between those receiving 24-week treatment and those receiving 48-week treatment (78% versus 42%, $P = 0.003$). Among the non-1H patients, HCV RNA relapsed in 14% (3/22) of those receiving 24-week treatment (Fig. 1) and 20% (1/5) of those receiving 48-week treatment.

3.2. *Timing of HCV RNA disappearance and efficacy of treatment*

The relationship between the timing of HCV RNA disappearance and SVR rate according to the duration of treatment was evaluated. As shown in Fig. 3A, in all patients receiving 24-week treatment, 71% (12/17) of the patients who had no detectable HCV RNA by week 4, 61% (11/18) by week 8 (beyond week 4), and 21% (4/19) by week 12 (beyond week 8) had SVR. Although 11 patients became negative for HCV RNA by week 24 (beyond week 12), none of them attained SVR. A tendency for a decrease in the SVR rate was observed as the timing of the HCV RNA disappearance was delayed. In the patients receiving 48-week treatment, 86% (6/7) of those who had no detectable HCV RNA by week 4, 100% (6/6) by week 8 (beyond week 4), 40% (4/10) by week 12 (beyond week 8), and 16% (3/19) by week 24 (beyond week 12) attained SVR.

Among the 1H patients, the same tendency was also observed (Fig. 3B). In the patients receiving 24-week treatment, 50% (3/6) of those who had no detectable HCV RNA by week 4, 40% (4/10) by week 8 (beyond week 4), and 18% (3/17) by week 12 (beyond week 8) attained SVR. None of the 10 patients who became negative for HCV RNA by week 24 (beyond week 12) showed SVR. In the patients receiving 48-week treatment, 80% (4/5) of those who had no detectable HCV RNA by week 4, 100% (5/5) by week 8 (beyond week 4), 38% (3/8) by week 12 (beyond week 8), and 16% (3/19) by week 24 (beyond week 12) had SVR. In spite of the small rate (16%), SVR was obtained from the patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment.

Fig. 4 shows the relationship between the timing of HCV RNA disappearance and the prediction value in 1H patients who received the combination therapy of interferon and ribavirin for 24 weeks. As the timing of the HCV RNA disappearance was late, the positive prediction value decreases and the negative prediction value increases. In particular, the negative prediction value at week 12 was 100%, that is, none

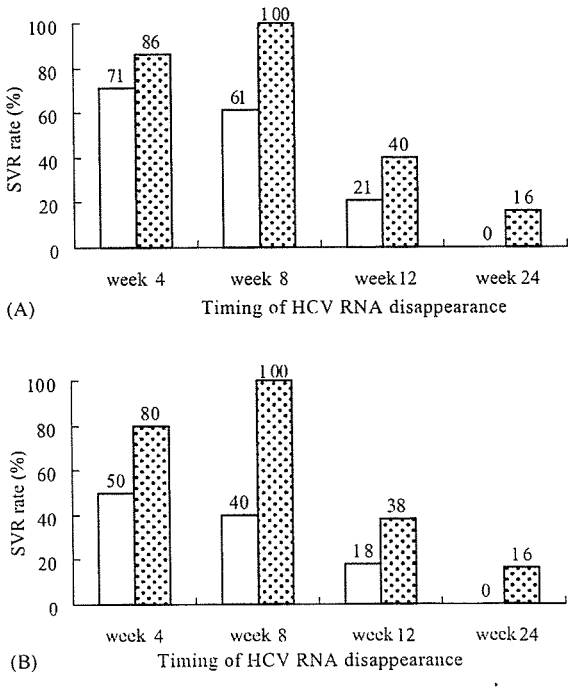

Fig. 3. Timing of HCV RNA disappearance and SVR rate (A) all patients, (B) patients with genotype 1 and high viral loads. (□) Combination therapy of interferon and ribavirin (24-week treatment); (▨) combination therapy followed by interferon monotherapy (48-week treatment).

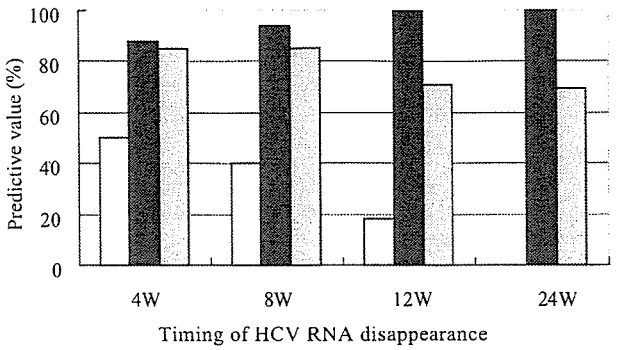

Fig. 4. Timing of HCV RNA disappearance and prediction value in patients with genotype 1 and high viral loads who received the combination therapy of interferon and ribavirin for 24 weeks. (□) Positive prediction value; (■) negative prediction value; (▣) predictive accuracy.

of the patients who were positive for HCV RNA at week 12 attained SVR.

4. Discussion

In Japan, randomized control studies were performed on the combination therapy of interferon and ribavirin for 24 weeks in patients with chronic hepatitis C, and the combination therapy was approved in November 2001. However, the duration of the combination therapy is limited to 24 weeks in the medical insurance because of the lack of clinical mega-trial evidence for the combination therapy for 48 weeks in Japan. From the results of international trials, the SVR rate in 1H patients treated by the combination therapy for 48 weeks has been shown to be higher than that of those treated for 24 weeks [10]. Moreover, for interferon monotherapy, prolonged interferon treatment was reported to suppress relapse after cessation of therapy and to lead to a higher SVR rate in patients with chronic hepatitis C [15]. Our strategy, the interferon and ribavirin combination therapy with an additional 24 weeks of interferon monotherapy, was conducted against this background.

Poynard et al. [22,23] evaluated the HCV RNA relapse rates after cessation of the combination therapy in naïve patients with chronic hepatitis C. Among patients with genotype 1, the relapse rates were 62% in those treated by interferon and ribavirin combination therapy for 24 weeks and 26% in those treated for 48 weeks: among patients with genotype 2/3, 21% in those for 24 weeks and 15% in those for 48 weeks. Among patients with genotype 1, the SVR rate increased due to suppression of the relapse rate by the combination therapy for 48 weeks. On the other hand, the patients with genotype 2/3 require only 24 weeks of therapy. In our study, patients with genotype 1 and high viral load (1H group) were evaluated, distinguishing them from others (non-1H group) since the efficacy of anti-viral therapy for the 1H patients has been known to be remarkably low. Among the 1H patients, the HCV relapse rate was significantly higher in those receiving 24-week combination treatment than in those receiving 48-week treatment, 24-week combination treatment followed by interferon monotherapy for 24 weeks (78% versus 42%, $P = 0.003$). Among the non-1H patients, no significant difference was found between those receiving 24-week treatment and those receiving 48-week treatment (14% versus 20%). These results indicate that our strategy of 48-week treatment is useful for the 1H group; the non-1H group seems to require only 24 weeks of therapy, similar to the patients with genotype 2/3 in the above-mentioned.

