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Apoptosis of neoplasm cell lines induced by hepatic peptides extracted from sucking porcine hepatocytes

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INTRODUCTION

Promoting hepatocyte growth factor (pHGF) extracted from the sucking pig liver is a series of polypeptides with molecular weight less than M_r 10 000 and has specific biological activities to stimulate the rat hepatocyte DNA synthesis after 1/3 partial hepatectomy and promote recovery of rat hepatic injuries induced by endotoxin and D-aminogalatose. These properties are similar to the results reported by Labrecque[1]. pHGF can effectively cure clinical acute fulminant hepatitis, chronic hepatitis and other hepatic injuries by significantly reducing serum alanine aminotransferase (sALT), eliminating jaundice and increasing the survival rate of fulminant hepatitis[2,3].

It was reported that three of the six fractions of pHGF purified by HPLC, can promote the DNA synthesis of rat primarily cultured hepatocytes[4,5]. Two of the fractions can inhibit the proliferation of BEL-7402 hepatoma cell line, their activities were not cross- affected, indicating that there exist at least two different active components in pHGF. To investigate the inhibitory mechanism of pHGF, we purified these inhibitory components and studied their physico-che mical properties and apoptosis inducing effects.

MATERIALS AND METHODS

Primarily cultured hepatocytes

Male Sprague Dowley rats of 250g±30g in weight were purchased from the Experimental Animal Center, Sun Yatsen University of Medical Sciences, Guangzhou, China. The animals were anaesthetized with ether, and their livers were removed, decapsulated, cut into small pieces, rinsed with PBS (pH 7.4) to wash away the blood. The liver pieces were incubated with 0.25% trypsin (Gibco) for 30min and gently homogenized. The hepatocytes were collected after centrifugation at 1000×*g* for 10min. Pellet parenchyma cells were washed three times with PBS (pH 7.4) by centrifugation, resuspended in RPMI-1640 medium containing 10 % FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin and placed to Costar (3596) 96 well culture plate at a density of 10.6 cells/ cm^3, 0.1mL each well. The cells were cultured at 37 ℃, 5% CO_2 until use.

Neoplasm cell lines

BEL-7402 human hepatoma cell, Hepe murine hepatoma cell and CNE-2 human nasopharyngeal carcinoma cell lines were gifts of Experimental Animal Center, Sun Yat -sen University of Medical Sciences. SMMC-7721, QGY-7703 human hepatoma cell lines, HCT_8 human colic adenocarcinoma cell and GLC-82 human lung adenocarcinoma cell lines were purchased from the Cellular Institute of Chinese Academy of Sciences. SGC-7901 human gastric carcinoma cell line was purchased from the Biochemistry Department of the Fourth Military Medical University. The cultured cells were grown to confluence, digested with 2.5 g/L trypsin containing 0.2 g/ L EDTA, washed with PBS (pH 7.4) and resuspended in DME/F12 or RPMI-1640 medium, added into Costar (3596) well culture plates at a density of 2.5-5.0×10^4 cells/mL in 0.05 mL each well, then incubated at 37 ℃, 5% CO_2 for 24 h. After remained at 4 ℃ for 1 h, the cells were immediately recovered at 37 ℃ for further use.

Proliferation inhibitory experiments

Various concentrations of HP (S4) in DME/F12 or RPMI-1640 medium containing 10% FCS were added to the cultures, incubated for another 12, 24, 48 and 72 h, MTT solution (1.5 g/L in PBS) was then added to all wells (10 μL per 100 μL medium) in the last 4 h-6 h, rinsed with PBS, DMSO was added and vibrated to dissolve the dark blue crystals. After placed at room temperature for a few minutes until all crystals were dissolved, the optic-metric density (OD) was read on Σ960 ELISA

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reader at test wavelength of 570 nm and referent wavelength of 630 nm.

