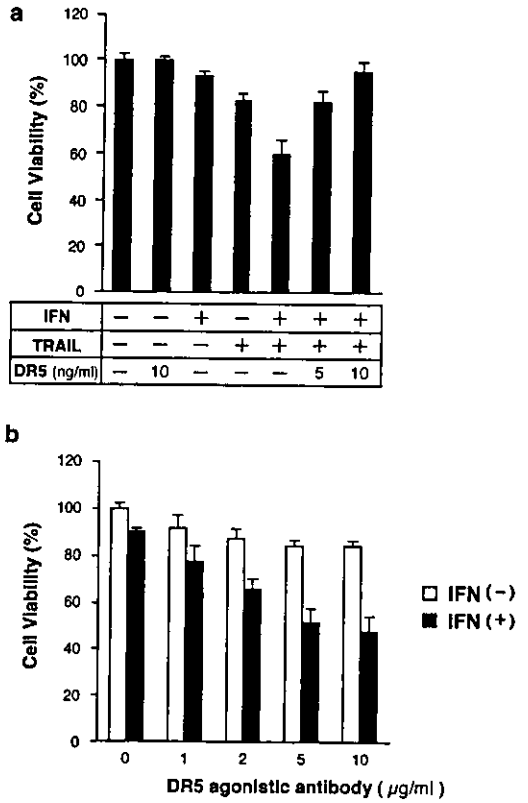

Figure 6 Effect of DR5-specific blocking chimera antibody on TRAIL/ IFN-α-induced apoptosis and effect of IFN-α on apoptosis triggered by DR5-specific agonistic antibody in HuH-7 cells. (**a**) HuH-7 cells were preincubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with or without 50 ng/ml of TRAIL for 24 h in the presence of indicated concentrations of DR5-specific blocking chimera antibody. (**b**) HuH-7 cells were preincubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with varying concentrations of DR5-specific agonistic antibody. Cell viability was determined by the colorimetric method. Results are expressed as a percentage of the control. Data represent the mean ± s.d. values of four experiments (**a** and **b**)

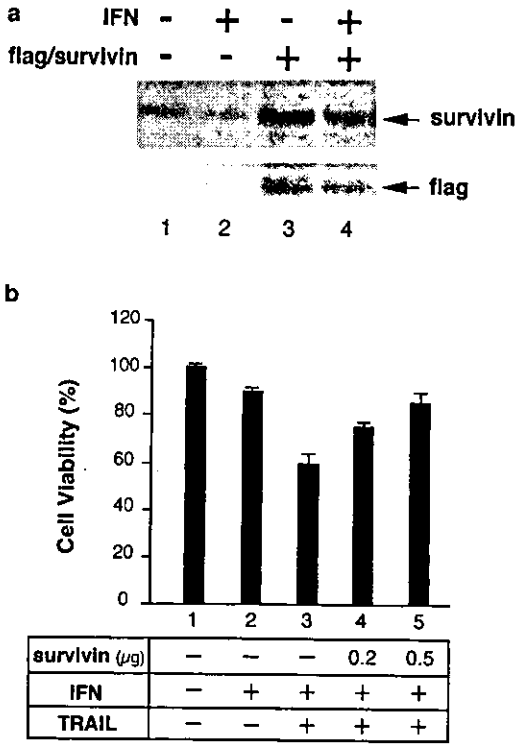

Figure 7 Effects of ectopic expression of survivin on TRAIL/IFN-α-induced apoptosis. (**a**) HuH-7 cells in six-well multiplates were transfected with 2 μg of flag/survivin expression vector (lanes 3 and 4) or vehicle vector alone (lanes 1 and 2) and incubated with or without 1000 IU/ml of IFN-α for 48 h. Then, the expression of survivin or flag/survivin fusion protein was detected by anti-survivin antibody (upper column) or anti-flag antibody (lower column), respectively. (**b**) HuH-7 cells in 24-well multiplates were transfected with indicated amount of survivin expression vector (lanes 4 and 5) or vehicle alone (lanes 1–3) and incubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with or without 50 ng/ml of TRAIL for 24 h. Cell viability was then determined by the colorimetric method. Results are expressed as percentages of the control. Data represent the mean ± s.d. values of four experiments

remained resistant to TRAIL-induced apoptosis even in the presence of IFN-α. Similar observations have been reported where IFN-α suppressed activation of RelA-p50 NF-κB and potentiated TNF-α-induced apoptosis of human cervical cancer cells and Jurkat lymphoma cells (Manna *et al.*, 2000; Suk *et al.*, 2001). Furthermore, Wen *et al.* (2000) have recently reported that overexpression of p202, an IFN-α-inducible protein, is capable of sensitizing breast cancer cells to TNF-α-induced apoptosis through inactivation of NF-κB by its interaction with p202. Taken together, these results indicate that IFN-α could possibly sensitize HuH-7 cells to TRAIL-induced apoptosis by inhibiting the activation of NF-κB including RelA. We also demonstrated that IFN-α stimulated TRAIL-mediated caspase-8 activity in HuH-7 cells, which could be explained by IFN-α-induced upregulation of DR5 in these cells. In addition, suppression of NF-κB activity by IFN-α may be also involved in the stimulation of caspase-8 activity, since the NF-κB-induced products, c-IAP, c-IAP2 and TNFR-associated factor-1 (TRAF1), are known to cooperatively block caspase-8 activity (Wang *et al.*, 1998). However, why IFN-α did not inhibit NF-κB activity in HepG2 cells, even though IFN-α can stimulate the expression of IFN-α-inducible genes in HepG2 and HuH-7 cells (Ichikawa *et al.*, 2001), remains to be clarified.

In conclusion, we have demonstrated in the present study that IFN-α potentiated the apoptotic effect of TRAIL in human hepatoma cells by regulating the expression of DR5 or survivin and by repressing TRAIL-mediated NF-κB activation. These results suggest that IFN-α may exhibit antitumor activity not only by endogenous TRAIL induction, but also by sensitizing cancer cells to TRAIL-mediated apoptosis, and that

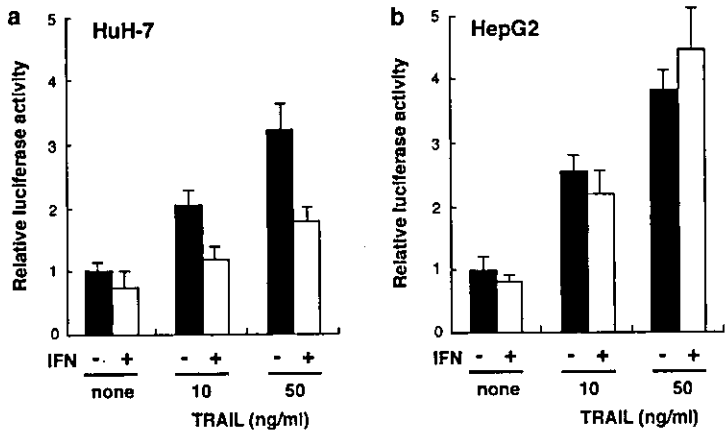

Figure 8 Effects of IFN-α on TRAIL-mediated transcriptional activity of NF-κB in HuH-7 and HepG2 cells. PNFκB-luc was cotransfected with pRL-CMV-luc into HuH-7 (a) and HepG2 (b) cells. After 6 h hours later, the cells were incubated with or without 1000 IU/ml of IFN-α for 36 h, followed by treatment with the indicated concentrations of TRAIL for 7 h. Luciferase activity in the cells was then analysed by dual-luciferase assay. Data represent the ratios of firefly-luc activity derived from pNFκB-luc over renilla-luc activity derived from pRL-CMV-luc relative to the control (no treatment), and are expressed as mean±s.d. of three separate experiments

TRAIL, in combination with IFN-α, may have therapeutic potential in the treatment of human hepatocellular carcinoma.

Materials and methods

Cell culture and detection of apoptosis

The human hepatoma cell lines, HepG2, Hep3B and PLC/PRF/5 were maintained in a chemically defined medium, IS-RPMI (Nakabayashi *et al.*, 1984) containing 10% fetal bovine serum (FBS). HuH-7 was maintained in IS-RPMI containing 5% FBS. Recombinant human TRAIL (leucine zipper construct) was supplied by Immunex Co. (Seattle, WA, USA). Recombinant human IFN-α 2a was provided by Nippon Roche Co. (Tokyo, Japan). To analyse cell viability, 5×10^3 cells were placed into 96-well multiplates. A day later, the medium was replaced with fresh medium in the presence or absence of 1000 IU/ml of IFN-α, and the cells were incubated for 48 h, followed by treatment with varying concentrations of TRAIL for 24 h. In some experiments, varying concentrations of recombinant human TRAIL R2/Fc chimera, DR5-specific blocking chimera antibody (R&D systems, Minneapolis, MN, USA), or goat anti-human TRAIL R2/DR5 antibody, and a DR5-specific agonistic antibody (R&D systems), were added to the cell cultures. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). DNA fragmentation was quantified by the percentage of cells with hypodiploid DNA. In brief, cells were fixed with 70% ethanol and treated with RNase (100 μg/ml, Sigma Chemical Co., St Louis, MO, USA) and then stained with propidium iodide (100 μg/ml, Sigma) for 30 min on ice. The stained cells were analysed by a flow cytometer (Epics XL, Beckman Coulter, Hialeah, FL, USA) to detect the presence of cells with hypodiploid DNA.