In the 1H patients receiving 48-week treatment, HCV RNA reappeared during interferon monotherapy in 11 out of 37 patients (30%) who were negative for HCV RNA at the end of 24-week combination therapy. The break through phenomenon should be taken into account when the efficacy of this treatment is evaluated. The SVR ratio in 1H patients receiving 48-week treatment can be calculated from the prevalence of undetectable HCV RNA at 24 weeks after the beginning of combination therapy of interferon and ribavirin (75%, 82/109), of break though (30%, 11/37) and of HCV relapse rate (42%, 11/26); the expected SVR is 30% ($(82/109) \times (1 - (11/37)) \times (1 - (11/26)) \fallingdotseq 0.30$). In the same manner, the SVR ratio in 1H patients receiving 24-week treatment is expected to be 17% $(82/109) \times (1 - (35/45)) \fallingdotseq 0.17$. In 1H patients, 48-week treatment, 24-week combination treatment followed by interferon monotherapy for 24 weeks, may be the useful treatment which can be actually performed in Japan.

The relationship between the timing of HCV RNA disappearance and the SVR rate according to the duration of treatment was evaluated. SVR rates decreased with a delay in the timing of HCV RNA disappearance in patients receiving 24-week treatment; the negative prediction value at week 12 was 100%, that is, none of the patients who were positive for HCV RNA at week 12 had SVR. In spite of the small rate (16%), SVR was attained for patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment. Accordingly, treatment withdrawal should be offered to patients who remain HCV RNA-positive after 12 weeks of therapy if the patient cannot continue treatment for 48 weeks for reasons including side effects and social issues. The patients who were positive for HCV RNA at week 24 should stop treatment because additional interferon monotherapy for 24 weeks could not clear HCV RNA in all five patients who were positive for HCV RNA at week 24.

Pol et al. [24] have reported the synergistic effect of ribavirin and interferon in 343 patients with the genotype 1b. In the study, ribavirin was administered for 4, 6, 12 months in combination with interferon-α for 12 months. A 12-month course of ribavirin achieved significantly greater virological efficacy than 6 or 4 months at the end of the 12-month course of interferon-α (59, 49, and 29%), the same trend seen at the end of follow-up duration (43, 36, and 21%). These results indicate that the maximum efficacy can be obtained

when ribavirin is administered for 12 months in combination with interferon. In our study, the break through ratio was expected to decrease with the administration of ribavirin for 48 weeks. In fact, a patient to whom ribavirin was given again after the break through, achieved marked decrease of HCV RNA (data not shown). Thus, in some patients who were negative for HCV RNA during the combination treatment, the additional ribavirin can be essential for eradicating HCV RNA. Longer duration of combination therapy with interferon and ribavirin is also most effective for suppressing HCV RNA relapse after 24 weeks of therapy [22,23]. Therefore, we would like to emphasize that combination therapy of ribavirin and interferon for 48 weeks should be permitted even in Japan. At present, 24-week combination therapy followed by 24-week interferon monotherapy is thought to be the most useful therapy that the medical insurance can be applied in Japan for suppressing the relapse rate of HCV RNA, leading to SVR.

Acknowledgements

In addition to the study authors, the following institutions and physicians were participants in the Osaka Liver Disease Study Group (Digestive Disease Study Group of Osaka Renaissance): Osaka National Hospital, R. Sakamori; Osaka Rousai Hospital, N. Kurashige, O. Nishiyama, and S. Shinzaki; Osaka Kouseinenkin Hospital, M. Kurokawa and A. Uemura; National Osaka South Hospital, T. Oze and N. Tsuda; Kansai Rousai Hospital, T. Yoshio; Osaka Police Hospital, K. Koga; Osaka Prefectural Hospital, A. Arimitsu; and Osaka University Graduate School of Medicine, S. Yamaguchi, M. Miyazaki, H. Miyatake, I. Itose, S. Egawa, and T. Nishida.

This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan.

References

[1] Davis GL, Balart LA, Schiff ER, et al. Treatment of chronic hepatitis C with recombinant interferon alpha. A multicenter randomized controlled trial. N Engl J Med 1989;321:1501–6.

[2] Di Bisceglie AM, Martin P, Kassianides C, et al. Recombinant interferon alpha therapy for chronic hepatitis C. A randomized, double blind placebo-controlled trial. N Engl J Med 1989;321:1506–10.

[3] Hiramatsu N, Hayashi N, Kasahara A, et al. Improvement of liver fibrosis in chronic hepatitis C patients treated with natural interferon alpha. J Hepatol 1995;22:135–42.

[4] Kasahara A, Hayashi N, Mochizuki K, et al. Pretreatment viral load and response to interferon therapy for liver cirrhosis caused by hepatitis C virus: a multicenter controlled study. Hepatol Res 2000;16:124–38.

[5] Kakumu S, Yoshioka K, Wakita T, et al. A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology 1993;105:507–12.

[6] Brillanti S, Garson J, Foli M, et al. A pilot study of combination therapy with ribavirin plus interferon alpha for alpha-interferon resistant chronic hepatitis C. Gastroenterology 1994;107:812–7.

[7] Lai MY, Kao JH, Yang PM, et al. Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. Gastroenterology 1996;111:1307–12.

[8] Bondenheimer Jr HC, Lindsay KL, Davis GL, et al. Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. Hepatology 1997;26:473–7.

[9] McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alpha-2b and ribavirin as initial treatment of relapse of chronic hepatitis C. N Engl J Med 1998;339:1485–92.

[10] Poynard T, Marcellin P, Lee SS, et al. Randomised trial of interferon alpha-2b and ribavirin for 48 weeks or for 24 weeks versus interferon alfa-2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998;352:1426–32.

[11] Reichard O, Norkrans G, Fryden A, et al. Randomized double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. Lancet 1998;351:83–6.

[12] Davis GL, Esteban-Mur R, Rustgi V, et al. Interferon alpha-2b alone or in combination with ribavirin for the treatment or relapse of chronic hepatitis C. N Engl J Med 1998;339:1493–9.

[13] Consensus statement. EASL international consensus conference on hepatitis C. Paris, 26–28 February 1999. J Hepatol 1999;30:956–61.

[14] Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. Hepatology 2002;36:S121–7.

[15] Kasahara A, Hayashi N, Hiramatsu N, et al. Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis C: a multicenter randomized controlled trial. Hepatology 1995;21:291–7.

[16] Yuki N, Hayashi N, Kasahara A, et al. Pretreatment viral load and response to prolonged interferon-α course for chronic hepatitis C. J Hepatol 1995;22:457–63.

[17] Lau YN, Davis G, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 1993;341:1501–4.

[18] Shiratori Y, Kato N, Yokosuka O, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Gastroenterology 1997;113:558–66.

[19] Hagiwara H, Hayashi N, Mita E, et al. Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. Gastroenterology 1993;104:877–83.

[20] Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology 1994;19:1347–53.

[21] Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431–5.

[22] Poynard T, Marcellin P, Lee SS, et al. Randomized trial of interferon α2b plus ribavirin for 48 weeks or for 24 weeks versus ineterferon α2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C. Lancet 1998;352:1426–32.

[23] Poynard T, McHutchison J, Goodman Z, et al. Is an "A la Carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? Hepatology 2000;31:211–8.

