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Generation of a liver targeting fusion interferon and its bioactivity analysis *in vitro*

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The objective of this study was to generate a liver targeting fusion interferon, galactosyl-human serum albumin-interferon α2b (G-HSA-IFN) and to evaluate its bioactivity *in vitro* on HepG2.2.15 cells which express hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg). The cell proliferation was determined by Sulpho Rhodamine B (SRB) staining method and flow cytometry (FCM) assay. Hochest33342 and Propidium Iodide (PI) double staining and Western blot analysis of Bcl-2/Bax were also performed to evaluate cell lethality and apoptosis. The concentrations of HBsAg and HBeAg secreted in culture supernatant were detected using Enzyme-Linked Immunosorbent Assay (ELISA). The results demonstrated that G-HSA-IFN could inhibit the proliferation of HepG2.2.15 cells and the cell cycle was arrested at G_0/G_1 phase. Western blotting results showed that the expression of Bcl-2 was inhibited in a dose-dependent manner while the expression of Bax was enhanced. The expression of HBsAg was inhibited by G-HSA-IFN in a dose-dependent manner, while no significant inhibiting effect on the expression of HBeAg was observed. Conclusively, G-HSA-IFN could not only significantly inhibit the HBsAg expression and the proliferation of HepG2.2.15 cells, but also induce the apoptosis of the target cells, rendering it a promising drug candidate for hepatitis B.

1. Introduction

As one of the two main antiviral pharmaceuticals for Hepatitis B viral infections (HB), interferon alpha (IFN-α) was used to 'switch' the immune system into infection clearing or remission, suppressing HBV DNA (Jones et al. 2009). But as a protein drug, IFN has a half-life of approximately 8 h, and, consequently, a 3-times-weekly dosing schedule may be insufficient to maintain adequate serum concentrations (Wills 1990). To overcome its common shortcomings such as low stability, short half-life and fateful antigenicity, IFN was modified to prolong its circulating half-life. One way was to attach an inert polyethylene glycol polymer to the IFN-α molecule to produce a larger molecule and to prolong the biological effect and thus fewer injections were necessary (Grace et al. 2005; Harris and Chess 2003). Another way was to couple IFN with human serum albumin (HSA) using genetic engineering technology (Sung et al. 2003; Osborn et al. 2002).

Abbreviations: G-HSA-IFN, galactosyl-human serum albumin-interferon α2b; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; SRB, Sulpho Rhodamine B; FCM, flow cytometry; PI, Propidium Iodide; ELISA, Enzyme-Linked Immunosorbent Assay; HBV, hepatitis B virus, ASGP-R, asialoglycoprotein receptor, TEMED, Tetramethylethylenediamine, PBS, phosphate buffer saline, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electropheresis.

Although the half-life could be prolonged, the modified IFNs are concentrated poorly in the liver which leads two results. First, the low concentration of IFNs in the liver is inefficient to suppress HBV. Second, IFNs constantly keeping high level in peripheral blood in the long period of HB treatment might induce side effects.

Directing IFNs to the liver is a possible option for HB therapy. Asialoglycoprotein receptor (ASGP-R) is a good hepatic target and has been used successfully in many pharmacologic studies (Li et al. 2008). ASGP-R is hepatocyte membrane receptor which recognizes and binds serum galactose-terminatal glycoproteins and transports them to the cytoplasm (Hudgin and Ashwell 1974). Zhong et al. (1995) have modified interferon with galactosyl so the galactose-terminated IFN can be targeting to ASGP-R. However, the affinity with ASGP-R relies on the number of the galactosyl residues (Takamatsu et al. 2003). There are only 11 primary-amino groups on the IFN molecule. When galactosylating, the galactosyl residues of galactosyl-IFN usually prepared are only 2–3 and they are not enough to direct to the liver very well. HSA-IFN fusion protein which has 69 lysine residues can provide much more primary amino groups (70) to be modified and the product will target to the liver more efficiently.