Western blotting

Cells were washed three times with PBS, lysed by addition of lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml of aprotinin, 1% NP40, and 0.5% sodium deoxycholate) for 10 min at 4°C and passed several times through a 25-gauge needle, and the protein concentration was determined using a Bio-Rad protein assay kit (Melville, NY, USA). The same amount of protein from each lysate (20 μg/well) was subjected to 8–16% SDS–PAGE. Proteins were transferred onto nitrocellulose membranes, that were then blocked for 1.5 h using 5% nonfat dried milk in PBS containing 0.1% Tween 20 (PBS-T), washed with PBS-T and incubated at room temperature for 1 h in the presence of each antibody (rabbit polyclonal anti-human DR4, Imgenex, San Diego, CA, USA; rabbit polyclonal anti-human DR5, R&D systems; mouse monoclonal anti-human caspase-8, MBL, Nagoya, Japan; rabbit polyclonal anti-human FLIP, PharMingen, San Diego, CA, USA; rabbit polyclonal anti-human Bax, Santa Cruz Biotechnology, Santa Cruz, CA, USA; mouse monoclonal anti-human Bcl-xL, Trevigen, Gaithersburg, MD, USA; mouse monoclonal anti-human XIAP, MBL; rabbit polyclonal anti-human survivin, Alpha Diagnostic International Inc., San Antonio, TX, USA; rabbit polyclonal anti-human c-IAP2, Santa Cruz Biotechnology; mouse monoclonal anti-flag antibody, Sigma; mouse monoclonal anti-human β-actin (as an internal control for Western blot analysis), Sigma). The membranes were washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 1 h. Following washing with PBS-T, immunoreactive bands were visualized by the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

Flow cytometry for DR4 and DR5

Cells were analysed for the surface expression of DR4 and DR5 by indirect staining with primary goat anti-human DR4 and DR5 (R&D systems), followed by phycoerythrin (PE)-conjugated rabbit anti-goat IgG (Sigma). Briefly, cells (5×10^5) were stained with 200 μl PBS containing saturating amounts of anti-DR4 or anti-DR5 on ice for 30 min. After incubation, cells were washed twice, and reacted with PE-conjugated rabbit anti-goat IgG on ice for 30 min. After double washing with PBS, the expression of DR4 and DR5 was analysed by FACScan (Epics XL, Beckman Coulter).

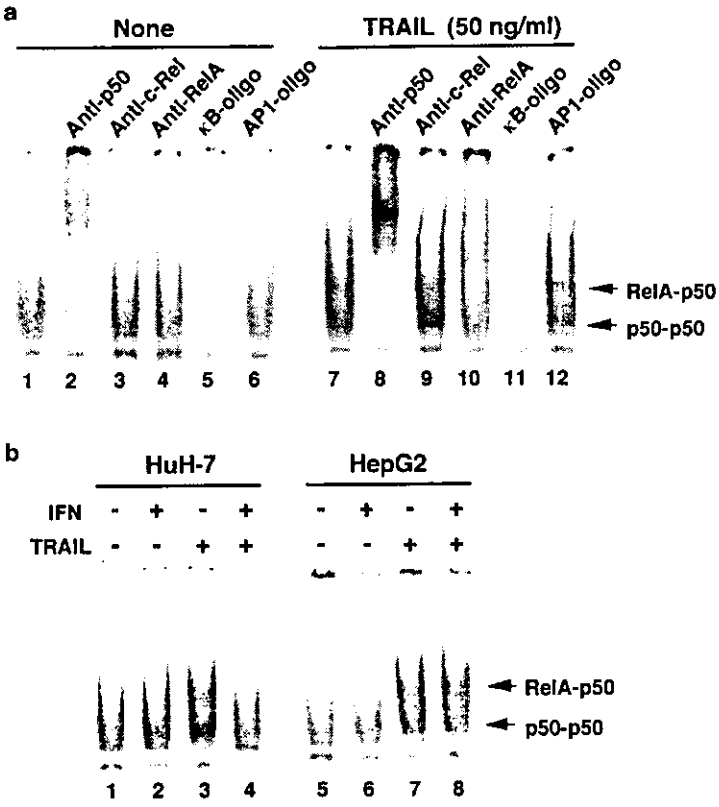

Figure 9 Effect of IFN-α on DNA-binding activity of NF-κB induced by TRAIL in HuH-7 and HepG2 cells. (**a**) Nuclear extracts from HuH-7 cells incubated in the absence (lanes 1–6) or presence (lanes 7–12) of 50 ng/ml TRAIL for 2 h were subjected to EMSA using a ^{32}P-labeled NF-κB oligonucleotide probe. To identify the subunits of NF-κB, the nuclear extract was preincubated for 30 min with the rabbit polyclonal anti-p50 (lanes 2 and 8), anti-c-Rel (lanes 3 and 9) and anti-RelA (lanes 4 and 10). For analysis of the specific binding to the κB sequence, a 100-times molar excess of the nonradiolabeled NF-κB oligonucleotides (lanes 5 and 11) or AP-1 oligonucleotides (lanes 6 and 12) was added to the sample as a competitor. (**b**) Nuclear extracts from HuH-7 and HepG2 cells preincubated with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) 1000 IU/ml of IFN-α, followed by treatment with (lanes 3, 4, 7 and 8) or without (lanes 1, 2, 5 and 6) 50 ng/ml of TRAIL for 2 h were subjected to EMSA using a ^{32}P-labeled NF-κB oligonucleotide probe. Results shown are from one representative experiment from a total of three performed

RNase protection assay

The RNase protection assay was performed using a RiboQuant Multi-Probe RNase Protection Assay System (PharMingen). According to the instructions provided by the manufacturer, hAPO3c (death receptors and death ligands) and hAPO5c (inhibitors of apoptosis) template sets, including a ribosomal protein L32 and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) template as internal controls, were labeled with [α^{32}P] UTP using T7 RNA polymerase. The labeled RNA probes were hybridized with 10 μg of total RNA from HuH-7 cells treated with or without 1000 IU/ml of IFN-α for 48 h. Samples were digested with RNase to remove single-stranded (nonhybridized) RNA. The remaining probes were resolved on 6% urea-polyacrylamide-bis-acrylamide gels. Gels were dried and analysed using an image analyser (BAS, Fuji Film Co., Tokyo).

Cell transfection and luciferase assay

The plasmids pNFκB-luc (Stratagene, La Jolla, CA, USA) containing four copies of the binding sequence of NF-κB and the firefly luciferase gene, and pRL-CMV-luc (Promega, Madison, WI, USA) containing the CMV immediate early enhancer/promoter and renilla luciferase gene were used in the assay. Cells were grown in 24-well multiplates in triplicate the day before transfection. In the next step, 200 ng of pNFκB-luc and 10 ng of pRL-CMV-luc were transfected into the cells by the lipofection method in serum-free medium. After a 6-h incubation, the medium was replaced with fresh medium containing 10% FBS with or without 1000 IU/ml of IFN-α, and the cells were incubated for 36 h, followed by treatment with varying concentrations of TRAIL for 7 h. Luciferase activity in the cells was then determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). In some experiments, pCR2FL; a flag/survivin fusion protein expression vector (Kobayashi *et al*., 1999), kindly provided by Prof. T Tokuhisa (Department of Developmental Genetics, Chiba University) or pCR3.1, a vehicle vector (Invitrogen, Carlsbad, CA, USA), was transfected into HuH-7 cells cultured in 6- or 24-well multiplates.

Electrophoretic mobility shift assay (EMSA)

Cells were incubated in the presence or absence of 1000 IU/ml of IFN-α for 48 h, followed by treatment with or without

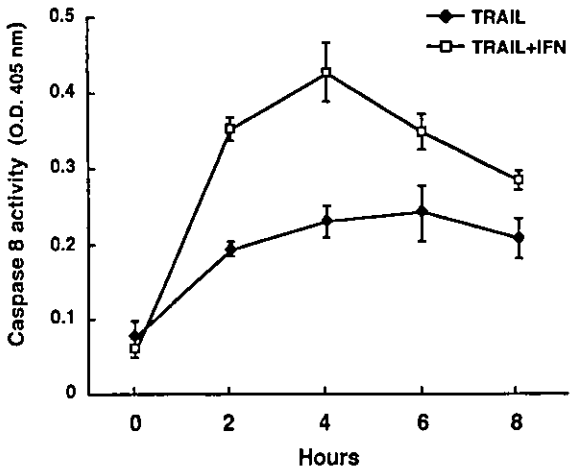

Figure 10 Effects of IFN-α on the TRAIL-mediated caspase-8 activity in HuH-7 cells. HuH-7 was preincubated with or without 1000 IU/ml of IFN-α for 48 h, followed by treatment with 100 ng/ml of TRAIL for the indicated periods, and caspase-8 activity cleaving IETD-pNA substrate was determined by caspase-8 colorimetric assay. Data represent the mean ± s.d. values of four experiments

50 ng/ml of TRAIL for 2 h, and the nuclear extract was prepared as described previously (Nakao *et al.*, 1999). Rabbit polyclonal anti-human p50, anti-human c-Rel and anti-human RelA were purchased from Santa Cruz Biotechnology. The following double-stranded oligonucleotides were used as probes or competitors in the assays (only the sense strand is shown): NF-κB oligonucleotide, AGTTGAGGGGACTTTCCCAGG; AP-1 oligonucleotide, CGCTTGATGAGTCAGCCGGAA. The probe was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extracts (4 μg) were incubated with 10 fmol of the labeled probe for 30 min at room temperature in the presence of 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly (dI-dC), and 8% glycerol. The reaction mixture was electrophoresed on a 4% polyacrylamide gel containing 25 mM Tris-borate and 0.25 mM EDTA. Gels were dried and analysed using an image analyzer.

Caspase-8 activity

Caspase-8 activity was determined using the ApoAlert Caspase-8 Colorimetric Assay kit (Clontech Laboratories, Palo Alto, CA, USA). In brief, cell pellets obtained from 2 × 10^6 cells were resuspended in 50 μl of chilled lysis buffer and incubated on ice for 10 min. The cell lysates were centrifuged at 12 000 r.p.m. at 4°C for 3 min, and the supernatants were collected. Reaction buffer (50 μl) containing 10 mM dithiothreitol and 5 μl of 1 mM IETD-pNA (N-acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline) substrate was added to the supernatants and incubated at 37°C for 2 h in the dark. The absorbance of each sample was measured at 405 nm with a microtiter plate reader.

Abbreviations

EMSA, electrophoretic mobility shift assay; FADD, Fas-associated death domain; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline; IFN-α, interferon-α; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, X-chromose-linked IAP.