[24] Pol S, Nalpas B, Bourliere M, et al. Combination of Ribavirin and interferon-alfa surpasses high doses of interferon-alfa alone in patients with genotype-1b-related chronic hepatitis C. Hepatology 2000;31:1338–44.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 321 (2004) 722–727
BBRC
www.elsevier.com/locate/ybbrc

Involvement of p38 signaling pathway in interferon-α-mediated antiviral activity toward hepatitis C virus

Hisashi Ishida [a], Kazuyoshi Ohkawa [a], Atsushi Hosui [b], Naoki Hiramatsu [a], Tatsuya Kanto [c], Keiji Ueda [d], Tetsuo Takehara [a], Norio Hayashi [a,*]

[a] *Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*
[c] *Department of Dendritic Cell Biology and Clinical Application, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*
[d] *Department of Microbiology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*

Received 12 March 2004

Abstract

We studied the involvement of the p38 signaling pathway in the interferon (IFN)-α-mediated antiviral activity toward hepatitis C virus (HCV) using HCV subgenomic replicon cells. When the cells were treated with IFN-α in the presence of p38 inhibitor, the suppressive effect of IFN-α on replicon RNA was reduced. Inhibition of p38 had almost no influence on phosphorylation of signal transducer and activator transcription factor 1 (STAT1) and interferon stimulatory response element-dependent gene expression after IFN-α treatment. This indicates that the anti-HCV activity through p38 may be independent of the Janus kinase -STAT pathway. Treatment with the inhibitor of the mitogen-activated protein kinase-activated protein kinase 2 (MK2) showed the same level of reduction in the IFN-α-mediated anti-HCV activity as that with the p38 inhibitor. Thus, MK2 may also be responsible for the anti-HCV activity through p38. In conclusion, the p38-MK2 signaling pathway may be substantially involved in the IFN-α-mediated anti-HCV activity.

Keywords: Hepatitis C virus; Subgenomic replicon; Interferon-α; p38 signaling pathway; Mitogen-activated protein kinase-activated protein kinase 2; Replication; Antiviral activity

Hepatitis C virus (HCV) often causes persistent infection, leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1,2]. Studies on the replicative manner of HCV have been hampered due to lack of an animal model or a tissue culture system sustaining efficient viral replication. Recently, the HCV subgenomic replicon system was developed by Lohmann et al. [3]. The replicon is a bicistronic construct composed of the HCV internal ribosome entry site (IRES), the neomycin phosphotransferase gene (Neo^r), the IRES of encephalomyocarditis virus (EMCV), the HCV NS proteins (NS3 through NS5B), and the HCV 3′X region. Human hepatoma-derived Huh-7 cells [4], which are transfected with in vitro synthesized replicon RNA and selected by G418, are capable of supporting the replication of replicon RNA. This system currently becomes a new tool to investigate the HCV replication and pharmacological mechanisms of anti-HCV drugs.

Interferon (IFN)-α has been widely used as an effective antiviral agent for persistent HCV infection [5]. However, approximately 40% of patients with chronic hepatitis C did not reveal the sustained eradication of serum HCV RNA even by the most effective combination therapy of PEG-IFN-α with ribavirin [6]. To improve the efficacy of IFN-α-based therapy, more detailed mechanisms,

* Corresponding author. Fax: +81-6-6879-3449.
E-mail address: hayashin@moltx.med.osaka-u.ac.jp (N. Hayashi).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.015

through which the IFN-α-mediated antiviral activity is exerted in HCV-infected hepatocytes, should be clarified. It is well known that the Janus kinase (JAK)-signal transducer and activator transcription factor (STAT) pathway plays a critical role in the generation of IFN-α-mediated signal transduction and transcriptional activation [7]. In response to IFN-α stimulation, JAKs and STATs are phosphorylated, followed by the complex formation of IFN-stimulated gene factor (ISGF)-3 composed by STAT1, STAT2, and the IFN regulatory factor-9. Then, ISGF-3 translocates to the nucleus, binds to the IFN-stimulated response element (ISRE), and triggers expression of a number of IFN-stimulated genes (ISGs). Antiviral activity of IFN-α is believed to be exhibited by gene products of ISGs, including the double-stranded RNA-activated protein kinase (PKR), 2′–5′ oligoadenylate synthetase (OAS), and Mx. Recent studies demonstrated that IFN-α activates other signal-transducing molecules, such as p38 [8], extracellular-regulated kinase (ERK) [9], phosphatidylinositol 3 kinase (PI3-kinase) [10], and protein kinase C (PKC)-δ [11]. However, it has not been fully elucidated whether these molecules are involved in the antiviral activity of IFN-α.

To clarify this, we studied the IFN-α-mediated anti-HCV activity using the HCV subgenomic replicon cells. The involvement of various signal transducing molecules in the anti-HCV activity was investigated by treating the cells with IFN-α and specific inhibitors.

Materials and methods

Cells and transfection. Huh-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glucose (1 mg/ml), penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Plasmid pNNeo/3-5B, which was a kind gift of Dr. Stanley M. Lemon (Department of Microbiology and Immunology, University of Texas Medical Branch) [12], contained the sequence of HCV subgenomic replicon, which was originated from the HCV-N strain (GenBank Accession No. AF139594). Plasmid pNNeo/3-5B(SI) was constructed from pNNeo/3-5B by introducing the adaptive mutation S2005I [12] by means of site-directed mutagenesis. After pNNeo/3-5B(SI) was linearized with *Xba*I, HCV replicon RNA was prepared using the Megascript T7 kit (Ambion). Then, Huh-7 cells were transfected with the replicon RNA by electroporation, followed by the culture in the medium containing 0.8 mg/ml G418 for 3 weeks. Subsequently, individual G418-resistant colonies were picked up, and one of them (designated Huh-repS2) was used for further experiments.

Antibodies and specific inhibitors. The p38 inhibitors (SB203580 and PD169316) and the PI3-kinase inhibitors (LY294002 and Wortmannin) were purchased from Calbiochem. The JAK-specific inhibitor, 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f] isoquinolin-7-one [13], also came from Calbiochem. The inhibitor of the mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2, MK2) (Calbiochem) is a synthetic 13-residue peptide (KKKALNRQLGVAA) corresponding to the phosphorylation site of HSP27, one of the known substrates of MK2. This peptide has been demonstrated to inhibit MK2 competitively with the substrate peptide [14]. Antibodies against whole STAT1, and phosphorylated STAT1 at Tyr^{701} and Ser^{727} residues (pY-STAT1 and pS-STAT1) were from Upstate Biotechnology. Antibodies against whole p38, phosphorylated p38 at Thr^{180}/Tyr^{182} (p-p38), whole MK2, and phosphorylated MK2 at Thr^{334} (p-MK2) were purchased from Cell Signaling Technology. An antibody against PKR was obtained from Santa Cruz Biotechnology. An antibody against NS5A was kindly provided by Dr. Y. Matsuura (Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University), and an antibody against NS3 was purchased from East Coast Biologics. Natural IFN-α was provided by Sumitomo Pharmaceuticals.