In previous research, we successfully generated a liver targeted fusion interferon, galactosyl-human serum albumin-interferon-α2b (G-HSA-IFN) which specifically targeted the ASGP-R and

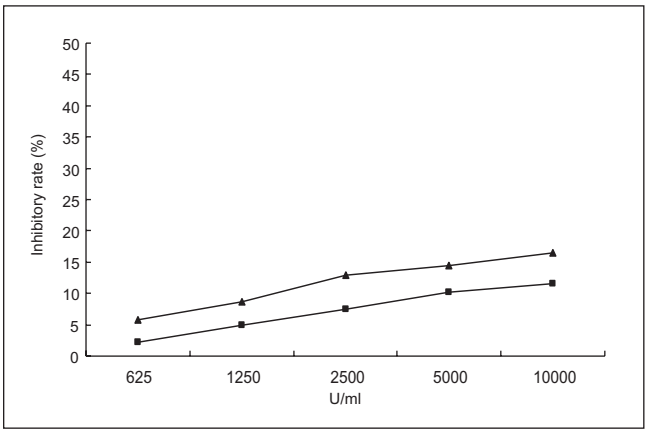

Fig. 1: Inhibitory effect of G-HSA-IFN on proliferation of HepG2.2.15 Inhibitory rate = $(1-A_{sample}/A_{control}) \times 100\,\%$ —■—3rd day; —▲—6th day

accumūlated effectively in the liver (> 45%/g) (Cai et al. 2009). To make a further research of its bioactivity, HepG2.2.15 cells which express HBsAg and HBeAg were used to evaluate the influence of G-HSA-IFN on HepG2.2.15 cells *in vitro*.

2. Investigations and results

2.1. Generation of G-HSA-IFN

G-HSA-IFN was prepared successfully with the methods reported. After separation with a PD-10 column, the product showed only one single main band with a molecular weight of about 91 kDa on SDS-PAGE and the average galactose residue number on each molecular was about 24. Biodistribution research in mice showed that the G-HSA-IFN prepared accumulated effectively in the liver (> 45%/g) (Cai et al. 2009).

2.2. Antiproliferation analysis

G-HSA-IFN in all the concentrations described (from 625 to 10000 U/ml) inhibited the proliferation of HepG2.2.15 cells. The inhibitory curves on the 3rd day and the 6th day post added drug were similar but the inhibitory effect on the 6th day was a little greater than that of on the 3rd day (Fig. 1, about 5.71% 16.42% and about 2.2% 11.57%, respectively).

2.3. Influence on the expression of HBsAg and HBeAg

G-HSA-IFN (from 625 to 10000 U/ml) obviously inhibited the expression of HBsAg in a dose-dependent manner. The inhibitory effect on the 6th day was greater than that on the 3rd day (Table, about 37.32%–90.49% and about 18.77%–41.46%, respectively, $p < 0.05$). The expression of HBeAg was gently inhibited by G-HSA-IFN with the concentrations we described no matter on the 3rd day or on the 6th day with the inhibitory rates were about 11.56%–22.16% and about 12.74%–25.06%, respectively. And there were no significant difference between the inhibitory rates on the 3rd day and on the 6th day (Table 1).

2.4. FCM Assay

After treatment with different concentrations of G-HSA-IFN for 6 days, the HepG2.2.15 cells were analyzed in a FACS caliber flow cytometry and analyzed with Modfit LT 3.2.1 software. And the results showed that the proportion of G_0/G_1 phase cells increased while the proportion of S phase cells reduced and it demonstrated that the cells might be blocked at G_0/G_1 phase by G-HSA-IFN (Fig. 2).

2.5. Cell apoptosis and death analysis with Double Staining

Treated with different concentrations of G-HSA-IFN 24 h later, the HepG2.2.15 cells were stained with Hochest33342 and PI. Observed with the fluorescence microscope, the normal cells were dyed with light blue and with the nuclear integrate. The apoptotic cells were dyed with clear blue or light red, and the nuclear was densely stained or broken to dense blocks while the dead cells were dyed with red. When the concentration of G-HSA-IFN was between 625 and 1250 U/ml, few apoptotic cells could be seen. With the concentration increased, the number of apoptotic cells increased too and there were few cells began to die (Fig. 3).

2.6. Western Blotting Analysis

Western Blotting analysis results with beta actin as internal reference confirmed that when the concentration of G-HSA-IFN was greater than 5000 U/ml, the concentration of Bcl-2, an antiapoptotic protein, was lower than that of control, while the concentration of pro-apoptotic protein, Bax, increased and was higher than that of control (Fig. 4).