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Gene Therapy (2003) 10, 1400–1403

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BRIEF COMMUNICATION

Enhanced antitumor effect of combined replicative adenovirus and nonreplicative adenovirus expressing interleukin-12 in an immunocompetent mouse model

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For cancer gene therapy, replicative adenovirus is a promising vector to overcome low infectivity and poor gene delivery of nonreplicative adenovirus in vivo, *but its therapeutic efficacy is still unsatisfactory because of the limited spread of replicative virus in a solid tumor. Therefore, the combined therapy with other antitumor agents may be necessary. Nonreplicative adenovirus expressing a therapeutic gene may be a promising candidate because E1 proteins expressed by replicative adenovirus would render nonreplicative adenovirus replicative, augmenting a transgene expression. In this study, we first found that mouse hepatoma Hepa 1-6 cells were permissive for the replication and cytopathic effect of human adenovirus, which enabled us to examine the potential of combined replicative adenovirus and nonreplicative adenovirus expressing an immunostimulator in an immunocompetent mouse-syngeneic Hepa 1-6 tumor model. Nonreplicative adenovirus expressing interleukin-12 (AdIL-12) was used as a model.* In vitro *coinfection of two adenoviruses produced higher concentrations of IL-12 than infection of AdIL-12 alone in this cell line.* In vivo *experiments with Hepa 1-6 tumors in syngeneic immunocompetent C57BL/6 mice showed higher concentrations of serum IL-12 and greater therapeutic efficacy in the combination therapy than infection of either adenovirus. These data indicate that the combination of replicative adenovirus and nonreplicative adenovirus expressing an immunostimulator appears to be very efficacious for cancer gene therapy.*
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Keywords: *replicative adenovirus; nonreplicative adenovirus; immunogene therapy; interleukin-12*

Recombinant adenovirus is being widely used as a vehicle for gene delivery in cancer gene therapy. *In vivo* therapeutic efficacy of nonreplicative adenovirus is however limited mainly because of its low infectivity and poor gene delivery to a solid tumor.[1] Use of replicative adenovirus may be a potential candidate to overcome this issue.[1,2] ONYX-015 is such an adenovirus with a deletion in E1B 55 kDa gene and reportedly replicates selectively in the cells defective in p53 gene.[3] Despite the initial expectation from the preclinical data with animal models, the antitumor effect of ONYX-015 as a single agent is minimal in head and neck cancers despite repeated daily injection, and no obvious response is observed in pancreatic, colorectal or ovarian carcinomas in clinical trials.[1,2,4,5] One of the reasons is thought to be the limited *in vivo* spread of the replicative virus in a solid tumor despite rapid spread in an *in vitro* cell culture system.

Hence multimodality treatments with other antitumor agents, which ideally exhibit no crossresistance and synergize with oncolytic adenovirus, have employed to improve the therapeutic efficacy of replicative adenovirus. The potentially synergistic interaction of replicative adenovirus with chemotherapy or radiotherapy has been demonstrated in several animal models and clinical trials.[2,4,5]

The combination with nonreplicative adenovirus expressing a transgene may also be promising because E1 proteins expressed by replicative adenovirus would render nonreplicative adenovirus replicative, thereby augmenting a transgene expression. Indeed, the enhanced efficacy of suicide gene therapy by replicative adenovirus has been demonstrated in animal models.[4,5] Although we have been interested in nonreplicative adenovirus expressing an immunostimulator as a candidate for combination, this type of study has been limited owing to the lack of a relevant immunocompetent animal model. Antitumor effect and safety issue of replicative adenovirus have all been studied in immunodeficient mouse–human xenograft models, because replication of human adenovirus that is used to construct recombinant adenovirus vectors is reportedly restricted to human cells.[4]

In this article, we first evaluated the replication of human replicative adenovirus in several rodent tumor cells. The replicative adenovirus we used has previously been constructed in our lab in which E1A and E1B 19 K proteins were expressed from CAG and CMV promoters, respectively, and E1B 55 K and E3 genes were deleted (formerly AdCALE1AL; we call it AdREP hereafter).[6] We found that Hepa 1-6 cells (a poorly immunogenic mouse

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hepatoma cell line derived from C57BL/6 mice) (ATCC, Manassa, VA, USA)[7] is highly sensitive to adenovirus infection and susceptible to viral replication, progeny production and cytopathic effect (CPE), although slightly less permissive as compared to a human hepatoma cell line HuH-7 as a control. Thus, more than 95% of Hepa 1-6 and HuH-7 cells were stained with x-gal when infected with adenovirus expressing β-galactosidase (AdLacZ)[6] at a multiplicity of infection (MOI) of 10 and 3, respectively (data not shown). In addition, infection of AdREP efficiently induced CPE (ie, cell ballooning) in both cells in dose- and time-dependent manners (Figure 1). For example, infection of AdREP at an MOI of 1 induced more than 95% of cell death in Hepa 1-6 and HuH-7 cells in 10–12 days. Infection of AdLacZ (as a negative control) at MOIs of up to 30 did not affect cell viability (data not shown). Efficient progeny production following AdREP infection, a most relevant marker for oncolytic virus, was also confirmed in Hepa 1-6 cells, although the levels were lower than those in HuH-7 cells (Figure 2). These results demonstrate the relatively high infectivity, replication and CPE of human adenovirus in mouse Hepa 1-6 cells, although we cannot completely exclude the possibility that the cytocidal effect of E1A protein overexpressed from a strong viral promoter may partly contribute to cell death. This cell line thus enabled us to study the feasibility of the combined therapy of replicative adenovirus and nonreplicative adenovirus expressing an immunostimulator.

Nonreplicative adenovirus expressing mouse interleukin-12 (AdIL-12) was used as a model in this study. IL-12 is reported to exhibit a potent antitumor effect by promoting NK cell activity and Th1 immune response.[8,9] Coinfection of AdREP and AdIL-12 into Hepa 1-6 and HuH-7 cells *in vitro* produced ~8-fold and ~15-fold, respectively, higher IL-12 than AdIL-12 alone (Figure 3a). Higher copy number of AdIL-12 vector in the cells infected with both adenoviruses than those with AdIL-12 alone was also demonstrated by polymerase chain reaction (PCR) analysis (Figure 3a). Thus, E1A proteins expressed by AdREP did render nonreplicative AdIL-12 replicative. In *in vivo* experiments with immunocompetent C57BL/6 mice bearing Hepa 1-6 tumors, intratumoral injection of AdREP, AdIL-12, or both, significantly suppressed tumor growth as compared to that of AdLacZ (Figure 4). The efficacy was the highest in the combined therapy, followed by AdREP alone and AdIL-12 alone. In addition, only the combined therapy induced complete tumor regression in three out of 10 (30%) mice. Indeed, serum concentrations of IL-12 in mice received intratumoral injection of AdREP and AdIL-12 were ~3-fold higher than those of AdIL-12 alone 24 h after injection (Figure 3b). These data clearly demonstrate the enhanced effect of the combined AdREP and AdIL-12. However, the tumors, which were treated with AdREP or AdREP and AdIL-12 and did not completely regressed, started to regrow ~2 weeks after injection (data not shown).

Replication of human adenovirus is generally poor in nonhuman cells. However, we found in this study that mouse Hepa 1-6 cells can support replication and progeny production of human adenovirus. A few other cell lines of mouse and hamster origin have also been previously identified to be permissive for human adenovirus replication.[10,11] Our finding allowed us to evaluate the therapeutic efficacy of the combined replicative adenovirus and nonreplicative adenovirus expressing an immunostimulator (IL-12 in this study) in immunocompetent mice. To our knowledge, this is the

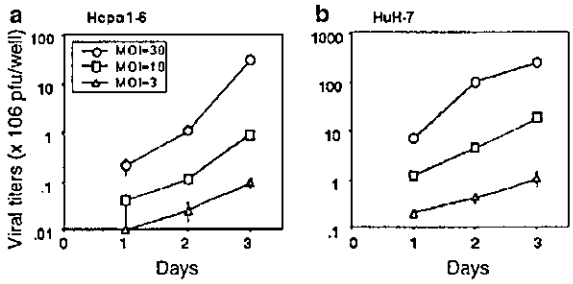

Figure 2 Progeny virus production in the cells infected with AdREP. The cells in a 12-well plate (5 × 10⁴ cells/well) were infected with AdREP at MOIs indicated (day 0). Aliquots of the culture media were centrifuged, and the supernatants were used to determine titers of adenovirus released into the culture medium on days 1, 2 and 3 by the plaque-forming assay with 293 HEK cells. Data are means±s.d. (n=3) from one of two separate experiments.

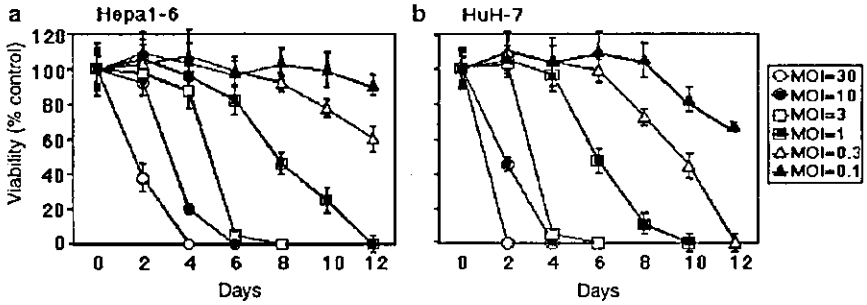

Figure 1 Time course and dose-response of CPE in the cells infected with AdREP. The cells, seeded at a density of 1 × 10⁵ (when MOIs ≤1) or 1 × 10⁵ (MOIs>1)/well in a 96-well microtiter plate, were infected with AdREP at the MOIs indicated. Recombinant adenovirus was propagated in 293 human embryonal kidney (HEK) cells and purified through two rounds of CsCl density gradient centrifugation.[6] The MOI was defined as the ratio of total number of plaque-forming units (PFU) used in a particular infection/number of cells. Cell viability was quantitated with a WST-1 assay (Cell Counting Kit; Wako, Osaka, Japan). Data are means±s.d. (n=3) from one of two separate experiments.