Detection of HCV replicon RNA by PCR. About 2×10^5 of Huh-repS2 cells were seeded in a 6-well culture dish and treated with various inhibitors of signal-transducing molecules for 1 h prior to the addition of IFN-α. After IFN-α treatment, total cellular RNA was extracted from the cells with the ISOGEN reagent (Nippon Gene) based on the guanidine-isothiocyanate method. Reverse transcription (RT) was performed with 1 μg of the RNA sample using a primer, 5′-GG AAATGGCCTATTGGCCTGGAGT-3′ (nt 9427–9404) and the mutated Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO). The cDNA was subsequently amplified using the KOD polymerase (TOYOBO) and a set of primers, 5′-TCGCACG GGCTGCGTGGGAAACAG-3′ (nt 8788–8811) and 5′-GTTTAGC TCCCCGTTCATCGATTGG-3′ (nt 9403–9379) by 29 PCR cycles involving denaturation at 94 °C for 15 s, annealing at 62 °C for 30 s, and extension at 68 °C for 1 min. The PCR products were subjected to the agarose gel electrophoresis and ethidium bromide staining. The nucleotide numbering is according to the HCV-N strain. As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was also examined. After cDNA synthesis with the oligo$(dT)_{20}$ primer (TOYOBO), the PCR was conducted using a commercially available set of primers to detect the G3PDH mRNA (TOYOBO).

Immunoblotting. The Huh-repS2 cells after IFN-α treatment were lysed in a RIPA buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 50 mM sodium fluoride in phosphate-buffered saline (pH 7.4). Fifty micrograms protein/lane was separated on the SDS–PAGE and transferred onto the nitrocellulose membrane (Hybond-P, Amersham–Pharmacia Biotech). Next, the membrane was incubated with the appropriate concentration of the specific antibody, followed by incubation with the horseradish peroxidase-conjugated second antibody. Then, the signals were developed by chemiluminescence (Supersignal, Pierce).

Reporter gene assay. The reporter plasmid pISRELuci, which was a kind gift of Dr. T. Hirano (Division of Molecular Oncology, Osaka University Graduate School of Medicine), carried the three tandem repeats of ISRE upstream of the minimal promoter and the firefly luciferase gene. pRLtk (Clontech) was the seapansy luciferase-expressing plasmid. For the assay, 3×10^5 of Huh-repS2 cells were seeded in a 6-well culture dish and transfected with 1 μg pISRELuci and 0.1 μg pRLtk using Lipofectin (Invitrogen). The cells were cultured for 2 days after transfection. After preincubation with the p38 inhibitors or the JAK inhibitor for 1 h, IFN-α was added at a concentration of 100 U/ml. After subsequent incubation for 6 h, the cells were lysed and subjected to the dual luciferase assay using luminometer (Lumat LB9507, EG&G Berthold). The relative light unit of the sample without IFN-α stimulation was regarded as 1, and the fold activity of each sample was determined. The assay was done three times and the mean value was calculated.

Results

HCV replicon RNA is autonomously replicated in replicon cells and susceptible to IFN-α

Huh-7 cells were transfected with in vitro transcribed replicon RNA, and four independent clones were obtained. One of them (Huh-repS2) was subjected to

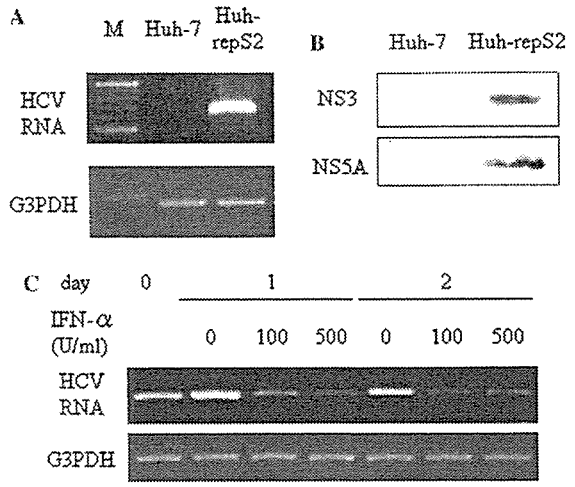

Fig. 1. Replication and susceptibility to IFN-α of HCV replicon RNA in Huh-repS2 cells. (A) HCV replicon RNA was detected by RT-PCR in Huh-7 and Huh-repS2 cells (upper panel). M, size marker. (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control. (B) Expression of HCV proteins NS3 and NS5A was examined by Western blot analyses in Huh-7 and Huh-repS2 cells. (C) Huh-repS2 cells were treated with 100 and 500 U/ml IFN-α, or left untreated. Then, the replicon RNA was detected by RT-PCR at days 0, 1, and 2 (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control.

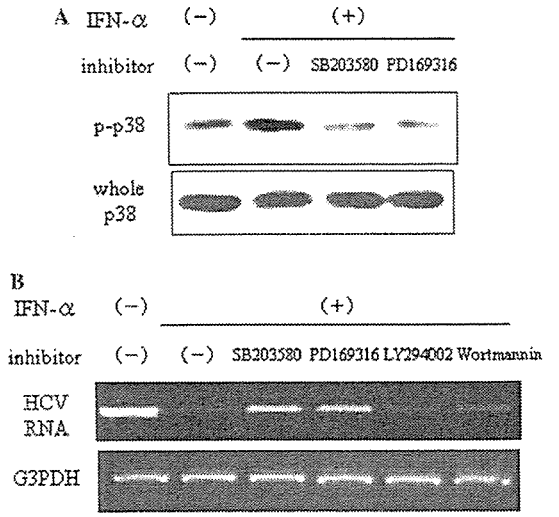

Fig. 2. Involvement of p38 in IFN-α-mediated anti-HCV activity in Huh-repS2 cells. (A) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 h, or left untreated in the presence or absence of SB203580 (10 μM) and PD169316 (10 μM). Then, the levels of p-p38 and whole p38 were examined by Western blot analyses. (B) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 days, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), LY294002 (10 μM), and Wortmannin (100 nM). Then, the replicon RNA was detected by RT-PCR (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control.

further experiments. The replicon RNA and HCV proteins were first detected in Huh-repS2 cells. As shown in Fig. 1A, Huh-repS2 cells specifically included the 3′ sequence of the replicon RNA, because the primers used in the PCR were located just upstream of the polyU stretch of HCV RNA. As for expression of NS3 and NS5A (Fig. 1B), these proteins were detected in the Huh-repS2 cells, but not in the parental Huh-7 cells. Next, the susceptibility of the Huh-repS2 cells to IFN-α was investigated. As shown in Fig. 1C, treatment with 100 U/ml IFN-α for 2 days was enough to suppress the replicon RNA. The Huh-repS2 cells could not survive after 1 week in culture in the presence of both G418 and IFN-α (data not shown), indicating that Neo^r was not constitutively expressed in the cells. According to this, it was denied that the replicon construct was integrated into the host genome. In the Huh-repS2 cells, the HCV replicon RNA was autonomously replicated and susceptible to IFN-α.

