23. Yamashita, T., et al., *EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features.* Gastroenterology, 2009. **136**(3):1012-1024.

24. Yang, Z.F., et al., *Significance of CD90+ cancer stem cells in human liver cancer.* Cancer Cell, 2008. **13**(2):153-166.

25. Libbrecht, L., *Hepatic progenitor cells in human liver tumor development.* World J Gastroenterol, 2006. **12**(39):6261-6265.

26. Yano, H., et al., *Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells.* Hepatology, 1993. **18**(2):320-327.

27. Hisaka, T., et al., *Interferon-alphaCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo.* J Hepatol, 2004. **41**(5):782-789.

28. Yano, H., et al., *Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines.* Hepatology, 1999. **29**(6):1708-1717.

29. Ueda, K., et al., *Aldehyde Dehydrogenase 1 Identifies Cells with Cancer Stem Cell-Like Properties in a Human Renal Cell Carcinoma Cell Line* . PLOS ONE, 2013. 8(10):e75463.

30. Burkert, J., W.R. Otto, and N.A. Wright, *Side populations of gastrointestinal cancers are not enriched in stem cells.* J Pathol, 2008. **214**(5):564-573.

31. Sell, S. and H.L. Leffert, *Liver cancer stem cells.* J Clin Oncol, 2008. **26**(17):2800-2805.

32. Breuhahn, K., T. Longerich, and P. Schirmacher, *Dysregulation of growth factor signaling in human hepatocellular carcinoma.* Oncogene, 2006. **25**(27):3787-3800.

33. El-Serag, H.B. and K.L. Rudolph, *Hepatocellular carcinoma: epidemiology and molecular carcinogenesis.* Gastroenterology, 2007. **132**(7):2557-2576.

34. Tannapfel, A., et al., *INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas.* Oncogene, 2001. **20**(48):7104-7109.

35. Teufel, A., et al., *Genetics of hepatocellular carcinoma.* World J Gastroenterol, 2007. **13**(16):2271-2282.

36. van Zijl, F., et al., *Epithelial-mesenchymal transition in hepatocellular carcinoma.* Future Oncol, 2009. **5**(8):1169-1179.

37. Villanueva, A., et al., *Genomics and signaling pathways in hepatocellular carcinoma.* Semin Liver Dis, 2007. **27**(1):55-76.

Figure legends

Fig. 1a. SP cells are considered to be abolished by reserpine. The ratio of SP cells in the HAK-1A cell line was 0.207%. There was almost no expression of CD133, CD90, EpCAM or CD13 in SP and NSP cells from HAK-1A.

Fig. 1b. The ratio of SP cells in the HAK-1B cell line was 0.9%. In the HAK-1B cell line, CD133 was expressed in 4.6～6.4% of SP cells and 3.9～5.3% of NSP cells. CD13 expression was higher in SP cells (21.7%) than in NSP cells (8.9%). CD90 and EpCAM expression was low in both SP and NSP cells. The experiments were repeated twice, and almost identical results were obtained.

Fig. 2. After culturing HAK-1A SP cells or HAK-1B SP cells for 1 week, the percentage of HAK-1A SP cells and HAK-1B SP cells decreased to 1.9% and 7.3%, respectively. In contrast, culture of HAK-1A and HAK-1B NSP cells generated a small population of SP cells in HAK-1A (0.1%) and HAK1B (0.7%). The experiments were repeated twice, and almost identical results were obtained.

Fig. 3a. Cell proliferation was significantly higher in SP cells than in NSP cells at 24 h, 48 h, 72 h, 96 h, or 120 h. The experiments were repeated twice, and almost identical results were obtained.

Fig. 3b. Cell cycle analysis found no apparent difference in G_0-G_1/ S/ G_2-M ratios between HAK-1B SP and NSP cells. The experiments were repeated twice, and almost identical results were obtained.

Fig. 3c. Drug resistance to CDDP, 5-FU or PEG-IFN-α2b was compared between SP and NSP cells from HAK-1B at 48 h and 96 h. The viability of SP cells was significantly lower than NSP cells after 96 h treatment with 0.75 μM or 1.5 μM 5-FU, or 96 h treatment with 500 IU/mL PEG-IFN-α2b. No other significant differences were observed between SP and NSP cells. The experiments were repeated at least three times, and almost identical results were obtained.

Fig. 3d. After exposure of HAK-1B cells to PEG-IFN-α2b for 72 h, the percentage of SP cells decreased as compared with control. Conversely, the percentage of SP cells increased when HAK-1B cells were treated with 5-FU for 72 h. The experiments were repeated at least three times, and almost identical results were obtained.

Fig. 4a. Colony formation assay found no significant difference between HAK-1B SP cells and NSP cells. The experiments were repeated at least three times, and almost identical results were obtained.

Fig. 4b. Sphere formation was significantly higher in HAK-1B SP cells than NSP cells. The experiments were repeated at least three times, and almost identical results were obtained.

Fig 4c. Injection of 1, 5, or 10 x 10^3 SP or NSP cells produced no tumors in NOD/SCID mice. In contrast, four mice that received 5 x 10^4 SP cells and five mice that received 10 x 10^4 SP cells developed tumors at 8 weeks. In addition, one mouse that received 5 x 10^4 NSP cells and two mice that received 10 x 10^4 NSP cells also developed small tumors

Fig. 5a and b. SP and NSP cells of HAK-1A and HAK-1B expressed mRNAs of CSC markers, such as CD13, CD133, CD24, CD44, EpCAM, ABCG2, Nanog, and Bmi-1, and hepatocyte marker, CYP3A4. The expression of CSC markers was slightly higher in SP cells than in NSP cells.

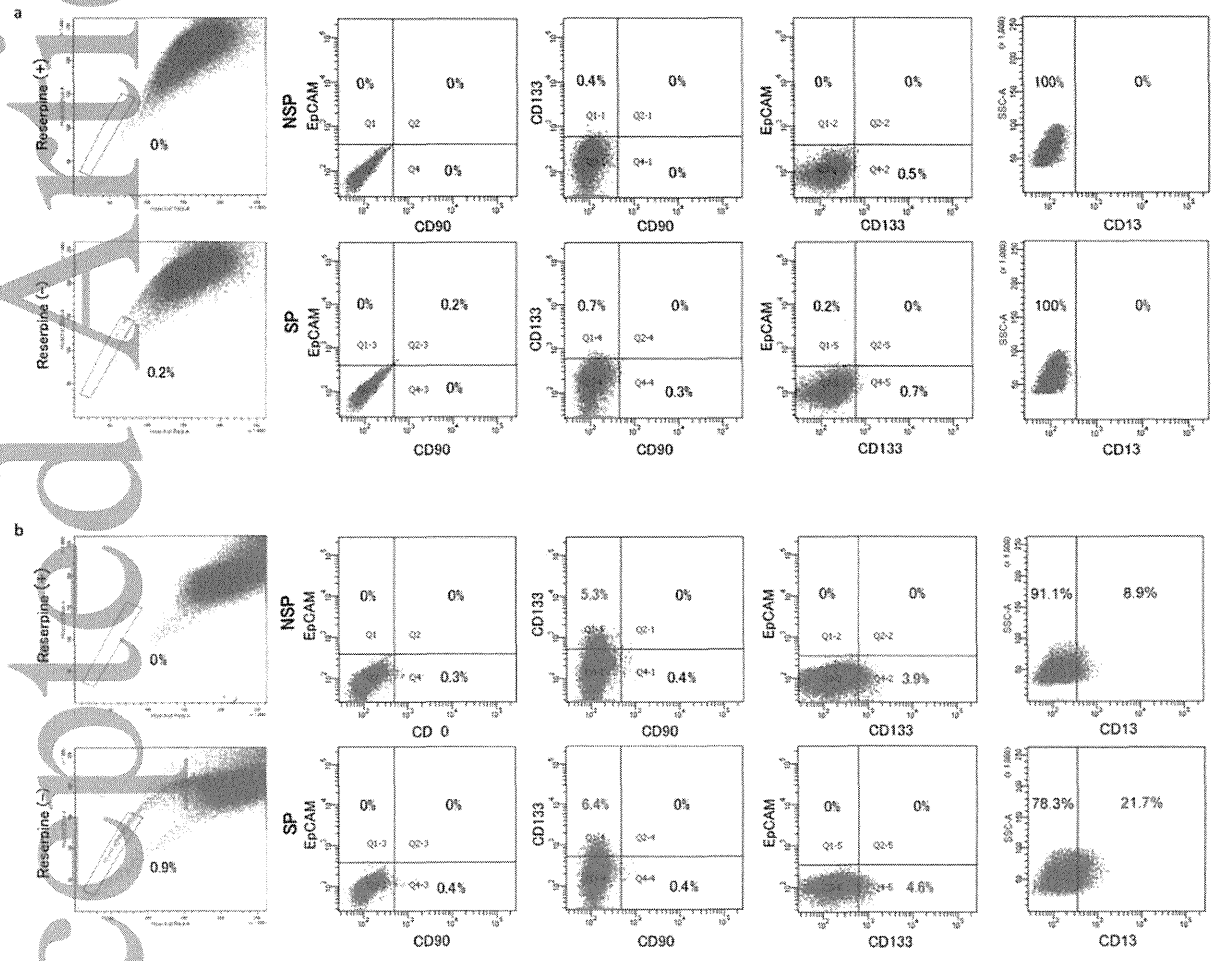
Table 1 Primer and probe mixes list for qRT-PCR analysis