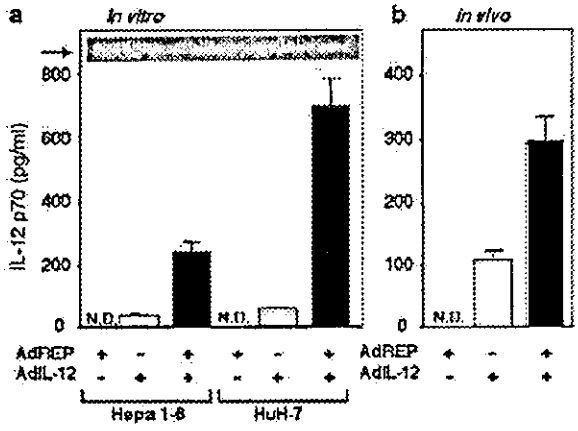

Figure 3 IL-12 production in the cells infected with AdREP, AdIL-12 or both in vitro and in vivo. AdIL-12, nonreplicative adenovirus expressing mouse IL-12, was constructed as previously described.[17,18] The DNA fragment containing cDNAs for IL-12 p35 and p40 linked with the internal ribosome entry site (IRES) gene of encephalomyocarditis virus was kindly provided by Dr H Yamamoto at Osaka University.[19] (a) The cells (1×10^5 cells/well in a 24-well plate) were infected with AdREP, AdIL-12 or both at MOIs of 3 and concentrations of IL-12 in culture supernatants were determined 2 days later with a mouse IL-12 p70 ELISA kit (Biosource International, Camarillo, CA, USA). Data represent means ± s.d. (n=3) from one of two separate experiments. Further, viral DNA together with genomic DNA was extracted from the cells with standard techniques,[20] and an aliquot (5%) was subjected to PCR (25 cycles of denaturation (94°C for 30 s), annealing (50°C for 30 s) and extenstion (72°C for 60 s)) with two primers specific for IL-12 p35 (sense; 5'-cac ccg cgt cgt gac cat-3') and p40 (antisense; 5'-aaa cgt ctt tct cca gct-3') cDNAs to estimate the relative amounts of AdIL-12 vector. Arrow indicates ~70 bp fragment including parts of p35 and p40 cDNAs and IRES, amplified with these two primers. Data are one of two independent experiments. (b) Hepa 1-6 cells (5×10^6/mouse) were subcutaneously injected into the right flank of C57BL/6 mice. Approximately 1 week later when tumor sizes were 75–100 mm³, AdREP (1×10^9 PFU/mouse), AdIL-12 (5×10^8 PFU) or both were intratumorally injected. Mice were bled for serum IL-12 assay 24 h after injection. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals in Nagasaki University. Data represent means ± s.d. of five mice in each group; ND, not detectable.

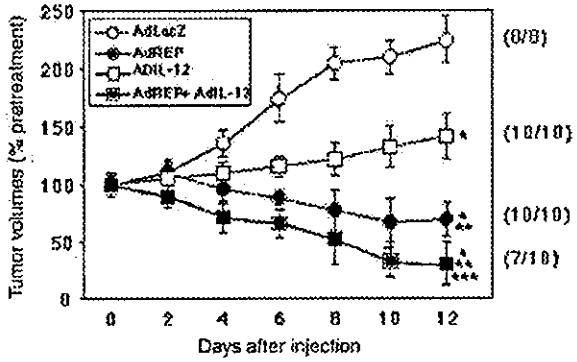

*Figure 4 In vivo antitumor effect of AdREP and/or AdIL-12 in Hepa 1-6 tumors in C57BL/6 mice. After adenovirus injection into Hepa 1-6 tumors (1×10^9 PFU AdREP plus 5×10^8 PFU AdLacZ, 5×10^8 PFU AdIL-12 plus 1×10^9 PFU AdLacZ or 1×10^9 PFU AdREP plus 5×10^8 PFU AdIL-12), tumor size was determined from caliper measurement using the standard formula (length × width²/2) and expressed as a percentage relative to that on day 0. Each bar represents mean ± s.e. (n=8–10). Numbers in parentheses, number of tumor-bearing mice/number of mice used. *, $P<0.01$ versus control; **, $P<0.05$ versus AdIL-12; ***, $P<0.05$ versus AdREP or AdIL-12 (Student's t-test).*

first report demonstrating that the combination of replicative adenovirus and nonreplicative adenovirus expressing IL-12 produced higher IL-12 *in vitro* and *in vivo*, and augmented the therapeutic efficacy as compared with either adenovirus in immunocompetent mice. The efficacy of the combination of replicative adenovirus and nonreplicative adenovirus expressing IL-2 has previously been demonstrated in *immunodeficient* mice.[12] Taken together, these results clearly indicate that nonreplicative adenovirus encoding an immunostimulator can be a good candidate for the combination therapy with replicative adenovirus.

Both replicative adenovirus therapy and immunogene therapy have shown somewhat limited therapeutic effects as a single agent.[4,5,13] By combining these two approaches, replicative adenovirus would provide an environment rich in tumor antigens by killing cancer cells, where immune cells recruited and activated by an immunostimulator expressed would elicit immune response against these tumor antigens. Moreover, expression level of an immunostimulator can be augmented by replication of nonreplicative adenovirus. Indeed, antitumor effects of most of cytokines are positively correlated with their expression levels.[13] Combination of replicative adenovirus and nonreplicative adenovirus expressing an immunostimulator is therefore highly rationale. Enhanced therapeutic efficacy of replicative herpes simplex virus encoding IL-12 has also been reported in immunocompetent mice.[14–16]

Finally, combination of replicative adenovirus and nonreplicative adenovirus encoding an immunostimulator described in this article is a promising approach for cancer gene therapy. However, a single replicative adenovirus armed with an immunostimulator will be more efficacious, because the need of double infection curtails the therapeutic efficacy when MOI is low or tumor cells are resistant to adenovirus infection.

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Short interfering RNA-directed inhibition of hepatitis B virus replication

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Abstract RNA interference (RNAi) is the process by which double-stranded RNA directs sequence-specific degradation of mRNA. In mammalian cells, RNAi can be triggered by 21-nucleotide duplexes of short interfering RNA (siRNA). We examined effects of siRNA on hepatitis B virus (HBV) replication. Human hepatoma cells were transfected with HBV DNA and siRNA against HBV-pregenome RNA. Transfection experiments demonstrated that the siRNA reduced the amount of HBV-pregenome RNA and resulted in reduction of the levels of replicative intermediates and viral protein. Our results indicate that siRNA-mediated gene silencing inhibits HBV replication through suppression of viral RNA, which may be useful as a potential therapeutic modality.

Key words: Hepatitis B virus; siRNA; RNAi

1. Introduction

RNA interference (RNAi) is a mechanism of gene regulation in plants, invertebrates and, more recently, in mammalian cells in which target mRNAs are degraded in a sequence-specific manner [1–3]. RNAi is initiated by the double-stranded RNA (dsRNA)-specific endonuclease Dicer, which promotes processive cleavage of long dsRNA into 21–23-mer short interfering RNA (siRNA) [4–8]. siRNAs are incorporated into a protein complex that recognizes and cleaves target mRNA [5–8]. RNAi has been shown to protect against invading genetic elements such as transposons, transgenes and viruses, which potentially share a long dsRNA trigger [9–12].

In mammalian cells, exposure to dsRNAs greater than 30 bp in length induces an antiviral interferon response that generally represses mRNA translation through the activation of dsRNA-dependent protein kinase [13]. However, introduction of shorter siRNA into mammalian cells leads to mRNA degradation with exquisite sequence specificity without activating the interferon system [3]. RNAi-mediated gene silencing offers a potentially powerful tool to inhibit viral replication. Therefore, we examined whether siRNA duplexes specific for hepatitis B virus (HBV) were capable of affecting the degradation of viral RNAs.

2. Materials and methods

2.1. Plasmids and synthesis of siRNA

Full-length HBV DNA was cloned via *Sac*I site into the pGEM-11Zf(+) vector (Promega, Madison, WI, USA) as described previously [14], registered in GenBank (accession number AB050018). According to Harborth's report for selection of siRNA duplexes for the target mRNA sequence [15], we searched for sequences of the type AA (N21) [N, any nucleotide (nt)] from the open reading frame of the 3.5-kb HBV-pregenome RNA, in order to obtain a 21-nt sense and 21-nt antisense strand with 2-nt 3′ overhangs. A selected sequence was also submitted to a BLAST search against the human genome sequence to ensure that the human genome was not targeted. siRNA with the following sense and antisense sequences was used: siHBV, 5′-CAUUGUUCACCUCACCAUATT-3′ (sense), 5′-UAUGGUGAGGUGAACAAUGTT-3′ (antisense), corresponding to HBV sequence in the core region from nt 2137 to nt 2157 (Fig. 1). siRNA against green fluorescent protein was used as control: siGFP, 5′-GGCUACGUCCAGGAGCGCACC-3′ (sense), 5′-UGCGCUCCUGGACGUAGCCTT-3′ (antisense). All siRNAs were purchased from Nihon Bioservice (Saitama, Japan).

2.2. Cells and transfections

The HuH7 and HepG2 cells, human hepatoma cell lines, were maintained in RPMI, supplemented with 10% fetal bovine serum. Prior to transfection, plasmid including HBV genome was cleaved with *Sap*I (New England Biolabs, Beverly, MA, USA) to release the heterologous primer sequences and to create linear HBV monomers with *Sap*I sticky ends, as described previously by Gunther et al. [16]. The digested DNA was purified by a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Transfection of cells using linear monomeric HBV genome with *Sap*I ends can initiate a full replication cycle, including production of viral RNAs, translation of viral proteins and release of virions [14,16]. The HuH7 and HepG2 cells were plated at a density of 2.5×10^5 and 4.0×10^5 cells per 35-mm-diameter dish, respectively. One day later, both cells were transfected with 0.5 μg of HBV DNA and 0.05 μg of pGL3-control vector expressing luciferase (Promega) together with 100 pmol of each siRNA, using oligofectamine (Gibco-Invitrogen, Rockville, MD, USA) according to the protocol provided by the manufacturer. The medium was replaced with a fresh medium 6 h after transfection, and the cells were harvested 2 days after transfection. Experiments were performed in triplicate. Transfection efficiency was standardized by measurement of the luciferase activity in the cell lysate using a luciferase assay system (Promega).