p38 is involved in the anti-HCV activity of IFN-α

There are several reports indicating that IFN-α activates not only the JAK-STAT pathway but also other signaling pathways [8–11]. To investigate the involvement of pathways other than JAK-STAT in the anti-HCV activity under IFN-α stimulation, p38 inhibitors (SB203580 and PD169316) or PI3-kinase inhibitors (LY294002 and Wortmannin) were added to the Huh-repS2 cells prior to IFN-α treatment. Fig. 2A represents the expression level of p-p38, as a marker of p38 activity, in the Huh-repS2 cells treated with IFN-α in the presence or absence of p38 inhibitors. Phosphorylation of p38 was induced by IFN-α stimulation, as reported previously in other cells [8,15,16], and the induction was diminished by pretreatment of SB203580 and PD169316. On the other hand, the whole p38 level was not affected by IFN-α stimulation. When the effect of p38 and PI3-kinase inhibitors on expression of replicon RNA was examined in the IFN-α-treated Huh-repS2 cells (Fig. 2B), the anti-HCV effect of IFN-α was considerably reduced (but not completely abolished) by SB203580 and PD169316. In contrast, PI3-kinase inhibitors, LY294002 and Wortmannin, did not affect the suppressive effect on expression of replicon RNA by IFN-α. The similar experiments using the PKC-δ inhibitor rottlerin and the ERK inhibitor PD98059 were also carried out. The level of the replicon RNA was not changed by pretreatment of these inhibitors in the IFN-α-treated Huh-repS2 cells (data not shown). These results indicate that activation of p38 may play a substantial role in the generation of the anti-HCV activity by IFN-α. On the other hand, PI3-kinase, PKC-δ, and ERK were not involved in the antiviral activity of IFN-α in the HCV replicon cells.

IFN-α-mediated anti-HCV activity through p38 is independent of JAK-STAT signaling pathway

We further studied the anti-HCV activity of IFN-α through p38 in relation to the JAK-STAT pathway.

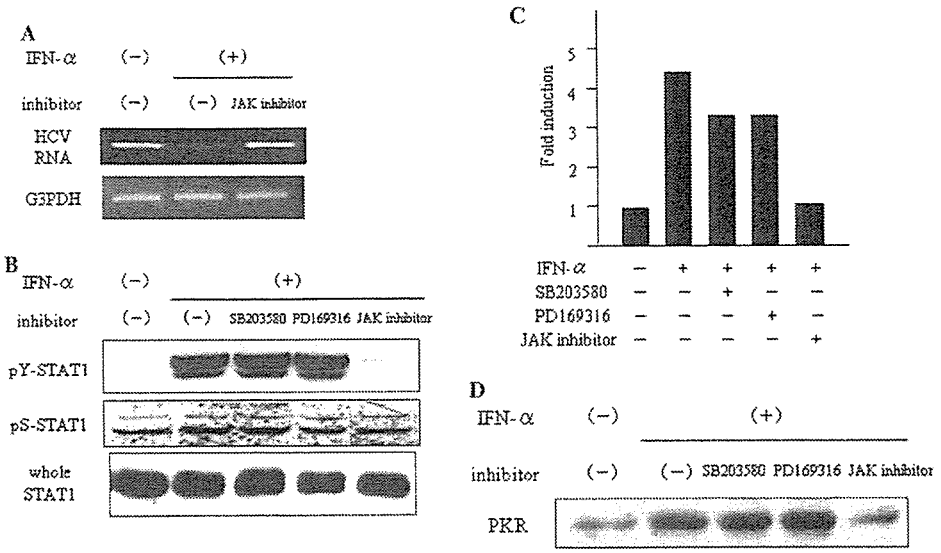

Fig. 3. Relationship of p38 with STAT1 phosphorylation and ISRE-dependent transcription in IFN-α-treated Huh-repS2 cells. (A) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 days, or left untreated in the presence or absence of JAK inhibitor (1 μM). Then, the replicon RNA was detected by RT-PCR (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control. (B) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 h, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), and JAK inhibitor (1 μM). Then, the levels of pY-STAT1, pS-STAT1, and the whole STAT1 were examined by Western blot analyses. (C) Huh-repS2 cells were cotransfected with pISRELuci and pRLtk. Two days after transfection, the cells were stimulated with IFN-α (100 U/ml) for 6 h, or left unstimulated with or without pretreatment of SB203580 (10 μM), PD169316 (10 μM), and JAK inhibitor (1 μM). Then, the cells were subjected to the dual luciferase assay. The assay was done three times and the mean value was calculated. (D) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 6 h, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), and JAK inhibitor (1 μM). Then, expression of PKR was examined by Western blot analysis.

Using the Huh-repS2 cells treated with IFN-α, the influence of the JAK inhibitor on expression of the replicon RNA was examined. As shown in Fig. 3A, the JAK inhibitor completely abolished the anti-HCV effect of IFN-α, indicating that JAK-STAT pathway is essential for the antiviral activity of IFN-α to be exhibited. STAT1 has been reported to possess two activating phosphorylation sites, Tyr^{701} and Ser^{727}. It has been shown that, under IFN-α stimulation, JAKs undergo the Tyr^{701} phosphorylation [17], whereas the Ser^{727} is phosphorylated by other kinases [18]. Especially, p38 has been suggested to be a candidate of the STAT1 kinase at the Ser^{727} [15]. Therefore, the levels of pY-STAT1 and pS-STAT1 were examined in the IFN-α-treated Huh-repS2 cells in the presence of p38 and JAK inhibitors (Fig. 3B). The strong induction of pY-STAT1 was observed by IFN-α treatment. This induction was prevented by the JAK inhibitor but not by the p38 inhibitors SB203580 and PD169316. In contrast, the pS-STAT1 was observed irrespective of IFN-α treatment, and its level was not influenced by both p38 and JAK inhibitors. Also, the whole STAT1 level was not affected by treatment with IFN-α and various inhibitors. Thus, contrary to the previous report [15], p38 did not participate in the STAT1 phosphorylation in the Hep-repS2 cells under IFN-α stimulation. As for the ISRE-dependent transcription activity, as determined by the reporter gene assay (Fig. 3C), JAK inhibitor completely decreased the activity to the baseline, whereas p38 inhibitor had a minimal influence on it. When expression of PKR, a member of ISGs, was also detected in the Hep-repS2 cells (Fig. 3D), it was induced by IFN-α stimulation. The induction of PKR expression by IFN-α was diminished by pretreatment of the JAK inhibitor but not by that of the p38 inhibitor, as was the case for the reporter gene assay. According to these results, the blockade of p38 had only a minimal effect on the JAK-STAT pathway under IFN-α stimulation. The IFN-α-mediated anti-HCV activity through p38 was almost independent of the JAK-STAT pathway.

p38-MK2 pathway is responsible for the anti-HCV activity of IFN-α

It has been demonstrated that MK2 retains in the nucleus interacting with p38, and that activation of p38 causes MK2 phosphorylation [19]. To further examine the downstream event of p38 in the anti-HCV efficacy of IFN-α, we focused on MK2 among the proteins identified as substrates of p38. The time course analyses in the expression levels of p-p38, p-MK2, whole p38, and whole MK2 were performed in Huh-repS2 cells after IFN-α stimulation. As shown in Fig. 4A, the boost of the p-p38 level was observed at 15 min after stimulation