Gene name	Assay IDs
CD13	Hs00174365_m1
CD133	Hs00195682_m1
CD24	Hs03044178_m1
CD44	Hs01075861_m1
CD90	Hs00174816_m1
EpCAM	Hs00158980_m1
ABCG2	Hs01053790_m1
Oct-4	Hs03666771_m1
Nanog	Hs04260366_m1
BMI1	Hs00201350_m1
Alb	Hs00910225_m1
CYP3A4	Hs00604506_m1
β-actin	Hs99999903_m1

Table 2. Summary of cDNA microarray of SP and NSP cells from HAK-1A and HAK-1B

Gene expression in SP cells and NSP cells sorted from HAK-1A or HAK-1B cells was analyzed by cDNA microarray, but no significant differences were observed between SP cells and NSP cells of either cell line with regard to stemness gene expression.

	HAK-1A	**HAK-1B**
Top Associated Network Functions	RNA Post-Transcription Modification, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair (e.g., ↑FAM124A, C1ORF35, etc.)	Cancer, Drug Metabolism, Molecular Transport (e.g., ↑ALDH3A1, ATF7, IL33, etc.)
Top 5 up-regulated molecules in SP vs. NSP	GBP5 (×10.5), BMP3 (×8.4), SLITRK2 (×8.1), TMEM90B (×7.8) CUGBP2 (×7.6)	FGF2 (×13.1), ZNF311 (×12.6), ADH6 (×8.2), HPCA (×7.9) AKR1B10＊ (×7.6)
Top 5 down-regulated molecules in SP vs. NSP	ZNF646 (×11), SAA3P (×10.1), HTR2C (×7.5), UGT2B7 (×7.4), CCR9 (×7.4)	CACNG3 (×47.1), HNMT (×20.3), GAK (×11.6), C14ORF126 (×11.5), GGT5 (×10.7)
Stemness gene expression in SP vs. NSP	CD44 (×1.04), Oct-4 (×0.95), Bmi-1 (×0.87), ABCG2 (×0.83), CD24 (×0.54), EpCAM (×1.02)	CD44 (×0.93), Oct-4 (×0.84), Bmi-1 (×0.97), ABCG2 (×1.47), CD24 (×1.27), EpCAM (×0.84)



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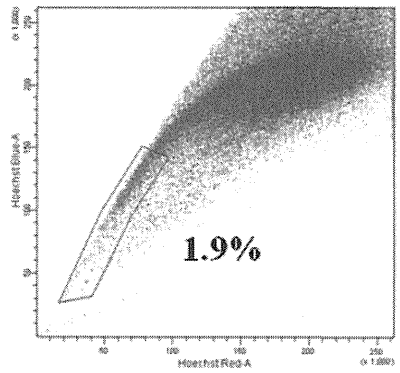
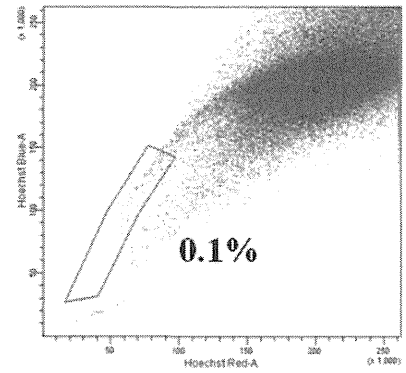
HAK-1A

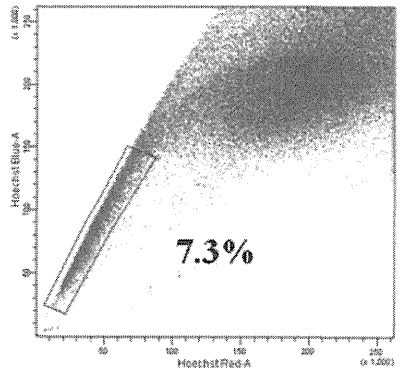


Sorted SP cells



Sorted NSP cells

HAK-1B



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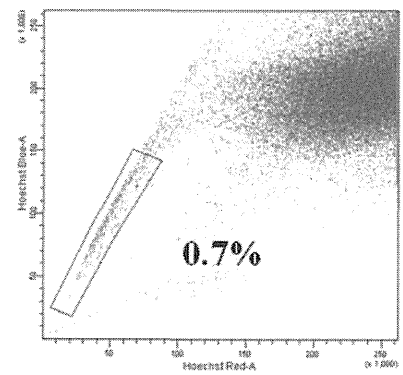

Sorted NSP cells

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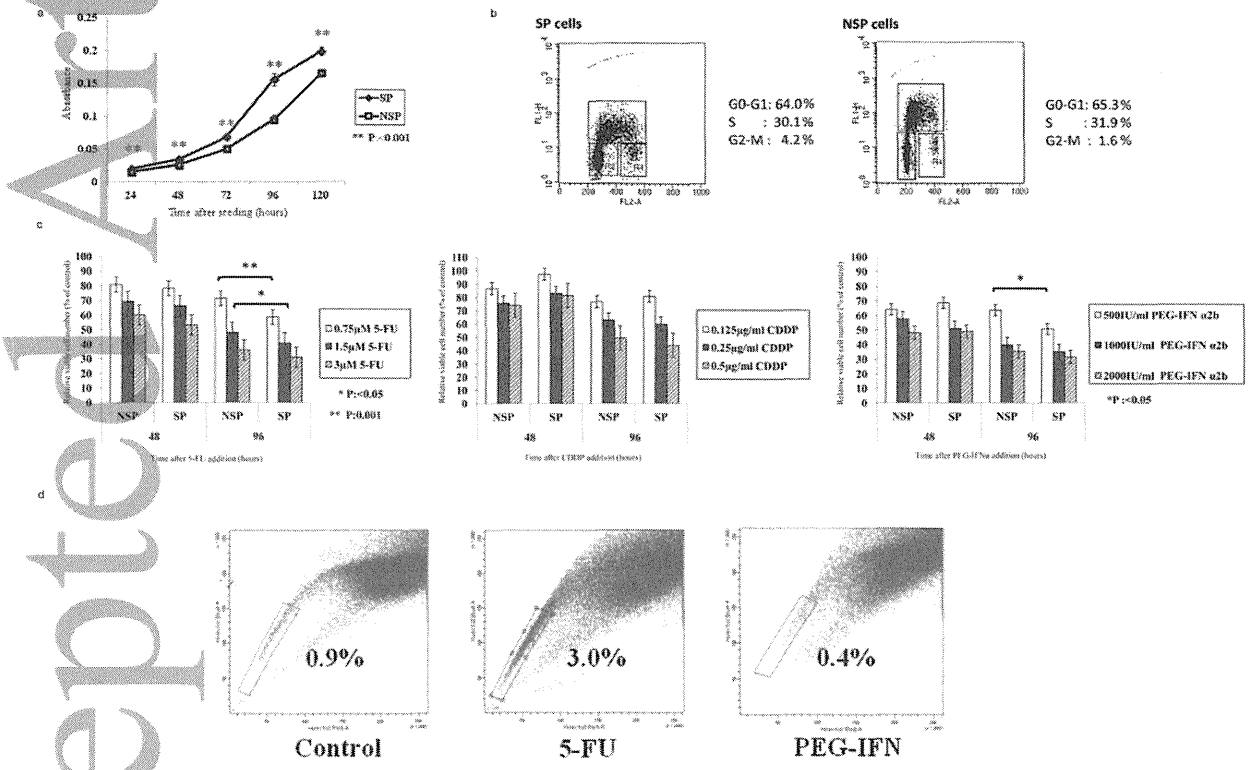