2.3. Quantitative assay of viral marker

The levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in culture media from transfected cells were quantitatively measured using chemiluminescent immunoassay and radioimmunoassay (Dainabot, Tokyo, Japan), respectively.

2.4. Northern blotting

Total RNA was isolated from the transfected HuH7 cells using the

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Abbreviations: dsRNA, double-stranded RNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; RNAi, RNA interference; siRNA, short interfering RNA

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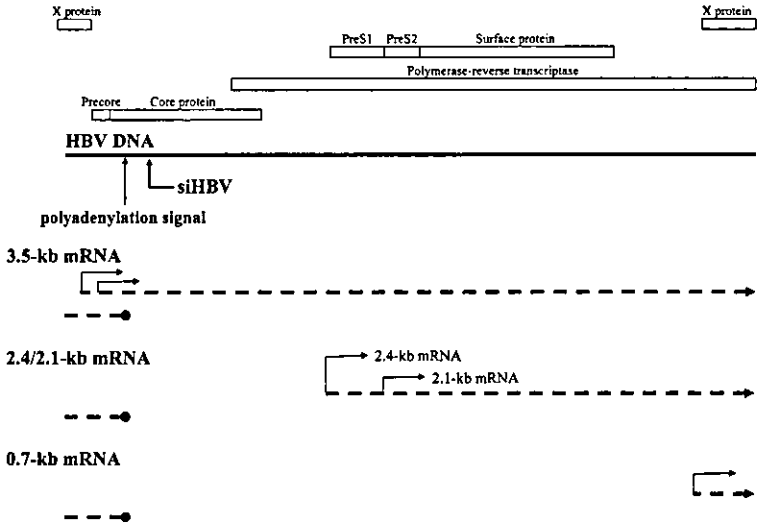

Fig. 1. A schematic diagram depicting the location of the siRNA in association with viral open reading frames and viral mRNAs within the HBV genome.

guanidium isothiocyanate method. The isolated RNA was fractionated on 1.5% formaldehyde agarose gel, transferred onto a nylon membrane (Hybond N$^+$, Amersham, Little Chalfont, UK), and hybridized with a ^{32}P-labeled full-length HBV fragment. The probe was generated with a random-primed labeling kit (Amersham). Autoradiography was performed and analyzed with a BAS2000 image analyzer (Fuji Photo Film, Tokyo).

2.5. Southern blotting

Purification of HBV DNA from intracellular core particles was performed using the method described by Gunther et al. [16]. Briefly, the cells were washed once with ice-cold phosphate-buffered saline and lysed in 300 μl of lysis buffer [50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% Nonidet P-40] per 35-mm-diameter dish. The cell lysates were transferred to 1.5-ml tubes, vortexed, and allowed to stand on ice for 15 min. Nuclei were pelleted by centrifugation for 1 min at 14000 rpm. The supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg ml^{-1} of DNase I for 30 min at 37°C. The reaction was stopped by addition of EDTA to a final concentration of 25 mM. Proteins were digested with 0.5 mg ml^{-1} of proteinase K and 1% sodium dodecyl sulfate for 2 h at 37°C. Nucleic acids were purified by phenol–chloroform (1:1) extraction and ethanol precipitation with glycogen. DNA isolated from the cytoplasmic core particles was separated on a 1.5% agarose gel, blotted onto a nylon membrane (Hybond N$^+$), and hybridized with a ^{32}P-labeled full-length HBV fragment.

3. Results

3.1. Quantitative analysis of viral markers in culture media

siRNA duplexes were co-transfected with a full-length HBV DNA into the HuH7 and HepG2 cells. HBsAg and HBeAg secreted into culture media were quantitatively assayed (Fig. 2). The amount of HBsAg in media of cells transfected with siHBV did not differ from that of cells transfected with siGFP (control siRNA). In contrast, medium HBeAg levels in cells transfected with siHBV decreased to 4.6-fold and 4.9-fold in comparison with cells transfected with siGFP in HuH7 and HepG2 cells, respectively.

3.2. Northern and Southern blot analysis

Northern blot analysis was performed 2 days after transfection (Fig. 3). The level of 2.4/2.1-kb mRNA of HBV was not suppressed by siHBV, whereas the level of 3.5-kb mRNA was reduced in cells transfected with siHBV relative to cells transfected with siGFP.

Using Southern blot analysis (Fig. 4), the level of open circular and single-stranded HBV-DNA, which is considered as an intracellular replicative intermediate, was clearly suppressed by siHBV. On the other hand, the level of double-stranded HBV-DNA, which is mostly derived from the transfected DNA, was slightly diminished.

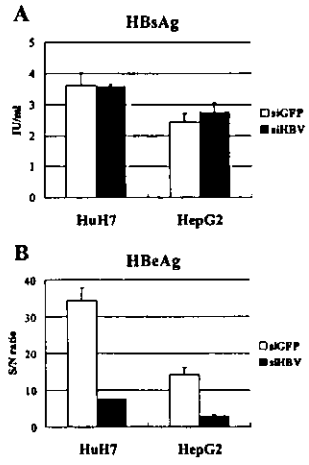

Fig. 2. Quantitative measurements of HBsAg (A) and HBeAg (B) in culture media from HuH7 cells and HepG2 cells co-transfected with HBV DNA and siRNA. Transfection experiments were performed in triplicate as described in Section 2. The levels of medium HBsAg and HBeAg are expressed as mean ± S.D. The S/N ratio denotes the signal-to-noise ratio.

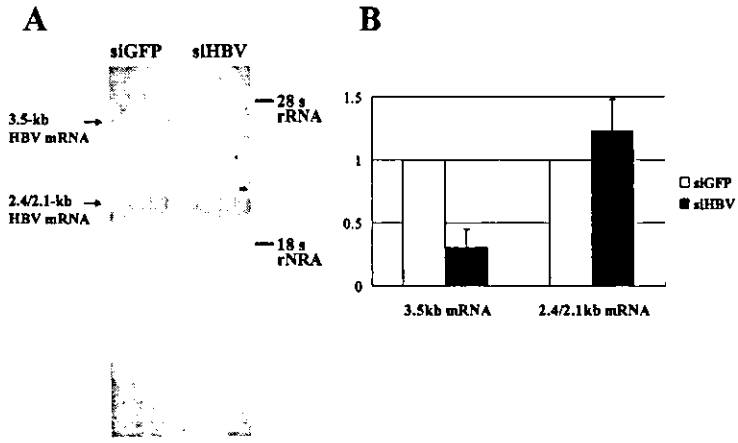

Fig. 3. Northern blot analysis of HBV in cells co-transfected with HBV DNA and siRNA. A: The HuH-7 cells were harvested 2 days after transfection. The amount of 3.5- and 2.4/2.1-kb HBV mRNA was determined by Northern blotting. B: The hybridization signals for the viral transcript were quantitatively evaluated by NIH image analysis software. Data are expressed as mean ± S.D. relative to the value of cells transfected with siGFP in three independent transfection experiments.

4. Discussion

Gene silencing mediated by siRNA is a sequence-specific and highly conserved mechanism in eukaryotes [9–11]. In plants, it serves as an antiviral defense mechanism [12]. Mammalian cells also possess this machinery but its specific function is unclear. More recently, several investigators demonstrated that siRNAs inhibit HIV-1 production by targeting various regions for its genome [17,18]. In this study, we showed that siRNAs directed against HBV genome could effectively block viral replication.

The HBV genome is a partially double-stranded 3.2-kb DNA molecule and is the template transcribed to generate the four viral RNAs [19]. The 3.5-, 2.4/2.1-, and 0.7-kb transcripts encode the core protein/HBeAg and polymerase-reverse transcriptase, HBsAg, and X protein, respectively. All viral transcripts utilize a common polyadenylation signal located within the core protein-coding region. The 3.5-kb mRNA not only serves for translation of the core protein/HBeAg and polymerase-reverse transcriptase but also represents the template for reverse transcription. In the present study, the effect of RNAi on HBV replication was examined using a siRNA specific for the 3.5-kb pregenome RNA, which did not bind to other viral transcripts. We demonstrated that the siRNAs reduced the level of 3.5-kb pregenome RNA and resulted in reduction of the levels of secreted HBeAg and replicative intermediates converted from the 3.5-kb pregenome RNA. These findings indicate that mammalian RNAi machinery can be programmed with siRNA corresponding to HBV genome to induce an effective antiviral response. Unsur-

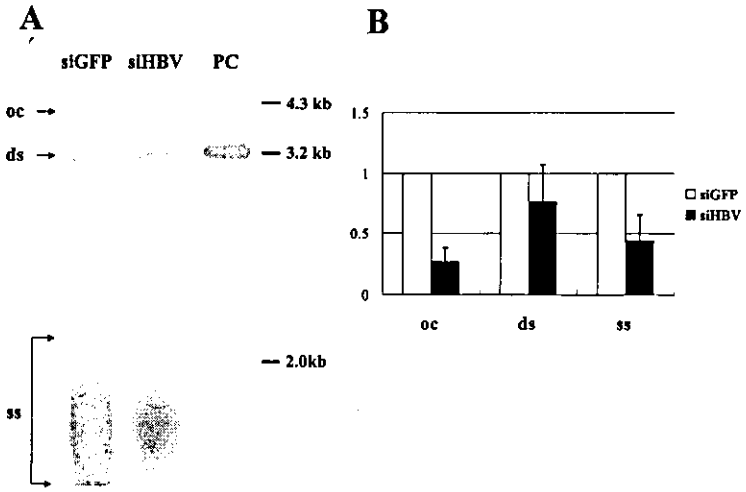

Fig. 4. Southern blot analysis of HBV in cells co-transfected with HBV DNA and siRNA. A: The HuH-7 cells were harvested 2 days after transfection. The level of open circular (oc), double-stranded (ds) and single-stranded (ss) HBV DNA was determined by Southern blotting. The lane denoted PC was loaded with linear full-length HBV. B: The HBV DNA signals obtained from three independent transfection experiments were quantitatively evaluated by NIH image analysis software. Data are expressed as mean ± S.D. relative to the value of cells transfected with siGFP.

prisingly, the levels of 2.4/2.1-kb mRNA and secreted HBsAg was not reduced in cells transfected with siHBV, since the 2.4/2.1-kb mRNA did not include the homologous sequence to siHBV.