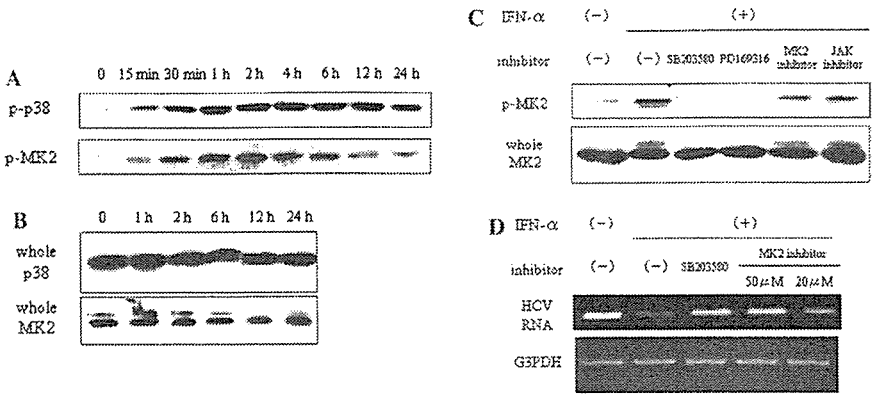

Fig. 4. Involvement of MK2 in IFN-α-mediated anti-HCV activity in Huh-repS2 cells. (A) Huh-repS2 cells were treated with IFN-α (100 U/ml), and the levels of p-p38 and p-MK2 were examined by Western blot analyses before and after the treatment. (B) Huh-repS2 cells were treated with IFN-α (100 U/ml), and the levels of whole p38 and MK2 were examined by Western blot analyses before and after the treatment. (C) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 4 h, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), MK2 inhibitor (50 μM), and JAK inhibitor (1 μM). Then, the levels of p-MK2 and the whole MK2 were examined by Western blot analyses. (D) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 days, or left untreated in the presence or absence of SB203580 (10 μM) and the MK2 inhibitor (50 μM and 20 μM). Then, the replicon RNA was detected by RT-PCR (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control.

and continued thereafter. Expression of p-MK2, as a marker of MK2 activity, followed this pattern, but its level showed attenuation after 12 h. It is speculated that this attenuation of p-MK2 might be due to the induction of some MK2 phosphatase(s). On the other hand, the levels of whole p38 and MK2 were unchanged by IFN-α stimulation (Fig. 4B). Next, the effects of p38, JAK, and MK2 inhibitors on the p-MK2 level were investigated in the IFN-α-treated Huh-repS2 cells (Fig. 4C). The induction of p-MK2 after IFN-α treatment was blocked by the p38 inhibitors SB203580 and PD169316, but not by the JAK inhibitor. The MK2 inhibitor did not also influence the level of p-MK2, because it was a peptide competitively inhibiting MK2 activity [14]. The whole MK2 level was not affected by treatment with IFN-α and various inhibitors. These findings indicate that MK2 may function as a downstream kinase of p38, apart from the JAK-STAT pathway. The effect of the MK2 inhibitor on expression of replicon RNA was compared with that of the p38 inhibitor in the IFN-α-treated Huh-repS2 cells. As shown in Fig. 4D, the addition of MK2 inhibitor (50 μM) showed the same level of reduction in the IFN-α-mediated anti-HCV activity as that of the p38 inhibitor SB203580. According to these, the p38-MK2 pathway may be responsible for the anti-HCV activity of IFN-α in the Huh-repS2 cells.

Discussion

Under IFN-α stimulation, the signal from the specific receptor is transmitted by the JAK-STAT pathway, and the antiviral effect is believed to be achieved by a number of gene products of ISGs including PKR, OAS, and Mx [20]. It has also been demonstrated that several signal transducing molecules, such as p38, ERK, PI3K, and PKC-δ, are activated in response to IFN-α [8–11]. The aim of this study was to investigate the contribution of pathways other than JAK-STAT to the anti-HCV activity by IFN-α. It was carried out by adding various inhibitors of the signal transducing molecules to the HCV replicon Huh-repS2 cells treated with IFN-α. Among them, the activation of p38 was found to play a role in the generation of IFN-α-mediated anti-HCV activity.

p38 belongs to a mitogen-activated protein kinase (MAPK) family and is activated by various cellular stresses and cytokines [21]. A few investigators have so far suggested that the p38 activation may be an upstream event of IFN-α-mediated gene transcription driven by ISRE. Goh et al. [15] showed that p38 underwent the STAT1 phosphorylation at Ser^{727}, resulting in the ISRE-dependent transcriptional activation. It has also been revealed by Li et al. [22] that the p38 activation did not affect the STAT1 phosphorylation and ISGF3 formation but was essential for ISRE-dependent transcription. In this study, we examined the relationship of p38 with the STAT1 phosphorylation and the ISRE-dependent transcription activity in the IFN-α-treated cells. In contrast to these previous reports, the level of the STAT1 phosphorylation was not affected by p38. Also, p38 had only a minimal effect on the ISRE-dependent transcription activity. These results indicate that the anti-HCV activity of IFN-α through p38 may be exhibited independent of the JAK-STAT pathway and the ISRE-dependent transcription at least in the Huh-repS2 cells.

As for functional relevance of p38 and JAK-STAT pathways to the anti-HCV efficacy of IFN-α, the activation of JAK-STAT and subsequently occurring transcriptional activation is primarily required, because the JAK inhibitor completely blocked the anti-HCV

activity of IFN-α in the Huh-repS2 cells. However, the activation of p38 may also play a substantial role in the generation of the anti-HCV effect of IFN-α. Our findings suggest that the two independent signaling pathways, JAK-STAT and p38, may be important for the anti-HCV activity of IFN-α to be fully exerted.

In the present study, we also addressed the role of MK2, one of the substrates of p38 [20], in the exhibition of anti-HCV activity by IFN-α. It was found that MK2 worked as a downstream kinase of p38 under IFN-α stimulation and was responsible for the IFN-α-mediated antiviral activity in the Huh-repS2 cells. Very recently, Li et al. [22] reported that the antiviral response by IFN-α was decreased in the MK2-deficient mouse embryo cells with the EMCV infection. Our result agreed with this report with respect to the functional importance of MK2 in the antiviral efficacy of IFN-α.

In conclusion, we showed that the activation of the p38-MK2 pathway may be substantially involved in the generation of anti-HCV activity by IFN-α. Further studies are required to clarify the mechanisms downstream of MK2 in the antiviral effect of IFN-α.

References

[1] L.B. Seeff, Natural history of hepatitis C, Hepatology 26 (1997) 21S–28S.

[2] K. Kiyosawa, T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R.H. Purcell, H.J. Alter, Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus, Hepatology 12 (1990) 671–675.

[3] V. Lohmann, F. Körner, J.-O. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[4] H. Nakabayashi, K. Taketa, K. Miyano, T. Yamane, J. Sato, Growth of human hepatoma cells lines with differentiated functions in chemically defined medium, Cancer Res. 42 (1982) 3858–3863.

[5] H. Hagiwara, N. Hayashi, E. Mita, T. Takehara, A. Kasahara, H. Fusamoto, T. Kamada, Quantitative analysis of hepatitis C virus RNA in serum during interferon alpha therapy, Gastroenterology 104 (1993) 877–883.

[6] M.W. Fried, M.L. Shiffman, K.R. Reddy, C. Smith, G. Marinos, F.L. Goncalcs Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, J. Yu, Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection, N. Engl. J. Med. 347 (2002) 975–982.

[7] G.R. Stark, I.M. Kerr, B.R.G. Williams, R.H. Silverman, R.D. Schreiber, How cells respond to interferons, Annu. Rev. Biochem. 67 (1998) 227–264.