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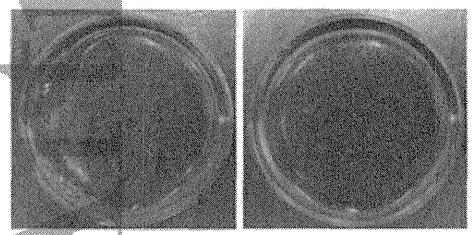
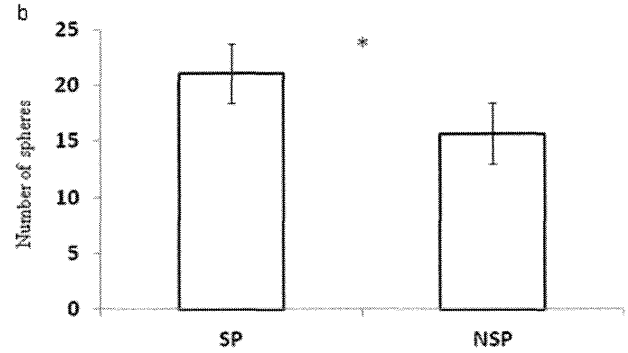
a



252 ±33.4 colonies / 1× 10^4 cells

243 ±70.1 colonies / 1 × 10^4 cells

b

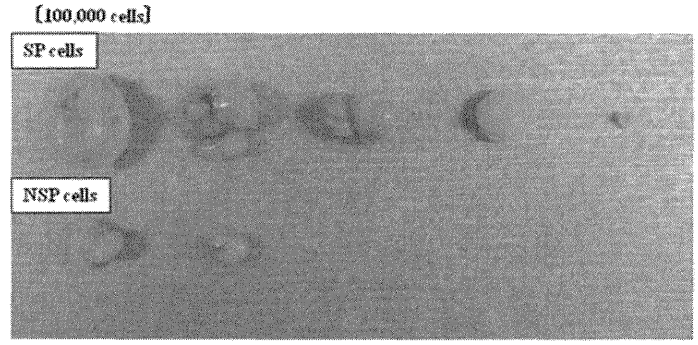


*P:<0.05

c

Tumorigenicity in NOD/SCID mice

Cell type	Injected cell number				
	1,000 cells	5,000 cells	10,000 cells	50,000 cells	100,000 cells
SP	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5 (80%)	5/5 (100%)
NSP	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	2/5 (40%)



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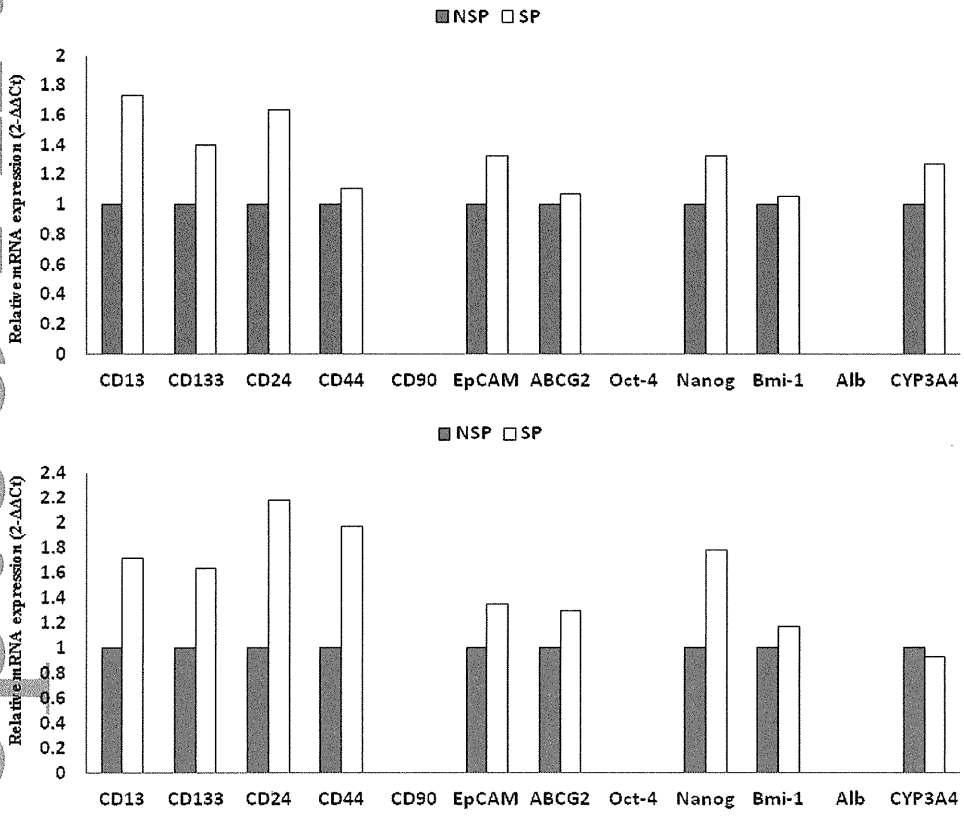

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Antiproliferative effects of sorafenib and pegylated IFN-α2b on human liver cancer cells *in vitro* and *in vivo*

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Abstract. Novel therapeutic strategies are needed to treat patients with advanced hepatocellular carcinoma (HCC). Combination therapy of sorafenib and type I interferon (IFN) has substantial activity in patients with metastatic renal cell carcinoma. We investigated the antiproliferative effects of sorafenib in combination with pegylated interferon-α2b (PEG-IFN-α2b) on human hepatocellular carcinoma (HCC) cells *in vitro* and *in vivo*. A poorly differentiated HCC cell line derived from a patient with hepatitis C virus infection, HAK-1B and the moderately differentiated HCC cell line KIM-1 were used in this study. We demonstrated a synergistic antiproliferative effect of combination therapy on HAK-1B cells *in vitro*. In the *in vivo* study, a significant reduction of tumor volume and weight were observed in the combination group in both HAK-1B and KIM1 tumors, although synergistic effects were not clearly observed. The density of CD34-positive microvessels was significantly lower and cleaved caspase-3-positive apoptotic cell numbers were higher, in the sorafenib group and the combination group compared to the control or PEG-IFN-α2b group in both HAK-1B and KIM-1 tumors. Ki67 labeling index was significantly lower in the combination group compared to the control group in KIM-1 tumors. In conclusion, our results suggest that the combination therapy may be more effective for the treatment of HCC cases with variable sensitivity to antitumor effects of single therapy with either sorafenib or PEG-IFN-α2b.