3. Discussion

The discovery and molecular understanding of the cellular mechanisms and clinical use of interferons has been a major advance in biomedicine over the past 50 years (Borden et al. 2007). With the shortcomings as protein pharmaceuticals, the clinical application of conventional IFNs are limited. Recently, IFN was modified with a lot of methods to change its behaviors *in vivo*. IFN was also be modified with galactosyl (Zhong et al. 1995) to target the asialoglycoprotein receptor (ASGP-R), a good hepatic target on hepatocytes and has been used successfully in many pharmacologic studies (Aramaki et al. 2003). However, the affinity with ASGP-R relies on the number of the galactosyl residues (Takamatsu et al. 2003). There are only 11 primary-amino groups on the IFNα2b molecule. When galactosylating, the galactosyl residues of galactosyl-IFN prepared usually are only 2–3 and they are not enough to direct to the liver very well. HSA-IFNα2b fusion protein, a long-term interferon, which has 69 lysine residues can provide much more primary amino groups (it is 70) to be modified and the product will target to the liver more efficiently.

Imidate is a water-soluble reagent that reacts rapidly with primary amino residues of proteins and is used as a bifunctional reagent for attaching thiogalactose residues to proteins to produce amidines (Lee et al. 1976). We chose thiogalactose instead of galactose because it forms a much more stable thioether bond than galactose. The reaction of imidates with ε-amino groups or α-amino groups was moderate and little change in protein tertiary structure occurred after modification. In our previously research, the average nūmber of thiogalactose residues of each G-HSA-IFN molecular was about 24. G-HSA-IFN could bind to the asialoglycoprotein receptor (ASGP-R) on hepatic cells specifically and biodistribution research in mice showed it could concentrate effectively in the liver (> 45%/g) (Cai et al. 2009).

HBV is a stringent hepatotropic DNA virus. Its adhesion, as well as viral DNA transcription and replication, is strict with the host. The HepG2.2.15 cell line established by Sureau et al. (1986) was a human hepatoma cell line which could express HBsAg and HBeAg. It could be used as a cell culture system for

Table: Inhibitory effect of G-HSA-IFN on the expression of HBsAg and HBeAg in HepG2.2.15 cells (%)

Concentration (U/ml)	Inhibition rates (%; n = 6, X ± s)			
	HBsAg		HBeAg	
	The 3rd day	The 6th day	The 3rd day	The 6th day
625	18.77 ± 8.63	37.32 ± 5.42	11.56 ± 3.31	12.74 ± 2.88
1250	25.91 ± 12.68	39.10 ± 7.40	12.84 ± 3.26	14.29 ± 1.73
2500	33.75 ± 5.32	42.13 ± 2.61	18.20 ± 3.24	14.61 ± 4.51
5000	39.36 ± 6.32	71.93 ± 1.13	19.72 ± 2.80	16.91 ± 2.87
10000	41.46 ± 11.28	90.49 ± 0.63	22.16 ± 2.18	25.06 ± 3.25