[8] S. Uddin, B. Majchrzak, J. Woodson, P. Arunkumar, Y. Alsayed, R. Pine, P.R. Young, E.N. Fish, L.C. Platanias, Activation of the p38 mitogen-activated protein kinase by type I interferons, J. Biol. Chem. 274 (1999) 30127–30131.

[9] T. Arora, G. Floyd-Smith, M.J. Espy, D.F. Jelinek, Dissociation between IFN-alpha-induced anti-viral and growth signaling pathways, J. Immunol. 162 (1999) 3289–3297.

[10] S. Uddin, E.N. Fish, D.A. Sher, C. Gardziola, M.F. White, L.C. Platanias, Activation of the phosphatidylinositol 3-kinase serine kinase by IFN-alpha, J. Immunol. 158 (1997) 2390–2397.

[11] S. Uddin, A. Sassano, D.K. Deb, A. Verma, B. Majchrzak, A. Rahman, A.B. Malik, E.N. Fish, L.C. Platanias, Protein kinase C-δ (PKC-δ) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727, J. Biol. Chem. 277 (2002) 14408–14416.

[12] M. Ikeda, M. Yi, K. Li, S.M. Lemon, Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells, J. Virol. 76 (2002) 2997–3006.

[13] J.E. Thompson, R.M. Cubbon, R.T. Cummings, L.S. Wicker, R. Frankshun, B.R. Cunningham, P.M. Cameron, P.T. Meinke, N. Liverton, Y. Weng, J.A. DeMartino, Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor, Bioorg. Med. Chem. Lett. 12 (2002) 1219–1223.

[14] K. Hayess, R. Benndorf, Effect of protein kinase inhibitors on activity of mammalian small heat-shock protein (HSP25) kinase, Biochem. Pharmacol. 53 (1997) 1239–1247.

[15] K.C. Goh, S.J. Haque, B.R.G. Williams, p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons, EMBO J. 20 (1999) 5601–5608.

[16] S. Uddin, F. Lekmine, N. Sharma, B. Majchrzak, I. Mayer, P.R. Young, G.M. Bokoch, E.N. Fish, L.C. Platanias, The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon α-dependent transcriptional activation but not serine phosphorylation of Stat proteins, J. Biol. Chem. 275 (2000) 27634–27640.

[17] K. Shuai, A. Ziemiecki, A.F. Wilks, A.G. Harpur, H.B. Sadowski, M.Z. Gilman, J.E. Darnell, Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins, Nature 366 (1993) 580–583.

[18] X. Zhu, Z. Wen, L.Z. Xu, J.E. Darnell, Stat1 serine phosphorylation occurs independently of tyrosine phosphorylation and requires an activated Jak2 kinase, Mol. Cell. Biol. 17 (1997) 6618–6623.

[19] A. Kotlyarov, M. Gaestel, Is MK2 (mitogen-activated protein kinase-activated protein kinase 2) the key for understanding post-transcriptional regulation of gene expression?, Biochem. Soc. Trans. 30 (2002) 959–963.

[20] C.E. Samuel, Antiviral actions of interferons, Clin. Microbiol. Rev. 14 (2001) 778–809.

[21] L.C. Platanias, The p38 mitogen-activated protein kinase pathway and its role in interferon signaling, Pharmacol. Ther. 98 (2003) 129–142.

[22] Y. Li, A. Sassano, B. Majchrzak, D.K. Deb, D.E. Levy, M. Gaestel, A.R. Nebreda, E.N. Fish, L.C. Platanias, Role of p38α map kinase in type I interferon signaling, J. Biol. Chem. 279 (2004) 970–979.

ELSEVIER

Journal of Hepatology 41 (2004) 637–643

Journal of Hepatology

www.elsevier.com/locate/jhep

Ninjurin1 increases p21 expression and induces cellular senescence in human hepatoma cells

Takashi Toyama[1,*], Yutaka Sasaki[2], Masayoshi Horimoto[1], Kenya Iyoda[2], Takayuki Yakushijin[2], Kazuyoshi Ohkawa[1], Tetsuo Takehara[2], Akinori Kasahara[3], Toshiyuki Araki[4], Masatsugu Hori[1], Norio Hayashi[2]

[1]*Department of Internal Medicine and Therapeutics, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[2]*Department of Molecular Therapeutics, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[3]*Department of General Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[4]*Department of Pathology and Immunology and Internal Medicine, Washington University Medical School, St. Louis, MO 63110, USA*

***Background/Aims*: Ninjurin1 is a novel adhesion molecule that has a role in promoting nerve regeneration. Although ninjurin1 is ubiquitously expressed in various human tissues, including the liver, the biologic functions of ninjurin1 in tissues other than the nervous system remain unknown. The aim of this study was to investigate the function of ninjurin1 in hepatocytes.**

***Methods*: The effect of ninjurin1 overexpression was examined in Huh-7 hepatoma cells. Ninjurin1 expression was examined by Western blot in human hepatocellular carcinoma tissues as well as their adjacent liver tissues.**

***Results*: Ninjurin1-overexpressing clones exhibited strong growth inhibition due to G1 cell cycle arrest, which is associated with a posttranscriptional increase in $p21^{WAF1/Cip1}$, a decrease of cyclin-dependent kinase 2 activity and the hypophosphorylation of Rb. The ninjurin1-overexpressing clones had increased senescence-associated β-galactosidase activity and autofluorescent pigment, characteristic features of cellular senescence. The levels of ninjurin1 expression were higher in hepatocellular carcinoma tissues than those in adjacent liver tissues.**

***Conclusions*: The present study provides the first evidence that ninjurin1 is able to induce the senescence program. Ninjurin1 may be involved in the regulation of cellular senescence in the liver during carcinogenesis.**

***Keywords*: Ninjurin1; Cellular senescence; p21; G1 Arrest; Carcinogenesis; Hepatocellular carcinoma**

1. Introduction

Ninjurin1 (nerve injury-induced protein 1) is a novel adhesion molecule first isolated as a gene induced in Schwann cells after nerve injury [1]. Ninjurin1 is located on the cell surface of Schwann cells and neurons, and has an important role in neurite regeneration via homophilic interactions between neuronal axons and Schwann cells [2]. In addition, ninjurin1 is ubiquitously expressed in various human tissues originating from epithelial cells (e.g. liver, kidney, thymus, adrenal gland) [3]. However, the biological relevance of ninjurin1 on tissues other than the nervous system remains unknown.

In the present study, we explored the biologic and biochemical consequences of ninjurin1 overexpression in a human hepatoma cell line. Ninjurin1 expression in Huh-7 hepatoma cells induced p21 elevation and G1 cell cycle arrest. Furthermore, ninjurin1-overexpressing cells exhibited the characteristic features of cellular senescence: increased senescence-associated (SA) β-galactosidase activity and the presence of autofluorescent pigment. In addition, the levels of ninjurin1 expression were higher in human hepatocellular carcinoma (HCC) tissues than in their

Received 15 January 2004; received in revised form 9 June 2004; accepted 25 June 2004; available online 20 July 2004

* Corresponding author. Address: Department of Molecular Therapeutics, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: +81-6-6879-3636; fax: +81-6-6879-3639.

E-mail address: toyamat@moltx.med.osaka-u.ac.jp (T. Toyama).