Introduction

Primary liver cancer, of which hepatocellular carcinoma (HCC) represents the major subtype accounting for between 85 and 90%, is the sixth most common tumor globally and the third most common cause of cancer-related death (1). Systemic treatment options for advanced HCC are limited and most deaths occur within 1 year of diagnosis (2-4).

Sorafenib is an oral multikinase inhibitor that was approved by the US Food and Drug Administration in December 2005 for the treatment of advanced renal cell carcinoma (RCC) and in November 2007 for the treatment of HCC. It has been shown to inhibit the activity of Raf kinase and several receptor tyrosine kinases, including vascular endothelial growth factor receptors (VEGFR)-1, 2 and 3, platelet-derived growth factor receptor (PDGFR)-α and β, FLT3, Ret and c-Kit. The intracellular signaling pathway Raf/MEK/ERK and the extracellular receptors VEGFR and PDGFR have been implicated in the molecular pathogenesis of HCC (5).

The Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial revealed efficacy of sorafenib in the treatment of HCC, i.e., both median survival and time to progression showed 3-month improvements by sorafenib therapy (6). Cheng *et al* (7) also reported the efficacy of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma. Combination therapy with sorafenib has a potential to improve the outcome of sorafenib monotherapy. Phase II trial of combination therapy of sorafenib and IFN-α has substantial activity in patients with metastatic RCC (8,9). The combination therapy of IFN-α and 5-fluorouracil is partly or completely effective in about 50% of the patients with advanced HCC (10). Type I interferon (IFN) has various effects, including anti-viral effects, antiproliferative effects and anti-angiogenic effects (11), and our laboratory previously reported the antiproliferative effect of IFN-α on human liver cancer cells *in vitro* and *in vivo* (12-14). In addition, type I IFN has suppressive effects on the occurrence of HCC, and the recurrence of HCC after curative treatment in patients with chronic hepatitis C virus infection (15-20). On the basis of above-described background, our current study examined the growth inhibitory effects of combination treatment of sorafenib and Pegylated IFN-α2b (PEG-IFN-α2b) on human HCC cell lines *in vitro* and *in vivo*.

Materials and methods

Cell line and cell cultures. This study used two HCC cell lines [KIM-1 (21) and HAK-1B (22)], which were originally established and characterized in our laboratory and previously confirmed to retain morphological and functional characte-

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Key words: hepatocellular carcinoma, pegylated interferon-α2b, sorafenib, combination therapy, microvessel density

ristics of the original tumor. Both of these two cell lines were established from surgically resected HCC nodules. KIM-1 is a moderately differentiated HCC cell line, and HAK-1B is a poorly differentiated HCC cell line which was derived from a patient with hepatitis C virus (HCV) infection.

The cells were grown in Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 2.5% heat-inactivated (56˚C, 30 min) fetal bovine serum (FBS, Bioserum, Victoria, Australia), 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco-BRL/Life Technologies Inc., Gaithersburg, MD, USA) and 12 mmol/l sodium bicarbonate, in a humidified atmosphere of 5% CO_2 in air at 37˚C.

Sorafenib and pegylated IFN-α2b. Sorafenib, kindly provided by Bayer Pharmaceutical Corporation (West Haven, CT, USA), was dissolved in dimethyl sulfoxide (DMSO) to create a 10 mM stock solution and stored at -20˚C for *in vitro* study. For the *in vivo* study, we prepared the solution at time of use.

PEG-IFN-α2b (PEG Intron®) was kindly provided by MSD K.K. (Tokyo, Japan). The specific activity of PEG-IFN-α2b was $6.4x10^7$ IU/mg protein.

Effect of sorafenib alone or combination treatment of sorafenib and PEG-IFN-α2b on the proliferation of HCC and CHC cell lines in vitro. The effects of sorafenib and/or PEG-IFN-α2b on the growth of the cultured cells were examined with colorimetry using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kits (Chemicon International Inc.) as described (12-14). Briefly, the cells ($1.5\text{-}5.5x10^3$ cells per well) were seeded on 96-well plates (Nunc Inc., Roskilde, Denmark), cultured for 24 h, and the culture medium was changed to a new one containing 0.2% DMSO (control) or sorafenib (0.3125, 0.625, 1.25, 2.5, 5, 10 or 20 μM), or both sorafenib (0, 1.25, 2.5 or 5 μM) and PEG-IFN-α2b (0, 2,000, 4,000, 8,000 IU/ml) (constant-ratio combination). After culturing for 72 h, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength at 570 nm and the reference wavelength at 630 nm. To keep the optical density within linear range, all experiments were performed while the cells were in the logarithmic growth phase.

Combination analysis was performed by using the method as described by Chou and Talalay (23), and the CalcuSyn software program (Biosoft, Cambridge, UK) for automated analysis. This program calculates the combination index (CI). A CI of 0.9-1.1 indicates a nearly additive effect, a CI of <0.9 a synergistic effect, a CI of >1.1 an antagonistic effect.

Morphological observation. For morphological observation under a light microscope, cultured HAK-1B cells were seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with or without 1.25 μM of sorafenib for 72 h, fixed for 10 min in Carnoy's solution, and stained with hematoxylin and eosin (H&E).

Quantitative analysis of apoptotic cells induced by sorafenib and/or PEG-IFN-α2b. HAK-1B and KIM-1 were cultured with the culture medium containing 0.02% DMSO or 2 μM of sorafenib for 72 h. For a study of combination therapy, HAK-1B cells were cultured with sorafenib (1.25 μM) or PEG-IFN-α2b (2,000 IU/ml), or both sorafenib (1.25 μM) and PEG-IFN-α2b (2,000 IU/ml) for 72 h. After incubation, the cells were stained with the Annexin V-EGFP (enhanced green fluorescent protein) using Apoptosis Detection Kits (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA), and the rate of Annexin V-EGFP-positive apoptotic cells was determined.

Effects of sorafenib and/or PEG-IFN-α2b on HCC cell proliferation in nude mice. This experiment was approved by the institutional committee for animal experiments and conducted according to the 'Guide for the Care and Use of Laboratory Animals' published and revised by the National Institute of Health in 1985.

Cultured HAK-1B or KIM-1 cells ($1.0x10^6$ cells/mouse) were transplanted subcutaneously (s.c.) to 4-week-old female BALB/c athymic nude mice (Clea Japan Inc., Osaka, Japan). On the 7th day when tumor size became 5 to 10 mm in diameter (day 0), the mice were divided into four groups (n=8 each) in a manner to equalize the mean tumor diameter of every group. Each group was assigned to one of the four treatments: i) control; ii) PEG-IFN-α2b alone; iii) sorafenib alone; and iv) sorafenib + PEG-IFN-α2b (combination).

Sorafenib was diluted with 12.5% Cremophor EL/12.5% ethanol/75% water for oral dosing in mice. Sorafenib (200 μg/day) was administered by tube feeding once a day for 14 days. PEG-IFN-α2b (1,920 IU) was subcutaneously injected twice a week for 14 days (days 1, 4, 8 and 11). In the control and the sorafenib alone groups, 0.1 ml of medium as the replacement of PEG-IFN-α2b was injected subcutaneously twice a week. In the control and the PEG-IFN-α2b alone groups, 0.2 ml of Cremophor EL/ethanol/water (12.5/12.5/75) as the replacement of sorafenib was administered by tube feeding once a day. The dose of sorafenib (200 μg) in the ratio to the average bodyweight of a mouse (20 g) was 10 mg/kg and this is almost comparable to a clinical dose (800 mg total daily dose). The clinical dose of PEG-IFN-α2b in chronic hepatitis C is 96,000 IU/kg per week. Because of species difference and different target which is not virus, but tumor, we used twice the dose per week in nude mice.