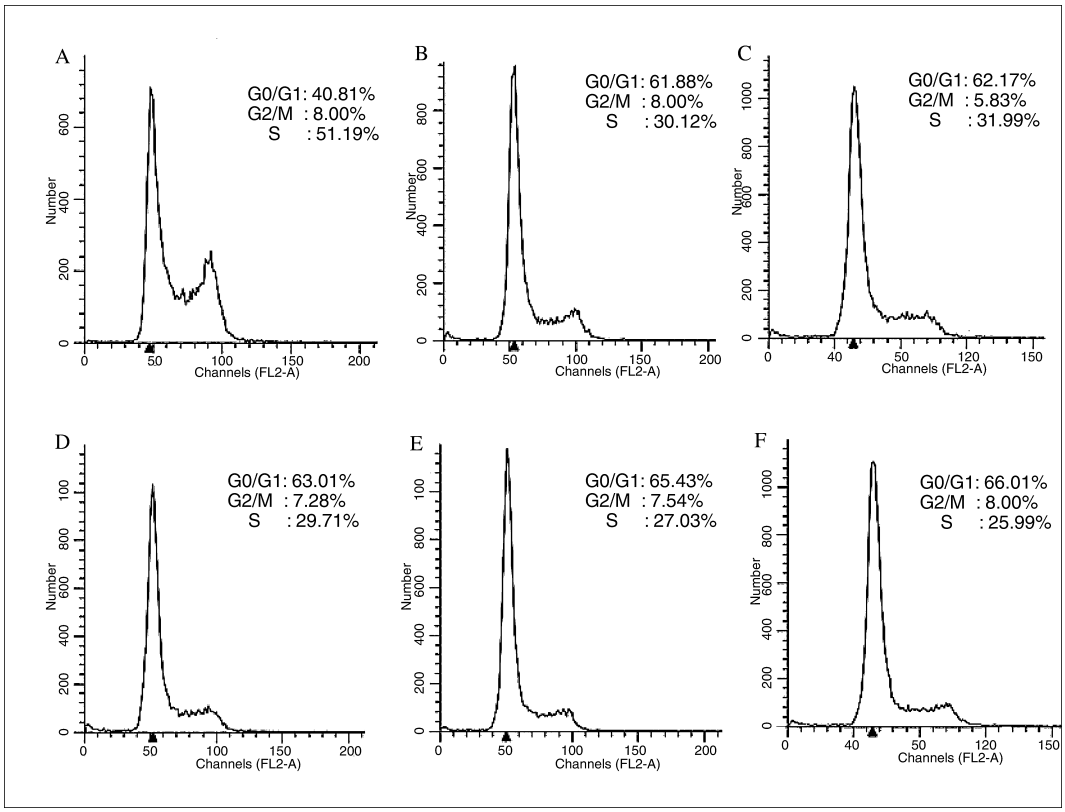

Fig. 2: FCM analyzed the cell cycle of HepG2.2.15 cells treated with G-HSA-IFN. HepG2.2.15 cells were treated with G-HSA-IFN (0, 625, 1250, 2500, 5000,10000 U/ml) for six days, dyed with PI and tested by flow cytometry. It showed that most cells were blocked in G_0/G_1 phase by G-HSA-IFN

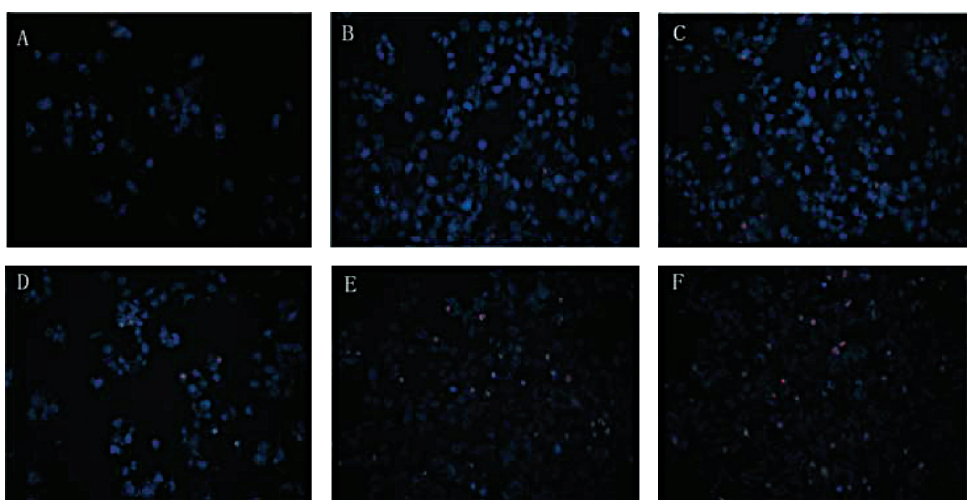

Fig. 3: Observations under a fluorescence microscope with Hoechst33342 and PI. HepG2.2.15 cells were treated with G-HSA-IFNα2b (0, 625, 1250, 2500, 5000,10000 U/ml) for 24 h, stained with Hochest 33342 and PI, and observed by microscope. Live cells will show only a low level of blue fluorescence, apoptosis cells will show bright blue or light red fluorescence, and necrotic cells will show bright red fluorescence

studying virus replication and virus-host cell interactions. Here we reported the influence of G-HSA-IFN on HepG2.2.15 cells which was used as a classical model *in vitro*.