Apoptosis inducing experiments

In situ cell death detection kits (POD) were purchased from Oncor Co., USA. The cells were adjusted to a density of 2.5 - 5.0×10^4 cells/cm^3, added to 24-well plates with cover glass-slides in 0.5 mL each well. Incubated at 37 ℃ 5% CO_2 for 24 h, the cells were remained at 4 ℃ for 1h, the tempera ture was then promptly recovered to 37 ℃. Various concentrations of S4 were added and incubated for different periods. The glass slides were taken out, rinsed, fixed and immunohistochemically stained. The negative control with omission of Tunel enzyme was designed according to the manufacturer's manual. The cells stained with dark yellow brown nucleus were considered as positive cells. Ten optical fields, about 500-1 000 cells were counted in each glass-slide under the high magnification (× 400) microscope. Results presented as ± indicated 5% slightly positively stained cells; +, >5 <10% positive cells; + +, >10 <20% positive cells; and +++, >20 <30% positive cells.

Effects of S4 on the expressions of 4 oncogenes in 8 neoplasm cell lines

The antibodies used in this study including p53, a murine monoclonal antibody IgG2b against human p53, and Bcl-2, a murine monoclonal antibody against human Bcl-2 -p25 oncoprotein were purchased from DAKO Co Inc. Fas, a rabbit polyclonal antibody against human Fas oncoprotein, and c-myc, a murine monoclonal antibody IgG2a against p67 oncoprotein purchased from Santa Cruz Biotechnology Inc, UK. Secondary antibody (DAKO products) was a rabbit antibody against murine immunoglobulin conjugated with horseradish peroxidase.

The neoplasm cells were adjusted to 2.5-5.0× 10^4cells/cm^3, added into 24 -well culture plates with cover glass-slides in 0.5 mL each well, incubated at 37 ℃ 5% CO_2 for 24 h, then remained at 4 ℃ for 1 h for cell growth at the same step. Immediately after the temperature was recovered to 37 ℃, S4 was added and continued to inoculate for 48 h, then the cells were fixed and immunohistochemically stained following the instruction of the manufacturer. The negative control with primary antibody was replaced by PBS, and observed under light microscope. The results were presented as "+ +" to "+ + +" which indicated the slight positive staining to the strongest positive staining.

Northern blot

The quantity of cells was adjusted to 1×10^7 per dish, and RNA in the cells was extracted rapidly with Guanidinium isothiocyanate. According to the method of random primer (Promega), the cDNA probe of human Bcl-2 gene was labeled with α-^{32}P-dCTP, and hybridized for 24 h at 68 ℃ in 6×SSC, 2 ×Denbart's reagent and 0.5% SDS. After the filters were washed, autorad iography was performed by exposing the filter to X-ray film at -70 ℃ for 48 h. The results were determined with an intensifying screen.

Apoptosis of BEL-7402 hepatoma cells transplanted in BALB/C nude mice induced by S4

Male BALB/C nude mice, weighing 18 g-22 g were provided by the Experim ental Animal Center of Sun Yat sen University of Medical Sciences. BEL-7402 he patoma cells were injected s.c. into the right neck (1.46×10^7 cells/mL) of BALB/C male nude mice. When growing to about 0.8 cm^3, tumors were cut into small pieces of about 1 mm^3 and transplanted into the renal capsulae of BALB/C nude mice, and their width and lengths were measured with micrographer. Twenty-six mice were divided into four groups. In the high-dose group, the six mice were injected ip with 1.0 mg S4/kg-per day and in the low-dose group, with 0.5 mg of S4/kg per day. In the positive control group, the six mice were injected ip with 1 mg Doxorubicin (Huaming Pharmaceutical Co, LTD)/kg per day, while in the negative control group, the eight mice were injected ip with 0.4 mL physical saline daily for 16 days. The nude mice were killed 2 days after the end of treatment. The width and lengths of the tumors were measured with a micrographer. All the data including body weight of mice were input to computer to calculate the growth inhibitory rate of tumors and T value.

Statistical analysis

Statistical significance was determined by using χ^2 test or Student's *t* test.

RESULTS

Homogeneous peak of hepatocyte extracts

DEAE-Sephadex A25 purified hepatocyte extract was desalted and lyophilized, the proliferation inhibitory activities were tested. The active fraction was further purified by Superdex 75 and C_{18} reversed-phase chromatography and lyophilized. The last purified fraction was called Subfraction 4 (S4). A combination of the above procedures produced an almost pure peak with 95.8% relative area, and molecular weight of M_r 4020.6 determined by HPLC and MALDI-TOF-MS mass spectrometer.