Tumor size was measured in two directions using calipers, and tumor volume (mm^3) was estimated by using the equation: length x $(width)^2$ x 0.5. This measurement was performed every two days. Mouse body weight was measured on days 0, 7 and 14. Mouse was sacrificed and the tumor was resected the next day after the completion of the 14-day treatment (day 15). The resected tumor was fixed in formalin after the weight measurement, prepared into paraffin sections, and underwent HE staining and immunohistochemistry.

Immunohistochemistry. Paraffin-embedded tissue samples were cut into 4-μm sections. Anti-mouse CD34 (Rat monoclonal, MEC14.7, Abcam, Cambridge, UK) (1:50 dilution) and Ki67 (Rabbit monoclonal, SP6, Abcam, Cambridge, UK) (1:100 dilution) staining were performed by standard avidin-biotin-peroxidase complex method and 3,3'-diaminobenzidine (DAB) solution was used for color development. Cleaved caspase-3 (rabbit polyclonal antibody, Cell Signaling Technologies, Beverly, MA, USA) (1:250 dilution) staining was

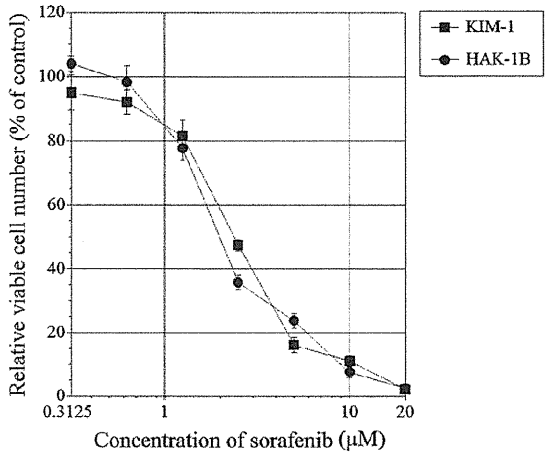

Figure 1. Seventy-two hours after adding 0.3125, 0.625, 1.25, 2.5, 5, 10 or 20 μM of sorafenib. Cell proliferation was suppressed in a dose-dependent manner in both KIM-1 and HAK-1B cell lines. The suppression was significant (P<0.001-0.05) in the range of 0.625-20 μM of sorafenib in KIM-1, 1.25-20 μM in HAK-1B. A total of 50% growth inhibitory dose was 2.5 μM in KIM-1 and 2.1 μM in HAK-1B. The values represent mean ± SD.

performed on the Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA) to detect the apoptotic cells. This automated system uses the streptavidin-biotin complex method with DAB as a chromogen (Ventana iView DAB detection kit).

Microvessel density (MVD) was evaluated within the tumor according to a modified method introduced by Tanigawa *et al* (24). Briefly the slides stained with CD34 were screened at low power field (x40 or x100) and the two or three most vascular areas were selected. Microvessel counts of these areas were performed at high power field (x200, 0.74 mm^2). All positive stained cells were counted as microvessels and every 40 μm length of vessel lumen was calculated as one point. The average microvessel counts of selected areas were regarded as MVD, which was expressed as the absolute number of microvessels per 0.74 mm^2. Immunohistochemically, cleaved caspase-3 was expressed perinuclearly and Ki67 was on the nuclear. The rate of apoptotic cells and Ki67 labeling index were evaluated by calculating the rate of cleaved caspase-3-positive cells and Ki67-positive cells, respectively.

Statistical analysis. Comparisons of estimated tumor volume and colorimetric cell growth were performed using two-factor factorial ANOVA and Student's t-test, respectively. The other data comparisons were performed using the Mann-Whitney U test.

Results

Effect of sorafenib alone or combination treatment of sorafenib and PEG-IFN-α2b on the proliferation of HAK-1B or KIM-1 HCC cells in vitro. Seventy-two hours after the addition of sorafenib, the relative viable cell number was suppressed in both HAK-1B and KIM-1 cell lines in a dose-dependent manner (Fig. 1). The 50% inhibitory concentration (IC_{50}) was 2.1 μM for HAK-1B and 2.5 μM for KIM-1.

Seventy-two hours after the addition of PEG-IFN-α2b and sorafenib, the relative viable cell number was suppressed to various degrees. The results are shown in Fig. 2. In HAK-1B cell line (Fig. 2A), significant difference in the relative viable cell number was observed between combination group and sorafenib or PEG-IFN-α2b alone groups, additionally, CI in all combination of PEG-IFN-α2b and sorafenib was <0.9. The CI was 0.879 in the combination of 2,000 IU/ml of PEG-IFN-α2b and

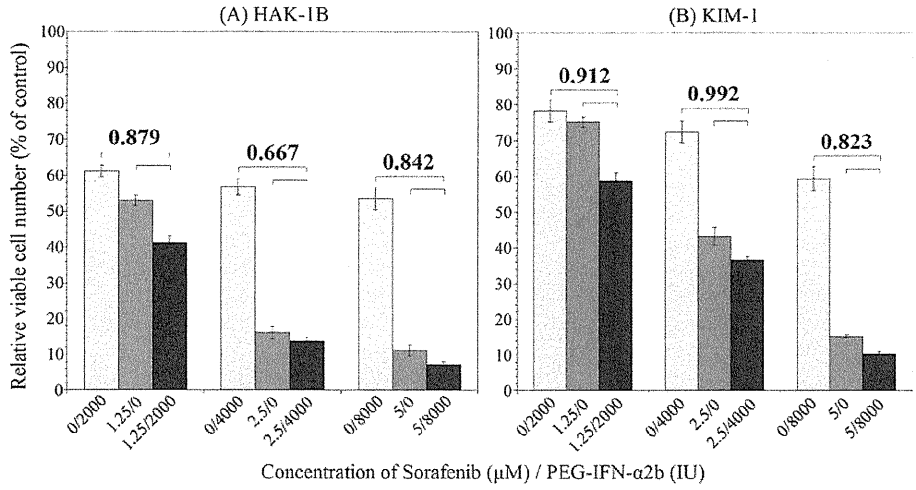

Figure 2. Effect of PEG-IFN-α2b and/or sorafenib on the proliferation of human HCC cell lines (A) HAK-1B and (B) KIM-1 in culture for 72 hours. Light gray bars are PEG-IFN-α2b alone group, dark gray bars sorafenib alone group, and black bars PEG-IFN-α2b + sorafenib group. All combination groups showed significant difference compared with monotherapy groups. The numbers above bars are CI. A CI of 0.9-1.1 indicates a nearly additive effect, a CI of <0.9 a synergistic effect, a CI of >1.1 an antagonistic effect. Representative data of two independent experiments are shown. The values represent mean ± SD.

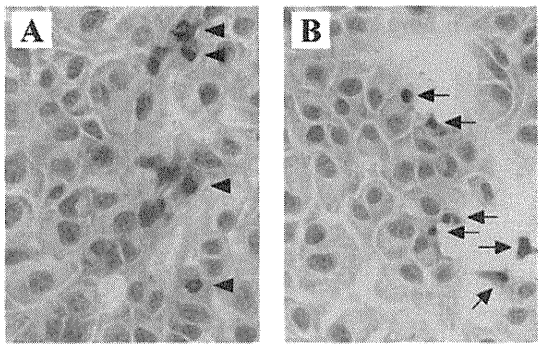

Figure 3. Photomicrograph of HAK-1B cells cultured for 72 h on Lab-Tek Chamber slide. (A) Without sorafenib in culture medium. Some mitotic figures were noted (arrowheads). (B) With 1.25 μM of sorafenib in culture medium. There were some apoptotic cells characterized by cytoplasmic shrinkage and nuclear chromatin condensation (arrows).