Sulforhodamine B (SRB) assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation for large-scale drug-screening applications. It performed similarly compared to other cytotoxicity assays such as MTT or clonogenic assay. But SRB assay possesses a colorimetric end point and is nondestructive and indefinitely stable (Voigt 2005). SRB assay and FCM assay showed that G-HSA-IFN could inhibit the proliferation of HepG2.2.15 cells and arrest the cells at the G_0/G_1 phase. Our research also found that G-HSA-IFN could inhibit the expression of HBsAg after treated the HepG2.2.15 cells 3 days later and the inhibition ratio could be 90% until the 6th day. The inhibition on the expression of HBeAg was weaker and it might be the suppression of cell proliferation which led to the reduction in expression of the supernatant.

As an important apoptosis regulatory gene, Bcl-2 was studied deeply. Bcl-2 is an anti-apoptotic factor within the cell, which can stable mitochondrial membranes, prevent mitochondrial from releasing caspase, and prevent oxygen free radicals from turning on the apoptosis signal pathway. Bax was the first homologous gene of Bcl-2 identified, and it had 45% homology with Bcl-2. The overexpression of Bax could antagonize the protective effect of Bcl-2 and lead to cell apoptosis. Our experiments also demonstrated that a small amount of cells apoptosed after treatment with G-HSA-IFN. As the concentration of drug increased, the number of apoptosed cells increased, and some of them began to die. Western Blotting analysis also proved that the expression of Bcl-2, the anti-apoptotic gene, decreased at some dose of G-HSA-IFN while that of Bax, a pro-apoptotic gene, increased.

All these *in vitro* results proved that G-HSA-IFN could inhibit the expression of HBsAg effectively. As a new drug for viral hepatitis, it did not only prolong the half-life of conventional interferon but also targeted to the liver. And it might be a hopeful drug candidate for hepatitis B.

In conclusion, the G-HSA-IFN we prepared could not only significantly inhibit the HBsAg expression and the proliferation of HepG2.2.15 cells, but also induce the apoptosis and death of the target cells, and might therefore be a promising drug candidate for hepatitis B.

4. Experimental

4.1. Materials

Cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside was prepared in our laboratory previously (Liang et al. 1998). Acrylic amide, *N*,*N* -methylene-bis-acrylamide, tetramethylethylenediamine (TEMED), Hochist 33342, propidium iodide (PI) and sulforhodamine B (SRB) were purchased from Sigma-Aldrich Co. Primary antibodies that anti Bcl-2 and anti Bax were purchased from Santa Cruz Biotechnology, Inc. and DMEM cell culture from GIBCO®. Long-term fusion protein HSA-IFN (electrophoresis grade) was kindly provided by Prof. Jianliang Yang, Wolwo Bio-Pharmaceutical Co., Ltd. RNase A, antibody anti Actin, HRP-labeled goat anti mouse and anti Rabbit IgG was purchased from Beyotime® Institute of Biotechnology. Neonatal Bovine Serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. and ELISA kits for the detection of HBsAg and HBeAg from Shanghai Kehua Bio-engineering Co., Ltd. G418 sulfate (Geneticin) was purchased from Shanghai Qianchen Biotechnology Company and HepG2.2.15 cell line was provided by Changsha Wanbio Biotechnology Development Center. Other reagents were analytical grade and purchased in China.

4.2. Generation of G-HSA-IFN

The synthetic approach to G-HAS-IFN was reported previously (Cai et al. 2009). 10 mg of HSA-IFN were modified with cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside and the reaction mixture were purified with a PD-10 column. Then it was equally distributed, lyophilized, and stored at −20 °C.

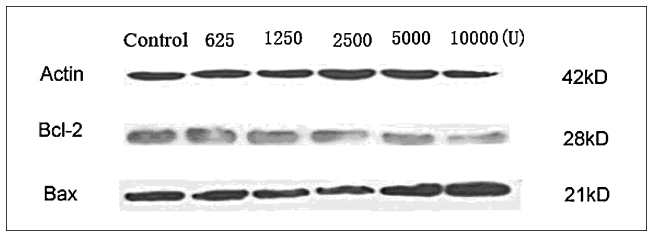

Fig. 4: Expressing levels of Bcl-2 and Bax in HepG2.2.15 cells after treatment with G-HSA-IFN. HepG2.2.15 cells were treated with G-HSA-IFN (0, 625, 1250, 2500, 5000,10000 U/ml) for 24 h. Cell lysates were prepared. The proteins in lysates were separated by 12% SDS-PAGE. After transferring onto the membrane, the blots were probed with anti- Bcl-2 and anti-Bax antibodies. Actin was used as internal reference