0168-8278/$30.00
doi:10.1016/j.jhep.2004.06.027

adjacent liver tissues. The present study suggests a possible role of ninjurin1 in regulating cellular senescence pathologically arising in the liver.

2. Materials and methods

2.1. Antibodies

Anti-human ninjurin1 mouse polyclonal antibody and the anti-pRb antibody (14001A) were obtained from Pharmingen/Transduction Laboratories (San Diego, CA). Polyclonal antibodies for p21 (C-19), p27 (F-8), cyclinD1 (M-20), cyclinE (M-20), cyclinA (H-432), cyclin-dependent-kinase2 (CDK2) (H-298), CDK4 (C-22), and CDK6 (C-21) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Establishment of ninjurin1-transfected Huh-7 cells

Huh-7 cells, a human hepatoma cell line, were obtained from the Japanese Cancer Research Resource Bank Cell Bank Center (Tokyo, Japan). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented fetal calf serum (FCS) and antibiotics. Human ninjurin1 cDNA was excised from a pT7T3 plasmid [2] and inserted into a cloning site of pcDNA3.1 plasmid (Invitrogen, Groningen, the Netherlands). The construction was verified by sequencing (ABI Prism310; Perkin–Elmer, Foster City, CA). The ninjurin1 expression plasmid was transfected into Huh-7 cells using a Lipofectin reagent (Gibco BRL/Life Technologies, Grand Island, NY). Selection was performed via the addition of 100 μg/ml Zeosin (Invitrogen, Co. Ltd., Japan). Using this method, the ninjurin1-transfected cells generated much fewer Zeosin-resistant colonies than cells transfected with vector alone (mock clones). In addition, because Zeosin-resistant clones grew slowly, it took over a month to pick the slowest growing colony. After the Zeosin-resistant clones were screened for ninjurin1 expression by immunoblotting, four positive single clones were selected. Two positive clones (Nin1 and Nin2) and mock clones were used for the experiments, but similar results were obtained using the other two clones, and the magnitude of the effects observed were proportional to the amount of ninjurin1 expressed by each clone.

2.3. Growth curves

The control cells and ninjurin1-overexpressing clones were seeded at a density of 1×10^4 cells/35 mm-diameter dish. At 1, 2, 3, and 4 days after plating, these cells were removed from the culture plate by short exposure to trypsin and EDTA (GIBCO-BRL/Life Technologies). The total cell number was determined by trypan blue staining via microscopy.

2.4. Cell-cycle analysis

Exponentially growing cultures of the control cells and ninjurin1-overexpressing clones were trypsinized and collected. Cell pellets were fixed in 70% ethanol and stored at −20 °C. On the day of the assay, the fixed cells were collected by centrifugation, and the pellets were resuspended in PBS containing 0.2 mg/ml propidium iodide, and 1 mg/ml RNase. After 30 min incubation, the cell suspension was filtered through a 60 μM Spectra mesh filter and analyzed with a FACS Caliber flow cytometer using CellQuest software (Becton Dickinson Co. Chino Hills, CA). The percentages of cells in different phases of the cell cycle were determined with the ModFit 2.0 computer program (Verity Software House, Inc., Topsham, ME).

2.5. Immunocytochemical detection of ninjurin1

Huh-7 cells were fixed with paraformaldehyde and permeabilized with 0.05% Triton X-100. After blocking with Block Ace (Snow Brand Milk Product Co., Sapporo, Japan) containing 10% normal goat serum, cells were incubated for 1 h with anti-ninjurin1 polyclonal antibody. The cells were further incubated with Alexa® 488 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR). Images were acquired by confocal laser microscopy using a Zeiss LSM510 (Carl Zeiss Co., Ltd., Germany).

2.6. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)

Cells were fixed with neutral buffered formaldehyde on day 3 after plating. Apoptotic cells were detected using an In situ Apoptosis Detection Kit (WAKO, Tokyo, Japan) according to the manufacturer's instructions. The percentage of TUNEL-positive cells relative to total cells was determined by counting 100–300 cells in 10 randomly chosen fields per cover slide using a Nikon Microphot-FXA (Nikon, Tokyo, Japan).

2.7. Western blot analysis

Whole cell lysates were prepared in Triton lysis buffer. Protein concentration was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL). Identical amounts of protein were separated on an acrylamide gel, and transferred to Hybond-P membranes (Amersham Pharmacia Biotech Co. Ltd., Buckinghamshire, UK). The membrane was blocked and then incubated with each antibody. The membrane was further incubated with the horseradish peroxidase-conjugated immunoglobulin. Detection was performed using the enhanced chemiluminescence (ECL) assay protocol (Pierce Chemical). The signal intensities were analyzed using an NIH image program (National Institutes of Health, Bethesda, MD).

2.8. RNA isolation and northern blot analysis

Total cellular RNA was prepared using Trizol reagent (GIBCO-BRL/Life Technologies) in accordance with the manufacturer's instructions. For Northern blot analysis, 20 μg of RNA was separated on 1.0% agarose formaldehyde gels and transferred onto Hybond-N membranes (Amersham Pharmacia Biotech Co. Ltd.), and crosslinked to the membrane with a GS GENE Linker (BioRad Laboratories, Hercules, CA). mRNAs were detected by hybridization with random-primer-labeled probes (Amersham Pharmacia Biotech Co. Ltd.).

2.9. Kinase assay

In vitro phosphorylation of Rb by CDKs was detected by the phosphoFind™ (Boston Biologicals, Inc, Bedford, MA) system following the manufacturer's instructions. Whole cell lysates were prepared in Triton lysis buffer, with 20 μg of total cell protein per sample being precleared with 15 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech Co. Ltd.). Immunoprecipitation of CDKs was performed with 15 μl of each anti-CDK polyclonal antibody. For the kinase reaction, immunocomplexes were incubated in kinase buffer supplemented with ATP-γ-S and Rb kinase substrate (Boston Biologicals). Samples were then separated on an acrylamide gel and transferred to Hybond-P membranes. The membrane was blocked and then incubated with PhosphoFind antibody (Boston Biologicals). The membrane was further incubated with horseradish peroxidase-conjugated antibody. Detection of the phosphorylated Rb was performed using the ECL assay protocol (Pierce Chemical).

2.10. Senescence-associated β-galactosidase

SA-β-galactosidase activity was detected using a SA-β-galactosidase staining kit (Cell Signaling Technology, Inc., Beverly, MA) according to the manufacturer's instructions. The control cells and ninjurin1-overexpressing clones were fixed and stained at pH 6.0 with X-gal. Clear blue cytoplasmic staining was regarded as positive. The percentage of SA-β-galactosidase activity-positive cells relative to total cells was determined by counting 100–300 cells in 10 randomly chosen fields per dish using phase-contrast microscopy (ECLIPSE TS 100, Nikon).

2.11. Autofluorescence

The control cells and ninjurin1-overexpressing clones cultured on Lab-Tek® four-chamber slides (Nalge Nunc International Corp, Naperville, IL) were fixed with neutral buffered formaldehyde. Images (×200) were then obtained using a green filter (FT510, Carl Zeiss Co., Ltd.), with 60-s exposure using a fluorescence microscope (Axioplan2, Carl Zeiss Co., Ltd.).