Although chronic HBV infection is a major health problem worldwide, there is no completely effective antiviral treatment. siRNA technology may provide a possible therapeutic strategy against chronic HBV infection. Further studies are necessary to determine the antiviral mechanism of siRNA on HBV replication.

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p48 Overexpression enhances interferon-mediated expression and activity of double-stranded RNA-dependent protein kinase in human hepatoma cells

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Background/Aims: **Double-stranded RNA-dependent protein kinase (PKR) is a key factor involved in interferon (IFN)-induced antiviral actions. Since p48, together with signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2), is an indispensable mediator in IFN-α signaling pathways, we investigated the effect of p48 gene transduction on PKR expression and its activity in HuH-7 human hepatoma cells.**

Methods: **HuH-7 cells were infected or transfected with p48 gene expression adenoviral vector or plasmid vector, respectively, and incubated with or without IFN-α, then PKR expression and phosphorylation of α-subunit of eukaryotic protein synthesis initiation factor-2 (eIF2α) in the cells were examined. In addition, PKR activity inhibiting protein translation was determined by the decrease of chloramphenicol acetyltransferase (CAT) gene translation or α-fetoprotein secretion.**

Results: **p48 overexpression itself could not stimulate PKR expression. However, p48 overexpression in combination with interferon-α treatment caused a marked increase in PKR expression and augmented the phosphorylation of eIF2α, by which the transfected CAT gene translation, as well as the endogenous α-fetoprotein synthesis, was blocked without affecting their mRNA levels.**

Conclusions: **These results suggest that p48 gene transduction may provide a strategy to enhance the IFN-mediated PKR expression and its activity in hepatocytes.**

Keywords: **RNA-dependent protein kinase; Interferon-α; p48; Interferon-stimulated regulatory element; Eukaryotic protein synthesis initiation factor 2α; Hepatitis C virus**

1. Introduction

Hepatitis C virus (HCV) cause acute and chronic liver diseases, and are closely linked to development of hepatocellular carcinoma (HCC) [1]. Interferon-α (IFN-α) has been used as an antiviral agent against HCV, and resulted in clinical improvement in patients with HCV [2]. However, more than a half of patients chronically infected by these viruses show only transient or no response to IFN-α treatment [2]. Therefore, several alternative treatment modalities with IFN-α are now on going in these patients [3,4].

Type I IFN (IFN-α and -β), after binding to its receptor, stimulates the intracellular IFN-signaling cascade, where the activation of receptor-associated Janus tyrosine kinases (Jak1 and Tyk2) induces the phosphorylation of signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2). The formation of interferon-stimulated gene factor 3 (ISGF3), which consists of phosphorylated STAT1 and STAT2, and p48 (ISGF3γ) [5],

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translocates into the nucleus and binds to the interferon-stimulated regulatory element (ISRE) in the promoter sequences of a variety of IFN-inducible genes, including antiviral proteins such as double-stranded RNA-dependent protein kinase (PKR) [6], 2′,5′-oligoadenylate synthetase (2′,5′-OAS) [7] and Mx proteins [8], and transactivates these gene expression. Interferon regulatory factor-1 (IRF-1), another important transcription factor, which also mediates the biological actions of IFN, binds to the ISRE-like sequences and activates the IFN-inducible gene transcription [9]. p48 and IRF-1 have a homology within their amino-terminal regions and are members of the IRF family together with other IRFs [10]. However, analysis in mice deficient for the p48 gene has shown that the induction of several IFN-inducible genes including PKR is largely p48 dependent, and that IRF-1 cannot compensate completely for the loss of p48 [11].

PKR, a serine/threonine kinase, is a key factor involved in the antiviral and antiproliferative actions of IFN-α [6]. PKR down-regulates protein neosynthesis through the phosphorylation of α-subunit of eukaryotic protein synthesis initiation factor-2 (eIF2α) [12]. Recent studies have indicated that some HCV products can suppress PKR function in vivo; in fact, envelope protein E2, and nonstructural 5A (NS5A) protein which contains the interferon sensitivity determining region directly interact with PKR to inhibit its kinase activity [13,14]. This probably allows HCV to escape from IFN-α treatment.

In the present study, we investigated that the influence of p48 overexpression on the IFN-α-induced PKR expression and its activity in HuH-7 human hepatoma cells.

2. Materials and methods

2.1. p48 expression vectors and cell culture

p48 expression plasmid, pcp48, was constructed by inserting the full-length p48 cDNA, which was kindly provided by Dr. D.E. Levy (Department of Pathology, New York University School of Medicine), into the expression vector plasmid, pcDNA3.1 (Invitrogen, Carlsbad, CA) [15]. p48 expression adenoviral vector, p48-Ad, was constructed by the in vitro ligation methods established recently [16]. In brief, p48 cDNA was inserted into a shuttle plasmid vector, pHMCMV6, which contains cytomegalovirus (CMV) promoter, and digested with I-CeuI and PI-SceI. The released fragment was inserted into I-CeuI/PI-SceI-digested adenoviral plasmid vector, pAdHM4, which contains a complete E1/E3-deleted adenovirus type 5 genome. The resultant p48-expressing adenoviral plasmid vector was digested with *Pac*I, and transfected into 293 human embryonal kidney (HEK) cells to yield p48-Ad. pHMCMV6 and pAdHM4 plasmids were kindly provided by Dr. H. Mizuguchi (National Institute of Health Sciences, Tokyo, Japan). LacZ-Ad, which was used as a vehicle adenoviral vector, was obtained from Dr. I. Saito (Tokyo University) [16].

The HuH-7 human hepatoma cell line was maintained in a RPMI with 5% fetal bovine serum. In each experiment, the medium was replaced with serum-free RPMI, and the cells were transfected and infected with pcp48 and p48-Ad, respectively, at the indicated concentrations. After 12–48 h incubation, the cells were treated with varying concentrations of recombinant human IFN-α 2a (Nippon Roche Co. Tokyo, Japan) for the indicated periods.

2.2. Western blotting

The cells were lysed by addition of lysis buffer (50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 100 μg/ml PMSF, 1 mmol/l sodium *o*-vanadate) for 10 min at 4 °C, and insoluble materials were removed by centrifugation at 14 000 rpm for 30 min at 4 °C. The supernatant was collected, and the protein concentration was determined using a Bio-Rad protein assay kit (Melville, NY). The samples were analyzed by electrophoresis on 8–12% SDS–polyacrylamide gels and electrotransferred to nitrocellulose membranes, blocked with 5% nonfat powdered milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h, and incubated with rabbit polyclonal antibodies raised against peptides corresponding to human p48, STAT1, STAT2, PKR (Santa Cruz Biotechnology, Santa Cruz, CA), eIF2α (New England Biolabs, Beverly, MA) and mouse monoclonal anti-human β-actin (Sigma Chemical Co., St. Louis, MO) respectively, overnight at 4 °C. Similarly, expression of phosphorylated STAT1 at tyrosine-701 and phosphorylated eIF2α at serine-51 was determined using the phosphospecific antibodies recognizing each phosphorylation (Upstate Biolab, Lake Placid, NY and Biosource, Camarillo, CA). The membranes were washed with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG for 1 h. Following washing with PBS-T, the immunoreactive bands were visualized by the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England). In addition, α-fetoprotein (AFP) secreted from the cells into culture media was detected by Western blotting using mouse monoclonal antibody against human AFP (kindly provided by Dr. Nishi, Department of Biochemistry, Hokkaido University School of Medicine).

2.3. Reporter gene transfection assay

For the luciferase assay, the pISRE-Luc vector containing five copies of the ISRE sequence and firefly luciferase gene, and pRL-SV40 vector containing SV 40 early enhancer/promoter and Renilla luciferase gene were obtained from Clontech (San Diego, CA) and Promega (Madison, WI), respectively. The HuH-7 cells were grown in 24-well plates in triplicate, and 1 μg of pISRE-Luc and 10 ng of pRL-SV40 were cotransfected with 1 μg of pcp48 or pcDNA3.1 (vehicle) into the cells by the lipofection method. One day later, the cells were treated with varying concentrations (0, 10 and 100 IU/ml) of IFN-α for 6 h. Then, the luciferase activities in the cells were determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). For the chloramphenicol acetyltransferase (CAT) assay, 3 μg of pSVAF2.4-CAT which contains a 2.4-kilobase pair full AFP enhancer region (−5.3 to −2.9) inserted at the 5′ end of the CAT gene in pSV1′-CAT [17] was cotransfected with 5 μg of pcp48 or pcDNA3.1 into HuH-7 cells grown in a 75-cm^2 flask. After 12 h incubation, the cells were treated with varying concentrations (0, 100 and 1000 IU/ml) of IFN-α for 24 h, and the cells were harvested and lysed through five cycles of freezing and thawing. The lysate was centrifuged at 15 000 rpm for 5 min, and the supernatant was used for determination of CAT activity.

2.4. Northern blotting and reverse transcription–polymerase chain reaction (RT–PCR)

For Northern blotting, total cellular RNA was isolated by guanidinium isothiocyanate method, and 10 μg of RNA was size-fractionated on an 1% formaldehyde agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled cDNA probe. PKR cDNA [18] and AFP cDNA [19] were used as probes.