Proliferation inhibitory effect of pHGF and its

fractions on normal hep atocytes and 7402 hepatoma cells

After the hepatocytes were removed from the livers of the SD rats, 7402 hepatoma cells were incubated for 24 h, 500, 1 000 and 2 000 mg/L of pHGF and 20 mg/L S4 purified by DEAE-Sephadex A25 were added into each well with 0.1mL medium. After incubation at 37 ℃, 5% CO_2 for 48 h, MTT was added and reincubated for 4 h-6 h, then DMSO was added and mixed thoroughly to dissolve the dark blue crystals. OD value was read on ∑960 ELISA reader. The inhibitory rate (%)=(100 test group OD value/control group OD value ×100%). pHGF apparently inhibited the proliferation of BEL-7402 hepatoma cells at concentrations of 500 mg/L - 2 000 mg/L, and promoted the activities of primarily cultured hepatocytes. Of six fractions of pHGF purified by DEAE- Sephadex A25, fractions 3 and 4 significantly inhibited the growth of 7402 hepatoma cells while fractions 1, 2 and 5 promoted the activities of primarily cultured hepatocyte dehydrogenase (Table 1).

The results showed that the fractions of pHGF both promoted the activities of normal hepatocyte dehydrogenase and inhibited the activities of hepatoma cell dehydrogenese. There were no cross effects between the two actions.

Inhibition of S4 on the proliferation of 8 neoplasm cell lines

S4 at concentrations of 1, 5, 10 mg/L were added into the culture plates (96 wells), each well containing 0.1 mL medium, and incubated at 37 ℃, 5% CO_2 for 12, 24, 48 and 72h. MTT solution (1.5 g/L in PBS) was added to all the wells (10 μL per 100 μL medium), and incubation was continued for h - 6 h, then 100 μL DMSO was added and mixed to dissolve MTT dark blue crystals. After vibrated at room temperature for 15min, the plates were read on ∑960 ELISA reader at test wavelength of 570 nm and referent wavelength of 630 nm. The results showed that S4 can significantly inhibit the proliferation of the 8 neoplasm cells with a clear dose and time dependent manner (Table 2).

Apoptosis of 8 neoplasm cells induced by S4

Based on the above results that S4 can induce tumor cells to die at concentrations of 1 mg/L-10 mg/L from 24 to 72 h, we selected the 5 mg/L of S4 and 48 h affecting period as experimental conditions so as to better compare the results of S4 on 8 neoplasm cell lines. The apoptosis inducing effect of pHGF and S4 on BEL-7402 hepatoma cells showed that the apoptosis inducing effect of S4 is 100 times as strong as that of pHGF calculated by their activities in weight. S4 induced apoptosis of 8 neoplasm cell lines with different activities, and apparently induced all the hepatoma cells to die, the effect on non-hepatoma cell lines being smaller than that of hepatoma cell lines. S4 had no apparent apoptotic effect on HCT-8 cell line, and even stagnated the apoptosis of CNE-2 cell line (Table 3). All these may be related to the histocellular sources and the signal differences tran smitted in the cells.

Effect of S4 on the 4 oncoprotein expressions of 8 neoplasm cell lines

Confluent cells digested by trypsin, adjusted to 2.5-5.0×10^4 cells/cm^3, added into 24 - well Costar plates with cover glass-slides in each well with 0.1mL medium and cultured in DME/F12 medium containing 10% FCS, at 37 ℃, 5% CO_2 for 24 h. To grow at the same step, the cells were placed at 4 ℃ for 1h before use. Temperature was quickly recovered to 37 ℃, 5 mg/L S4 was added and incubated for an other 48 h. Immunohistochemistry staining was performed following the manufacturer's manual and positive and negative controls were designed. The results (Table 4) showed that S4 significantly up -regulated the expression of P53, Fas and depressed Bcl-2, but had no apparent effect on the expression of c-myc in 4 hepatoma cell lines, and the most prominent effect fell upon the BEL-7402 cell line. Effects of S4 on regulating oncogene expressions of 4 nonhepatoma neoplasm cell lines differed. S4 slightly promoted the expression of Fas and depressed Bcl-2 and c-myc in 7901 cell line; slightly down-regulated Bcl-2 and c-myc in GLC-82 cell line; but promoted the expression of Bcl-2 in CNE-2 cell line. Based on the results in 8 neoplasm cells, the apoptotic induction of S4 on the cells was considered to be exerted via affecting the Bcl-2 oncogene expression.