4.3. Antiproliferation analysis

HepG2.2.15 cells were cultured in DMEM medium with 380 mg/l G418, 10% Neonatal Bovine Serum, 2 mmol/l Glutamine, 1×10^5 U/l penicillin, and 1×10^5 U/l streptomycin. The exponential growth phase cells were harvested, rinsed, diluted to 8×10^4 cells/ml, and then seeded 100 µl/well on 96-well plates. After the HepG2.2.15 cells were cultured over night at 37 °C with 5% CO_2, G-HSA-IFN 100 µl/well diluted to appropriate concentrations (625–10,000 U/ml) with culture medium was added, and the cells were cultured additionally at 37 °C. Every three days, the supernatant was gently removed and the new culture medium containing fresh drugs was added. Each G-HSA-IFN concentration contained three parallel wells. After 3 or 6 days, the supernatant was aspirated to be measured the expression of HBsAg and HBeAg respectively. The surviving cells in each well were dyed with Sulpho Rhodamine B (SRB, Sigma Production), which stained living cells specifically (Skehan et al. 1990) and then been measured at OD_{531} (Vichai and Kirtikara 2006).

4.4. Influence on the expression of HBsAg and HBeAg

Treated with different concentrations G-HSA-IFN 3 or 6 days later, the supernatants of cultured HepG2.2.15 cells were gently aspirated and measured the concentrations of HBsAg and HBeAg expressed and secreted into the culture medium with ELISA kits for the detection of HBsAg and HBeAg according the protocols provided by the kits.

4.5. Cell cycle analysis with PI staining

The exponential growth phase cells 4 ml/well (2×10^5 cells/ml) were seeded on 6-well plates and cultured over night. Then different concentrations of G-HSA-IFN were added, respectively. Every third day, the supernatant was gently removed and the new culture medium containing fresh drugs was added. Six days later, the cells were dissociated, washed with 1 mmol/l PBS, pH 7.4 and fixed over night with PBS containing 75% cold ethanol. Then the cells were washed 3 times with 1 mmol/l PBS, pH 7.4 to prepare single cell suspension. 0.5 ml of PI staining solution containing 50 mg/l PI, 0.1% sodium citrate, 1.0% Triton X-100 and 1 mg/l RNase A was added and placed at 4 °C for 30 min. Then it was measured with FACS caliber Flow Cytometry (Becton, Dickinson and Company) and analyzed with Modfit LT 3.2.1 software.

4.6. Anti apoptosis analysis with double staining

The exponential growth phase cells 100 µl/well (1×10^5 cells/ml) were seeded on 96-well plates and cultured over night. Then different concentrations of G-HSA-IFN were added, respectively. Incubated 24 h later, Hochest 33342 and PI with the final concentration both of 10 mg/l were added and incubated at 37 °C for 10 min, the cellular morphology was analyzed with IX51 Inverted System Microscope (Olympus Corporation).

4.7. Western blotting analysis

The exponential growth phase cells 4 ml/well (5×10^5 cells/ml) were seeded on 6-well plates and cultured over night. Different concentrations of G-HSA-IFN were added, respectively. Incubated 24 h later, the cells were harvested, washed twice with 0.9% NaCl solution. Then the cold cell lysate was added and the cells were lysed for 30 min on ice. The cleavages were centrifuged at 12,000 rpm for 5 min and the protein concentrations of liquid supernatants were measured with the methods of Bradford. Liquid supernatants with same protein content were loaded and SDS-PAGE was performed. After separated, the proteins were transferred on to PVDF membrane by electroblotting.

Following protein transfer, the protein membrane was incubated in blocking buffer 1 h at room temperature. The membrane was then incubated over night at 4 °C with the appropriate primary antibody diluted with the diluting solution according to the manufacturer's recommendations. The following primary antibodies were used: anti-β-actin antibody, anti-Bax antibody, anti-Bcl-2 antibody. The incubation was followed by three washes in washing solution for 10 min each. The appropriate secondary antibodies conjugated with horseradish peroxidase were diluted in diluting solution, added and then incubated for 2 h at room temperature, followed by 3 washes, 10 min each. The membrane was then washed and the visualization of immunocomplexes was achieved by exposed to X-ray film.