For the detection of CAT mRNA by RT–PCR, total RNA was extracted from the cells transfected with pSVAF2.4-CAT. The extracted RNA was treated with DNase I to eliminate the contamination of CAT DNA in RNA preparation, and 0.5 μg of total RNA was subjected to the first strand cDNA synthesis using superscript II reverse transcriptase and oligo(dT) primer in the Superscript preamplificaton system (Life technologies, Rockville, MD). To standardize the CAT mRNA levels in the transfected cells, purified CAT RNA with a known concentration was serially diluted to yield 0.05 pg to 5

pg per sample, and subjected to the first strand cDNA synthesis. The synthesized cDNAs were simultaneously amplified by PCR using Taq DNA polymerase and CAT gene-specific primers (sense: 5′-CGACCGTTCAGCTGGATATTAC-3′ and anti-sense: 5′-GACATGGAAGCCATCACA GAC-3′) to yield a 523 bp product. The amplification was performed for 30 cycles in a programmable DNA thermal cycler. The PCR products were electrophoresed on an 1.2% agarose gel and visualized by ethidium bromide staining.

3. Results

3.1. p48 overexpression enhances IFN-α-mediated ISRE-driven gene transcription

The HuH-7 cells were infected with varying multiplicities of infection (MOI) of p48-Ad or transfected with varying concentrations of pcp48. Two days later, p48 expression in the cells was analyzed by Western blotting (Fig. 1A). The baseline level of p48 in HuH-7 cells was very low. However, its expression was dose-dependently increased by p48-Ad infection (Fig. 1A, upper column) or pcp48 transfection (Fig. 1A, lower column). Expression of STAT1, STAT2 or the phosphorylated STAT1 at tyrosine-701 in the cells could not be induced by infection with p48-Ad at a MOI of 50 (Fig. 1B, lanes 1 and 4). In contrast, IFN-α treatment caused the phosphorylation of STAT1 in p48-Ad-infected cells, as well as in the vehicle vector (LacZ-Ad)-infected cells (Fig. 1B, lanes 2, 3, 5 and 6).

For the functional analysis of p48 overexpression, the HuH-7 cells were cotransfected with pISRE-Luc containing five repeats of the ISRE sequence in its promoter and the pcp48 or pcDNA3.1 (vehicle plasmid), and incubated with varying concentrations of IFN-α for 6 h (Fig. 1C). The ISRE-luciferase activity in pcp48-cotransfected cells was significantly higher than that in vehicle plasmid-cotransfected cells; in fact, the elevated ISRE-luciferase activity in pcp48-cotransfected cells treated with 10 IU/ml of IFN-α was almost similar to that in vehicle plasmid-cotransfected cells treated with 100 IU/ml of IFN-α (lanes 3 and 5).

3.2. p48 overexpression enhances IFN-α-mediated PKR expression and its activity

To clarify whether p48 overexpression could influence the IFN-α-mediated PKR gene expression, HuH-7 cells infected with p48-Ad at a MOI of 50 were incubated with varying concentrations of IFN-α for 8 h. By Northern blotting, the levels of both 6.0 and 2.5 kb PKR mRNA were dose dependently induced by IFN-α, and the elevated levels were further enhanced by p48 overexpression (Fig. 2A). Similarly, Western blot analysis showed that the IFN-α-induced PKR expression (Fig. 2B, lanes 1–3) was clearly augmented by p48 overexpression (Fig. 2B, lanes 4–6).

The eIF2α acts as a downstream effector of PKR, which is phosphorylated at serine-51 by PKR and functions to inhibit protein neosynthesis. As shown in Fig. 2C, IFN-α, similar to double-stranded RNA (dsRNA), induced the phosphorylation of eIF2α at serine-51 (Fig. 2C, lanes 3 and 6), while p48-Ad infection apparently enhanced the phosphorylation of eIF2α induced by IFN-α (Fig. 2C, lane 5). Interestingly, p48-Ad infection itself could induce the phosphorylation of eIF2α (Fig. 2C, lane 4), to some degree similar to the level induced by IFN-α treatment (Fig. 2C,

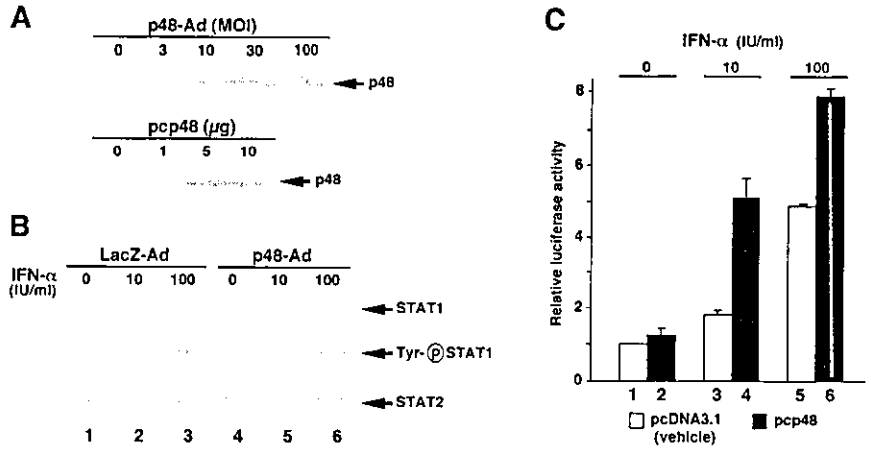

Fig. 1. Effect of p48 overexpression on IFN-α-mediated ISRE-driven gene transcription. (A) HuH-7 cells were infected with p48-Ad (upper column) or transfected with pcp48 (lower column) at the indicated concentrations. Two days later, p48 expression was analyzed by Western blotting. (B) HuH-7 cells infected with LacZ-Ad (lanes 1–3) or p48-Ad (lanes 4–6) at a MOI of 50, respectively, were incubated for 48 h, followed by treatment with IFN-α at the indicated concentrations for 1 h. Expression of STAT1 (upper column), phosphorylated STAT1 at tyrosine-701 (middle column) and STAT2 (lower column) was analyzed by Western blotting. (C) One microgram of pISRE-Luc and 10 ng of pRL-SV40 were cotransfected with 1 μg of pcDNA3.1 (vehicle) (lanes 1–3) or pcp48 (lanes 4–6) into HuH-7 cells. One day later, the cells were treated with IFN-α at the indicated concentrations for 6 h. Dual luciferase assay was performed to correct the transfection efficacy in each experiment. Data represent the ratios of firefly-luc activity derived from pISRE-Luc/renilla-luc activity derived from pRL-SV40 relative to the control (lane 1), and are expressed as mean ± SD of three separate experiments.

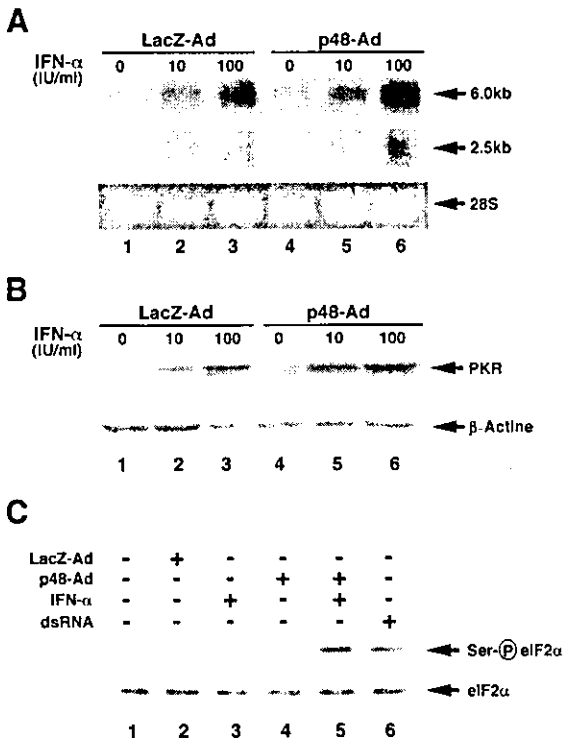

Fig. 2. Effect of p48 overexpression on IFN-α-mediated PKR expression and the phosphorylation of EIF2α. (A) HuH-7 cells infected with LacZ-Ad (lanes 1–3) or p48-Ad (lanes 4–6) at a MOI of 50, respectively, were incubated for 48 h, and treated with IFN-α at the indicated concentrations for 8 h. Expression of PKR mRNA was analyzed by Northern blotting using a PKR cDNA probe. Upper and lower bands correspond to 6.0 and 2.5 kb of PKR mRNA, respectively. Ethidium bromide-stained 28S ribosomal RNAs are shown as control. (B) HuH-7 cells infected with LacZ-Ad (lanes 1–3) or p48-Ad (lanes 4–6) at a MOI of 50, respectively, were incubated for 48 h, and treated with IFN-α at the indicated concentrations for 24 h. PKR expression was analyzed by Western blotting. Expression of β-actin was used as an internal control. (C) HuH-7 cells infected with none (lanes 1, 3 and 6), LacZ-Ad (lane 2) or p48-Ad (lanes 4 and 5) at a MOI of 50, respectively, were incubated for 48 h, and further incubated in the absence (lanes 1, 2 and 4) or presence (lanes 3 and 5) of 100 IU/ml of IFN-α for 24 h. For the positive control, HuH-7 cells were treated with dsRNA at a concentration of 100 μg/ml of poly (rI)-(rC) for 8 h (lane 6). Expression of phosphorylated EIF2α at serine-51 and total EIF2α was analyzed by Western blotting.

lane 3), but vehicle adenoviral vector (Lacz-Ad) infection could not (Fig. 2C, lane 2).