1.25 μM of sorafenib, 0.667 in 4,000 IU/ml of PEG-IFN-α2b and 2.5 μM of sorafenib, and 0.842 in 8,000 IU/ml of PEG-IFN-α2b and 5.0 μM of sorafenib. According to the definition of the CI, these results indicate that a combination of PEG-IFN-α2b and sorafenib may produce a synergistic growth inhibitory effect in HAK-1B cell line. In KIM-1 cell line (Fig. 2B), there was also a significant difference in the relative viable cell numbers between combination group and monotherapy groups. The CI was 0.912 in the combination of 2,000 IU/ml of PEG-IFN-α2b and 1.25 μM of sorafenib, 0.992 in 4,000 IU/ml of PEG-IFN-α2b and 2.5 μM of sorafenib, and 0.823 in 8,000 IU/ml of PEG-IFN-α2b and 5.0 M of sorafenib. These results indicate that combination therapy may produce an additive or synergistic growth inhibitory effect in KIM-1 cell line.

Morphologically, HAK-1B cells showed characteristic features of apoptosis, such as cytoplasmic shrinkage and nuclear chromatin condensation at 72 h after adding 1.25 μM of sorafenib (Fig. 3).

Table I. The weight of subcutaneous tumors of HAK-1B cells or KIM-1 cells in nude mice at sacrifice.

	Tumor weight (g)	
Treatment group	HAK-1B	KIM-1
Control	0.333±0.03	0.504±0.17
PEG-IFN-α2b alone	0.078±0.02[a]	0.379±0.18
Sorafenib alone	0.236±0.06	0.206±0.04[c]
PEG-IFN-α2b + sorafenib	0.113±0.04[b]	0.185±0.12[c]

Tumor weight represents mean ± SE (g). [a]P<0.0001 vs. control, P<0.05 vs. sorafenib alone. [b]P<0.001 vs. control. [c]P<0.05 vs. control.

The rate of Annexin V-EGFP positive apoptotic cells was increased by adding 2 μM of sorafenib in HAK-1B cells (5.8% of the control and 37.8% of the sorafenib). In KIM-1 cells, however, the increase was relatively small (7.9% of the control and 9.5% of the sorafenib) (Fig. 4A). In another setting, the combination group with PEG-IFN-α2b showed higher rate of apoptosis than control or monotherapy groups in HAK-1B (4.8% of control, 37.4% of the PEG-IFN-α2b, 14.3% of the sorafenib, 42.8% of the combination) (Fig. 4B).

Effects of sorafenib and/or PEG-IFN-α2b on HAK-1B or KIM-1 cell proliferation in nude mice. Chronological changes in estimated tumor volume after subcutaneous injection of cultured HAK-1B cells or KIM-1 cells to nude mice are summarized in Fig. 5. The actual tumor weights at the time of sacrifice are shown in Table I. In the experiment of HAK-1B tumors, the tumor volume of mice receiving PEG-IFN-α2b, sorafenib, and sorafenib+PEG-IFN-α2b was 34, 73 and 36%, respectively, of the control volume and the tumor weight was 23, 71 and 34%, respectively, of the control weight. Statistically, there were significant differences both in tumor volume and weight

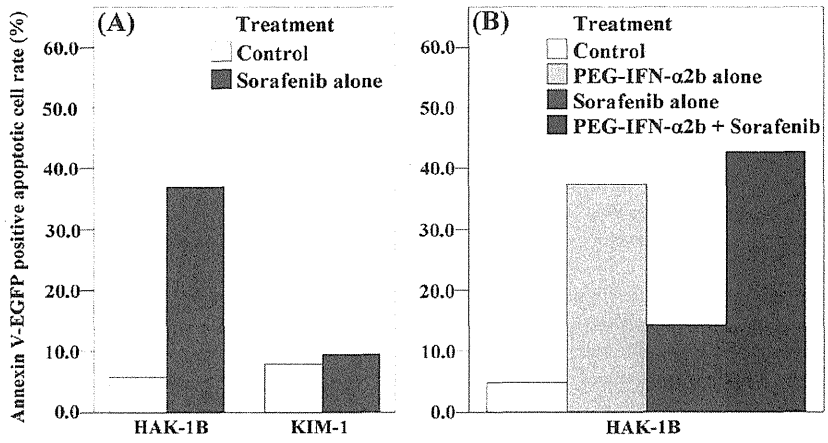

Figure 4. Quantitative analysis of Annexin V-EGFP positive apoptotic cells. (A) Apoptosis of HAK-1B or KIM-1 cells induced by 2 μM of sorafenib. (B) Apoptosis of HAK-1B cells induced by 2,000 IU/ml of PEG-IFN-α2b and/or 1.25 M of sorafenib. Representative data of three independent experiments are shown.

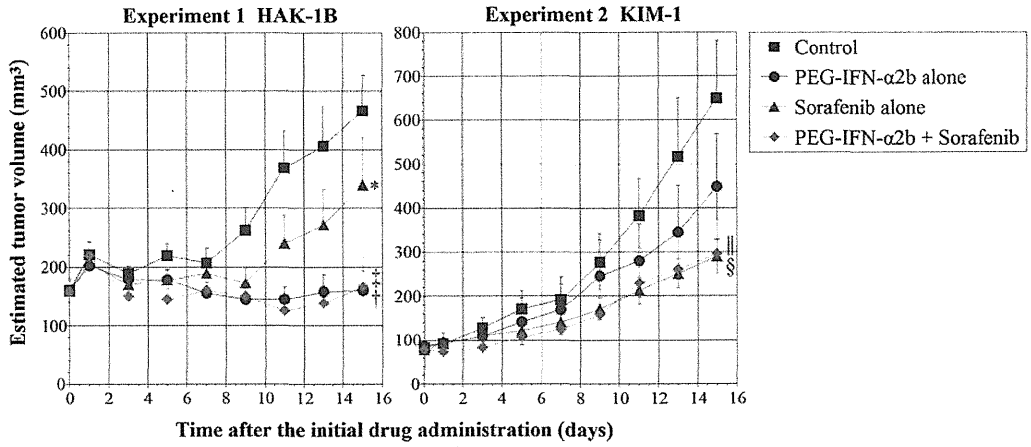

Figure 5. Chronological changes on the estimated volume of HAK-1B (Experiment 1) or KIM-1 (Experiment 2) tumor that was developed subcutaneously on nude mice. The PEG-IFN-α2b alone group (●) received subcutaneous injection of 1,920 IU twice a week for 14 days. The sorafenib alone group (▲) received 10 mg/kg/mouse/day orally every day for 14 days. The PEG-IFN-α2b + sorafenib group (◆) received 1,920 IU of PEG-IFN-α2b twice a week and 10 mg/kg of sorafenib every day for 14 days. The control group (■) received subcutaneous injection of 0.1 ml of medium twice a week and 0.2 ml of Cremophor EL/ethanol/water (12.5/12.5/75). The values represent mean ± SE. *P<0.05 vs. control. †P<0.0001 vs. control, P<0.005 vs. sorafenib alone. ‡P<0.0001 vs. control, P<0.001 vs. sorafenib alone. §P<0.001 vs. control. ‖P<0.0001 vs. control.

Table II. MVD and the ratio of apoptotic cells and Ki67 positive cells in human HCC tumors subcutaneously transplanted in nude mice.