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References

Aramaki Y, Lee I, Arima H, Sakamoto T, Magami Y, Yoshimoto T, Moriyasu F, Mizuguchi J, Koyanagi Y, Nikaido T, Tsuchiya S (2003) Efficient gene transfer to hepatoblastoma cells through asialoglycoprotein receptor and expression under the control of the cyclin A promoter. Biol Pharm Bull 26: 357–360.

Borden EC, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR (2007) Interferons at age 50: past, current and future impact on biomedicine. Nat Rev Drug Discov. 6: 975–990.

Cai G, Jiang M, Zhang B, Zhou Y, Zhang L, Lei J, Gu X, Cao G, Jin J, Zhang R (2009) Preparation and biological evaluation of a glycosylated fusion interferon directed to hepatic receptors. Biol Pharm Bull 32: 440–443.

Grace Mj, Lee S, Bradshaw S, Chapman J, Spond J, Cox S, Delorenzo M, Brassard D, Wylie D, Cannon-Carlson S, Cullen C, Indelicato S, Voloch M, Bordens R (2005) Site of pegylation and polyethylene glycol molecule size attenuate interferon-alpha antiviral and antiproliferative activities through the JAK/STAT signaling pathway. J Biol Chem 280: 6327–6336.

Harris JM, Chess RB (2003) Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov 2: 214–221.

Hudgin RL, Ashwell G (1974). Studies on the role of glycosyltransferases in the hepatic binding of asialoglycoproteins. J Biol Chem 249: 7269–7272.

Jones J, Shepherd J, Baxter L, Gospodarevskaya E, Hartwell D, Harris P, Price A (2009). Adefovir dipivoxil and pegylated interferon alpha for the treatment of chronic hepatitis B: an updated systematic review and economic evaluation. Health Technol Assess 13: 1–172, iii.

Lee YC, Stowell CP, Krantz MJ (1976) 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. Biochemistry 15: 3956–3963.

Li Y, Huang G, Diakur J, Wiebe Li (2008) Targeted delivery of macromolecular drugs: asialoglycoprotein receptor (ASGPR) expression by selected hepatoma cell lines used in antiviral drug development. Curr Drug Deliv 5: 299–302.

Liang GL, Jin J, Hu MY, Zhang RJ, Yu YH (1998). Synthesis of sialoglycoprotein. Chin J Pharmaceut (in Chinese) 6: 248–249.

Osborn BL, Olsen HS, Nardelli B, Murray JH, Zhou JX, Garcia A, Moody G, Zaritskaya LS, Sung C (2002) Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys. J Pharmacol Exp Ther 303: 540–548.

Skehan P, Storeng R, Scudiero D, Monks A, Mcmahon J, Vistica D, Warren Jt, Bokesch H, Kenney S, Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112.

Sung C, Nardelli B, Lafleur DW, Blatter E, Corcoran M, Olsen HS, Birse CE, Pickeral OK, Zhang J, Shah D, Moody G, Gentz S, Beebe L, Moore PA (2003) An IFN-beta-albumin fusion protein that displays improved pharmacokinetic and pharmacodynamic properties in nonhuman primates. J Interferon Cytokine Res 23: 25–36.

Sureau C, Romet-Lemonne JL, Mullins JI, Essex M (1986). Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell. 47: 37–47.

Takamatsu S, Fukuta K, Asanagi M, Abe R, Yokomatsu T, Fujibayashi Y, Makino T (2003) Monitoring biodistribution of glycoproteins with modified sugar chains. Biochim Biophys Acta 1622: 179–191.

Vichai V, Kirtikara K (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 1: 1112–1116.

Voigt W (2005) Sulforhodamine B assay and chemosensitivity. Methods Mol Med 110: 39–48.

Hepatitis B: Fact sheets WHO., Http://Www.Who.Int/Mediacentre/Factsheets/Fs204/En.

Wills RJ (1990). Clinical pharmacokinetics of interferons. Clin Pharmacokinet 19: 390–399.

Zhong Y, Fu X, Guan C (1995) Preparation of galactosyl-interferon α-1 and its hepatic targeting property. Chin J Med Chem (in Chinese) 163–168.