For the functional analysis of eIF2α, pSVAF2.4-CAT containing the full AFP enhancer sequence and pcp48 or vehicle plasmid (pcDNA3.1) were cotransfected into HuH-7 cells, followed by incubation with varying concentrations of IFN-α for 24 h. As shown in Fig. 3A, CAT activity in the cells cotransfected with pSVAF2.4-CAT and vehicle plasmid was slightly suppressed by 1000 IU/ml of IFN-α. In contrast, CAT activities in the cells cotransfected with pSVAF2.4-CAT and pcp48 were suppressed by 50% by 100 IU/ml and by 70% by 1000 IU/ml of IFN-α, respectively (Fig. 3A, lanes 5 and 6). To elucidate whether this suppression of CAT activity was due to the decreased translation or transcription of CAT gene, the CAT mRNA levels in the transfected cells were determined by RT–PCR, in which a linear correlation was found between the amounts of standard CAT RNA and the densities of RT–PCR products from the standard CAT RNA (Fig. 3C). When the CAT mRNA levels were compared in pcp48- or vehicle plasmid (pcDNA3.1)-cotransfected cells, the levels could not be influenced by IFN-α treatment irrespective of p48 overexpression (Fig. 3B), indicating that p48 overexpression plus IFN-α treatment could not alter CAT gene transcription, although CAT activity in the p48 overexpressing cells was clearly reduced by IFN-α treatment (Fig. 3A). In addition, to investigate the eIF2α activity on the endogenous protein synthesis, the amount of secreted AFP in HuH-7 cells and its transcript in the cells were determined by Western blotting and Northern blotting, respectively (Fig. 4). IFN-α treatment did not influence the levels of AFP mRNA or the amounts of secreted AFP in the vehicle vector (LacZ-Ad)-infected cells (Fig. 4, lanes 2 and 3). IFN-α did not affect the levels of AFP mRNA in p48-Ad-infected cells, but it dose-dependently suppressed the AFP secretion in these cells (Fig. 4, lanes 5 and 6), similar to the results of the transfected CAT gene expression (Fig. 3A).

4. Discussion

Previous studies have shown that priming with IFN-γ enhances the IFN-α-mediated ISGF3 formation in HeLa cells through the induction of p48 [20], and that lack of response to IFN-α in adenovirus E1A-expressing HT1080 cells and transitional cell carcinoma cells is attributed to low levels of functional p48 protein in these cells, which is restored by the exogenous p48 gene overexpression [21,22]. Similarly, priming with a low dose of interleukin-6 has been found to enhance the cellular responses to IFN-α through the induction of p48 in HeLa M cells [23]. These observations suggest that the cellular level of p48 is involved in a key regulator of the IFN-α-mediated pathway. Lau et al. have recently reported that p48, like other IRF family proteins, has a nuclear localization signal in its amino-terminal DNA-binding domain, and that p48 is capable of translocating to the nucleus in the absence of cytoplastic retention factors such as STATs, in which STAT2 rather than STAT1 can retain p48 in the cytoplasm through interacting with the carboxyl-terminal STAT-binding domain of p48 [24]. Moreover, several investigators suggest that a STAT2-p48 complex formation in the cytoplasm contributes to the cellular ability to rapidly generate the ISGF3 complex in response to IFN-α stimulation [24,25]. In HuH-7 cells, the baseline levels of STAT1 and STAT2 were thought to be more abundant than the level of p48 (shown in Fig. 1). Therefore, p48 overexpression in HuH-7 cells probably results in the increased cytoplasmic

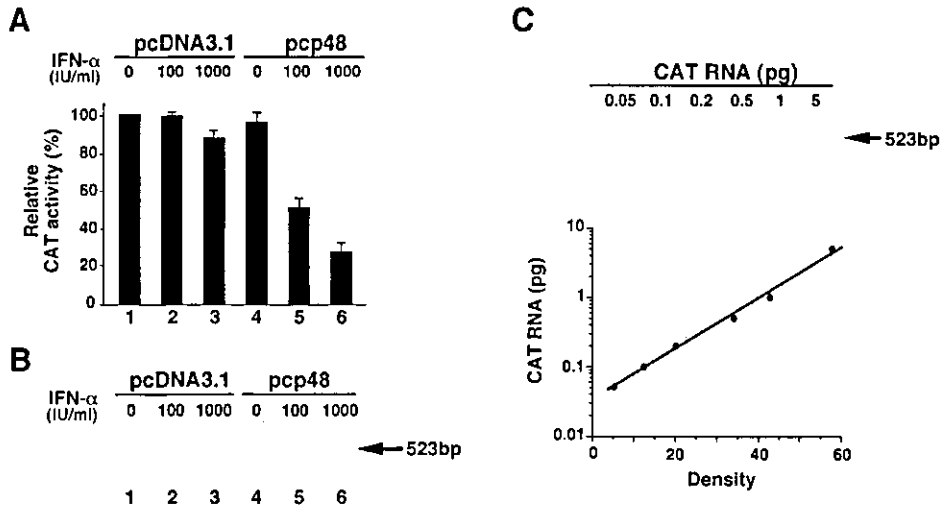

Fig. 3. Effects of IFN-α and p48 overexpression on CAT expression from pSVAF2.4-CAT and its mRNA levels in HuH-7 cells. pSVAF2.4-CAT was cotransfected with pcDNA3.1 (vehicle) (lanes 1–3 in A,B) or pcp48 (lanes 4–6 in A,B) into HuH-7 cells. After 12 h incubation, the cells were treated with IFN-α at the indicated concentrations for 24 h. The harvested cells were divided in two and subjected to analyze CAT expression and its mRNA levels, respectively. (A) In the CAT assay, the amount of extracts and incubation time were 10 μg of protein and 2 h, respectively. Data represent CAT activities relative to the control (lane 1), and are expressed as mean ± SD of three separate experiments. (B) For the analysis of CAT mRNA levels, 0.5 μg of total RNA from each sample was subjected to RT–PCR. The RT–PCR products from the samples were electrophoresed in an 1.2% agarose gel and visualized by ethidium bromide staining. (C) To standardize the CAT mRNA levels in the transfected cells, purified CAT RNA at the indicated amounts was also subjected to RT–PCR. The RT–PCR products were electrophoresed in an 1.2% agarose gel and visualized by ethidium bromide staining. The densities of bands in RT–PCR from standard CAT RNA were quantified by densitometry with an image program. There was a linear correlation between the densities of bands and the amounts of standard CAT RNA.

level of a STAT2-p48 complex which, in turn, leads to the enhanced ISGF3 formation in response to IFN-α stimulation, and strengthens the ISRE-driven gene transcription including PKR in the cells.

PKR is a well-known IFN-α-inducible protein, which mediates antiviral and antiproliferative activities of IFN-α. PKR inhibits translation initiation through the phosphorylation of eIF2α at serine-51 [12]. In addition, recent studies have shown that PKR regulates the activation of several transcription factors such as NF-κB [26], p53 [27], IRF-1 [28] and STATs [29], and also participates in apoptosis induced by many different stimuli, including tumor necrosis factor-α and viral infection [30–32]. Although PKR mediates the antiproliferative activity of IFN-α, it has been reported that PKR expression is upregulated in HCC [33], cholangiocarcinoma [34] and breast cancer cells [35]. These observations suggest that PKR might have a tumor promoting activity in some cancer cells. In this context, Deb et al. [36] have recently proposed that PKR is an upstream regulator of activation of STAT3 and mediates both growth-promoting and growth-inhibitory signals. Thus, PKR is now characterized by a multi-functional protein controlling a variety of cellular responses. In this study, p48 overexpression enhanced the IFN-α-mediated PKR expression at a transcriptional level. PKR contains two copies of a dsRNA-binding motif in the region of amino-terminal 171 amino acids, and is activated by binding of dsRNA produced in the life cycle of viral replication [6]. However, PKR is also activated in the absence of viral replication, in which several cellular mRNAs are proposed to activate PKR, such as the 3′ untranslated region (UTR) of α-tropomyosin mRNA [37], 3′ UTR of TNF-α mRNA [38], 5′ UTR of IFN-γ mRNA [39] and PKR mRNA itself [40]. Furthermore, PKR activating protein (PACT), a 35-kDa human protein, has been recently identified to directly activate the latent form of PKR in response to stress signals [41]. In our study, p48 overexpression augmented the IFN-α-

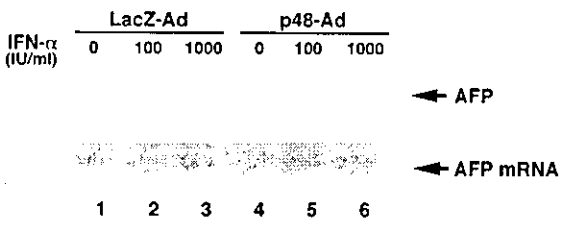

Fig. 4. Effects of IFN-α and p48 overexpression on the levels of AFP gene transcription and translation in HuH-7 cells. HuH-7 cells infected with LacZ-Ad (lanes 1–3) or p48-Ad (lanes 4–6) at a MOI of 50, respectively, were incubated for 48 h, and the medium was replaced with serum-free medium containing varying concentrations of IFN-α every 24 h (0, 100 and 1000 IU/ml). After 48 h stimulation, the medium was collected, and the cells were harvested. The levels of AFP secreted from the cells into culture media for the last 24 h were analyzed by Western blotting (upper column). Expression of AFP mRNA in the cells was analyzed by Northern blotting using a human AFP cDNA probe (lower column).

mediated phosphorylation of eIF2α, which caused the inhibition of protein synthesis from the transfected CAT gene and the endogenous AFP gene in HuH-7 cells. These results indicate that p48 overexpression and IFN-α can cooperatively activate PKR. In addition, p48 overexpression itself by p48-Ad infection could induce the phosphorylation of eIF2α, suggesting that the overexpressed p48 mRNA may interact with PKR and activate it, similar to tropomyosin, TNF-α and IFN-γ mRNAs, although further experiments are necessary to confirm such mechanism. Cellular stresses including viral infection are known to facilitate the PKR activation [6]. However, control adenoviral infection (LacZ-Ad) had little effect on the phosphorylation of eIF2α in the current study. Thus, p48 is likely to be involved in not only the PKR gene induction but also the cellular process of PKR activation.

Recent studies have shown that NS5A or E2 of HCV products can directly bind to PKR and inhibit its kinase activity [13,14], which may, in part, account for a resistant mechanism of HCV against IFN-α therapy. Based on our findings, the increased expression and activity of PKR by p48 overexpression in combination with IFN-α treatment may overcome such inhibitory effects of HCV products, although the relationship between p48 expression and IFN-α responsiveness in HCV-infected patients is not yet clear.

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