Cell line	Treatment group	MVD	Apoptotic cells	Ki67 positive cells
HAK-1B	Control	100.8±7.7	3.8±0.4	36.8±2.0
	Peg-IFN-α2b alone	114.9±16.7	4.4±0.4	37.5±4.6
	Sorafenib alone	53.8±4.3[a]	6.7±1.3[b]	38.3±2.0
	Peg-IFN-α2b + soragfenib	69.4±10.1[b]	5.6±1.3[b]	35.3±2.2
KIM-1	Control	125.9±16.2	4.6±0.4	6.7±0.2
	Peg-IFN-α2b alone	97.4±10.4	5.1±0.4	7.5±0.8
	Sorafenib alone	85.1±6.6[b]	6.5±0.7[b]	5.7±0.4
	Peg-IFN-α2b + soragfenib	79.0±7.2[b]	6.3±0.6[b]	4.6±0.5[c]

Scores represent mean ± SE. [a]P<0.0005 vs. control, P<0.01 vs. Peg-IFN-α2b alone. [b]P<0.05 vs. control. [c]P<0.005 vs. control, P<0.05 vs. Peg-IFN-α2b alone.

between the control group and the PEG-IFN-α2b alone group (P<0.0001 vs. control in tumor volume, P<0.0001 vs. control in tumor weight) or the combination group (P<0.0001 vs. control in tumor volume, P<0.001 vs. control in tumor weight) and between the sorafenib alone group and the PEG-IFN-α2b alone group (P<0.005 vs. sorafenib alone in tumor volume, P<0.05 vs. sorafenib alone in tumor weight). Although there was a significant difference between the sorafenib alone group and the combination group in tumor volume (P<0.001), this was not the case in the actual tumor weight (P=0.099). In the experiment of KIM-1 tumors, the tumor volume of mice receiving PEG-IFN-α2b, sorafenib, and sorafenib+PEG-IFN-α2b was 69, 45 and 46%, respectively, of the control volume and the tumor weight was 75, 41 and 37%, respectively, of the control weight. Statistically, there were significant differences in both tumor volume and weight between the control and the sorafenib alone group (P<0.0001 vs. control in tumor volume, P<0.05 vs. control in tumor weight) or the combination group (P<0.001 vs. control in tumor volume, P<0.05 vs. control in tumor weight).

The results of immunohistochemical examination are summarized in Table II. The significant decrease of MVD and increase of apoptotic cells were observed in the sorafenib group (P<0.0005 and 0.05 respectively vs. control in HAK-1B, P<0.05 and 0.05 respectively vs. control in KIM-1) and the combination group (P<0.05 and 0.05 respectively vs. control in HAK-1B, P<0.05 and 0.05, respectively, vs. control in KIM-1) compared to the control group in both HAK-1B and KIM-1 tumors, although there was no significant difference between the combination group and monotherapy groups. Ki67 labeling index was significantly lower in the combination group (P<0.005 vs. control, P<0.05 vs. PEG-IFN-α2b group) than in the control group or the PEG-IFN-α2b group only in KIM-1.

Discussion

In this study, we showed the synergistic effect of sorafenib and PEG-IFN-α2b on HAK-1B cells *in vitro*. We previously reported that PEG-IFN-α2b induced apoptosis on both HAK-1B and KIM-1 cells *in vitro* (14). We found that sorafenib also induced apoptosis on HAK-1B *in vitro*. On the other hand, the increase of apoptotic cells was not clearly observed on KIM-1 cells in spite of the fact that the proliferation of KIM-1 cells was inhibited by sorafenib in MTT assay. A possible explanation is that cell proliferation might be inhibited by other antiproliferative mechanisms. The blockade of Raf signaling which is the main effect of sorafenib can lead to the repression of transforming growth factor α-epidermal growth factor receptor autocrine loops of tumor cells (5). Such a mechanism could have inhibited the growth of KIM-1 cells. In addition, a limitation of *in vitro* study is that we are not able to assess the indirect anti-angiogenic effect against endothelial cells.

In the *in vivo* study, there was a significant reduction of tumor volume and weight in the combination group on both HAK-1B and KIM-1 tumors compared with the control group. However, there was no significant difference between the combination and the monotherapy groups, and it seemed that HAK-1B tumors were sensitive to PEG-IFN-α2b and KIM-1 tumors to sorafenib. Only in KIM-1 tumors that might be sensitive to sorafenib, Ki67 labeling index was lower in the combination group than in the control group. Recently Wang *et al* (25) reported that combination therapy of sorafenib with recombinant human INF-α2a was effective *in vitro* and *in vivo* on two HCC cell lines, Huh-7 and Sk-Hep-1. In their study, the significant differences between combination and monotherapy groups were clearly observed. This partial difference might be due to the different experimental settings, such as different cell lines and different dose of drugs. One of the greatest differences, we surmise, is the site of IFN administration. They injected IFN directly into subcutaneous tumors, whereas we did subcutaneously but not into the tumors.

Since sorafenib is a multikinase inhibitor, it is considered that sorafenib has both direct antiproliferative effect due to the blockade of Raf kinase on tumor cells themselves and indirect effect due to the blockade of receptor tyrosine kinases, such as VEGFR-2, on endothelial cells followed by the inhibition of angiogenesis (5). Therefore we also evaluated MVD of xenografts and confirmed the significant decrease of MVD in the sorafenib alone and the combination group in both HAK-1B and KIM-1 tumors. It has been repeatedly shown that IFN suppresses the growth of various types of human tumors that were transplanted into mice through the anti-angiogenic effect. Tedjarati *et al* (26) reported that the subcutaneous injection of 7,000 IU per week of PEG-IFN-α2b into nude mice bearing human ovarian cancer cells induced a significant decrease of CD31-positive endothelial cells and Huang *et al* (27) showed similar results with the subcutaneous injection of 70,000 IU per week of PEG-IFN-α2b on human prostate cancer cells. PEG-IFN-α2b administered at higher or lower doses was less effective. In our current study, however, there was no significant decrease of MVD in the PEG-IFN-α2b group compared with the control group. Moreover, in our previous report, the decrease of artery-like blood vessels was not observed in the same HAK-1B tumors by the administration of PEG-IFN-α2b at either higher or lower doses (14).

Another notable finding regarding the MVD in this study is the discrepancy between MVD and tumor weight or size. Interestingly, the reduction of tumor weight and size was not so much in sorafenib monotherapy group in HAK-1B tumors despite the most prominent decrease of MVD was observed in this group. On the other hand, there was a significant reduction of tumor weight and size in PEG-IFN-α2b alone group in HAK-1B, although this group did not show any significant decrease of MVD. This result supports our previous findings in which we showed there was no relationship between tumor shrinkage and the number of artery-like blood vessels in HAK-1B tumors after the administration of the various concentration of PEG-IFN-α2b (14). Hlatky *et al* (28) mentioned in their review article that the efficacy of anti-angiogenic agents cannot be simply visualized by alterations in microvessel density during treatment because of the difference of the tightness of the coupling between vessel drop-out and tumor-cell drop-out after the treatment. In addition, Yao *et al* (29) recently reported that the expression of VEGFR-1 in tumor cells which is normally expressed specifically in endothelial cells were strongly associated with anti-PlGF antibody efficacy, but not with anti-angiogenesis. More studies are needed to investigate new approaches to assess the efficacy of anti-angiogenic drugs *in vivo* and molecular mechanisms of their action of 'anti-angiogenic' drugs.

In conclusion, we demonstrated the synergistic antiproliferative effect of combination therapy on HAK-1B cells *in vitro*. Although, *in vivo* the synergistic effects of the combination therapy were not clearly observed, the combination therapy induced nearly maximal antitumor effects, independent of the HCC cell sensitivity to antitumor effects of single therapy with either PEG-IFN-α2b or sorafenib. These findings suggest that PEG-IFN-α2b might be a promising candidate for use in combination therapy with sorafenib and warrant further investigation.

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References

1. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2008.
2. Llovet JM, Burroughs A and Bruix J: Hepatocellular carcinoma. Lancet 362: 1907-1917, 2003.
3. Bruix J and Sherman M: Management of hepatocellular carcinoma. Hepatology 42: 1208-1236, 2005.
4. Llovet JM, Bustamante J, Castells A, Vilana R, Ayuso Mdel C, Sala M, Brú C, Rodés J and Bruix J: Natural history of untreated nonsurgical hepatocellular carcinoma: rationale for the design and evaluation of therapeutic trials. Hepatology 29: 62-67, 1999.
5. Wilhelm SM, Adnane L, Newell P, Villanueva A, Llovet JM and Lynch M: Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol Cancer Ther 7: 3129-3140, 2008.

6. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D and Bruix J; SHARP Investigators Study Group: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
7. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D and Guan Z: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 10: 25-34, 2009.
8. Gollob JA, Rathmell WK, Richmond TM, Marino CB, Miller EK, Grigson G, Watkins C, Gu L, Peterson BL and Wright JJ: Phase II trial of sorafenib plus interferon alfa-2b as first- or second-line therapy in patients with metastatic renal cell cancer. J Clin Oncol 25: 3288-3295, 2007.
9. Escudier B, Szczylik C, Hutson TE, Demkow T, Staehler M, Rolland F, Negrier S, Laferriere N, Scheuring UJ, Cella D, Shah S and Bukowski RM: Randomized phase II trial of first-line treatment with sorafenib versus interferon alfa-2a in patients with metastatic renal cell carcinoma. J Clin Oncol 27: 1280-1289, 2009.
10. Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, Kawata S, Imai Y, Iijima S and Monden M: Combined intraarterial 5-fluorouracil and subcutaneous interferon-α therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. Cancer 94: 435-442, 2002.
11. Pestka S, Langer JA, Zoon KC and Samuel CE: Interferons and their actions. Annu Rev Biochem 56: 727-777, 1987.
12. Yano H, Iemura A, Haramaki M, Ogasawara S, Takayama A, Akiba J and Kojiro M: Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. Hepatology 29: 1708-1717, 1999.
13. Hisaka T, Yano H, Ogasawara S, Momosaki S, Nishida N, Takemoto Y, Kojiro S, Katafuchi Y and Kojiro M: Interferon-αCon1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. J Hepatol 41: 782-789, 2004.
14. Yano H, Ogasawara S, Momosaki S, Akiba J, Kojiro S, Fukahori S, Ishizaki H, Kuratomi K, Basaki Y, Oie S, Kuwano M and Kojiro M: Growth inhibitory effects of pegylated IFN alpha-2b on human liver cancer cells in vitro and in vivo. Liver Int 26: 964-975, 2006.
15. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H and Kawanishi M: Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. Hepatology 29: 1124-1130, 1999.
16. Ikeda K, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, Tsubota A, Chayama K, Murashima N and Kumada H: Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer. Hepatology 32: 228-232, 2000.
17. Mazzella G, Accogli E, Sottili S, Festi D, Orsini M, Salzetta A, Novelli V, Cipolla A, Fabbri C, Pezzoli A and Roda E: Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. J Hepatol 24: 141-147, 1996.
18. Nishiguchi S, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K and Otani S: Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 346: 1051-1055, 1995.
19. Nishiguchi S, Tamori A and Kubo S: Effect of long-term postoperative interferon therapy on intrahepatic recurrence and survival rate after resection of hepatitis C virus-related hepatocellular carcinoma. Intervirology 48: 71-75, 2005.
20. Sakaguchi Y, Kudo M, Fukunaga T, Minami Y, Chung H and Kawasaki T: Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. Intervirology 48: 64-70, 2005.
21. Murakami T: Establishment and characterization of human hepatoma cell line (KIM-1). Acta Hepatol Jpn 25: 532-539, 1984.
22. Yano H, Iemura A, Fukuda K, Mizoguchi A, Haramaki M and Kojiro M: Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. Hepatology 18: 320-327, 1993.
23. Chou TC and Talalay P: Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27-55, 1984.
24. Tanigawa N, Lu C, Mitsui T and Miura S: Quantification of sinusoid-like vessels in hepatocellular carcinoma: its clinical and prognostic significance. Hepatology 26: 1216-1223, 1997.
25. Wang L, Jia D, Duan F, Sun Z, Liu X, Zhou L, Sun L, Ren S, Ruan Y and Gu J: Combined anti-tumor effects of IFN-α and sorafenib on hepatocellular carcinoma in vitro and in vivo. Biochem Biophys Res Commun 422: 687-692, 2012.
26. Tedjarati S, Baker CH, Apte S, Huang S, Wolf JK, Killion JJ and Fidler IJ: Synergistic therapy of human ovarian carcinoma implanted orthotopically in nude mice by optimal biological dose of pegylated interferon alpha combined with paclitaxel. Clin Cancer Res 8: 2413-2422, 2002.
27. Huang SF, Kim SJ, Lee AT, Karashima T, Bucana C, Kedar D, Sweeney P, Mian B, Fan D, Shepherd D, Fidler IJ, Dinney CP and Killion JJ: Inhibition of growth and metastasis of orthotopic human prostate cancer in athymic mice by combination therapy with pegylated interferon-alpha-2b and docetaxel. Cancer Res 62: 5720-5726, 2002.
28. Hlatky L, Hahnfeldt P and Folkman J: Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 94: 883-893, 2002.
29. Yao J, Wu X, Zhuang G, Kasman IM, Vogt T, Phan V, Shibuya M, Ferrara N and Bais C: Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. Proc Natl Acad Sci USA 108: 11590-11595, 2011.

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Pegylated Interferon-α2a Inhibits Proliferation of Human Liver Cancer Cells *In Vitro* and *In Vivo*

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Abstract

Purpose: We investigated the effects of pegylated interferon-α2a (PEG-IFN-α2a) on the growth of human liver cancer cells.

Methods: The effect of PEG-IFN-α2a on the proliferation of 13 liver cancer cell lines was investigated *in vitro*. Cells were cultured with medium containing 0–4,194 ng/mL of PEG-IFN-α2a, and after 1, 2, 3, or 4 days of culture, morphologic observation and growth assay were performed. After hepatocellular carcinoma (HCC) cells (HAK-1B and KIM-1) were transplanted into nude mice, various doses of PEG-IFN-α2a were subcutaneously administered to the mice once a week for 2 weeks, and tumor volume, weight, and histology were examined.

Results: PEG-IFN-α2a inhibited the growth of 8 and 11 cell lines in a time- and dose-dependent manner, respectively, although the 50% growth inhibitory concentrations of 7 measurable cell lines on Day 4 were relatively high and ranged from 253 ng/mL to 4,431 ng/mL. Various levels of apoptosis induction were confirmed in 8 cell lines. PEG-IFN-α2a induced a dose-dependent decrease in tumor volume and weight, and a significant increase of apoptotic cells in the tumor. Subcutaneous administration of clinical dose for chronic hepatitis C (3 μg/kg, 0.06 μg/mouse) was effective and induced about 30-50% reduction in the tumor volume and weight as compared with the control.

Conclusions: Although *in vitro* anti-proliferative effects of PEG-IFN-α2a were relatively weak, PEG-IFN-α2a induced strong anti-tumor effects on HCC cells *in vivo*. The data suggest potential clinical application of PEG-IFN-α2a for the prevention and treatment of HCC.

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Introduction

Interferons (IFNs) are types of cytokine that are produced by host cells, such as leukocytes, in response to inflammation. Since IFNs possess antiviral activity, antiproliferative activity and various immunoregulatory activities, IFN therapy is used to treat patients with chronic viral hepatitis or certain types of cancer including malignant melanoma, acquired immunodeficiency syndrome-related Kaposi's sarcoma and some hematopoietic malignancies [1,2]. Lai et al also showed that recombinant IFNα is useful in prolonging survival among patients with inoperable hepatocellular carcinoma (HCC) [3]. In addition, some studies showed IFN therapy might prevent either occurrence or recurrence after initial curative therapy of HCC, such as liver resection and radiofrequency ablation, in patient with chronic viral hepatitis [4–7]. This cancer preventive effect of IFNs is regarded mainly as results of their antiviral effect and the consequent suppression of inflammation, and might be due to their direct antitumor effect against clinically undetectable HCC as well. The detailed mechanism of the antitumor effect of IFNs, however, remains obscure.

Pegylated interferon-α2a (PEG-IFN-α2a) and pegylated interferon-α2b (PEG-IFN-α2b), which are used to treat patients with chronic hepatitis C virus (HCV) or B virus (HBV) infection, are modified IFNs that have longer serum half-life in body than non-pegylated forms of IFNs, therefore they can be given to