Table 1 Effect of pHGF and its fractions on normal hepatocytes an d hepatoma cells ($\bar{x}$±s, *n* = 8)

	pHGF (OD, mg/L)			Fractions (OD, 20mg/L)						Control
	500	1000	2000	1	2	3	4	5	6	
7402 cells	0.422±0.11[b]	0.410±0.09[b]	0.401±0.07[b]	0.545±0.03	0.601±0.11	0.427±0.067[b]	0.411±0.09[b]	0.580±0.08	0.649±0.21	631±0.12
Primary hepatocytes	0.390±0.07[b]	0.398±0.09[b]	0.388±0.06[b]	0.293±0.06[b]	0.308±0.06[b]	0.223±0.07	0.236±0.06	0.280±0.10[b]	0.219±0.11	0.218±0.07

[b]P<0.01, *vs* control.

Table 2 Effects of S4 on 8 neoplasm cell lines at different time points

Cells	IC50 (mg/L)		
	24h	48h	72h
BEL-7402	8.2	5.0	3.9
SMMC-7721		8.4	4.2
QGY-7703		24.2	9.5
Hepe	15.2	8.9	6.5
SGC-7901	30.2	19.7	14.3
GLC-821	2.4	9.9	5.4
CNE-2			8.9
HCT-8			9.9

IC_{50}: the half inhibitory concentration.

Table 3 Apoptosis of 8 neoplasm cell line induced by S4 (5mg/L, 48h)

	Control	S4 Treatment
BEL-7402	+	++
SMMC-7721	+	++
QGY-7703	±	++
Hepe	+	++
HCT-8	±	±
GLC-82	±	+
SGC-7901	±	+
CNE-2	++	+

Table 4 Effects of S4 on the 4 oncogene expressions of 8 neoplasm ce ll lines (S4, 5μg/well, 48h)

	P53		Fas		Bcl-2		c-myc	
	C	T	C	T	C	T	C	T
BEL-7402	+	++	+	++	+	±	+	+
SMMC-7721	+	++	+++	+++	+	±	++	++
QGY-7703	±	+	+	+++	±	±	+	++
Hepe	+	++	++	++	++	±	++	+
HCT-8	+	+	++	++	±	±	++	+
GLC-82	++	+	+++	+++	+	±	++	+
SGC-7901	+	+	±	+	+	±	++	+
CNE-2	+	++	+++	++	±	+	++	+

C: control group; T: test group.

Northern blot

The changes of Bcl-2 mRNA in BEL-7402 cell line after incubation with 5 mg/ L S4 were observed by blot hybridization. The results showed that S4 can apparently inhibit the Bcl-2 mRNA transcription determined by the quantity measure ment of intensifying screen. Relative value of the test group was related to the dose of S4. This result was concordant with the oncoprotein expression and demo nstrated that S4 can inhibit the Bcl-2 oncogene expression at mRNA transcript level.

Experiments of BALB/C nude mice transplanted with BEL-7402 hepatoma cells

The mice transplanted with BEL-7402 hepatoma cells were injected ip with S4 at d3 after tumor transplantation, once daily for 16 days. The results showed that S4 can apparently inhibit the growth of BEL-7402 hepatoma cells innude mice. This inhibitory effect was also induced by apoptosis (Tables 5 and 6). S4 had no apparent effect on the mitosis and differentiation of BEL-7402 hepatoma cells observed under the optical microscope. Cell shrinkage and condensation, microvillus disappearance, condensed chromatin margination and apoptotic body formation were found under electronic microscope.

Table 5 Inhibition of S4 on hepatoma cells in nude mice ($\bar{x}\pm s$, n=6)

	Dose (mg/kg)	Do (mm^3)	Dn (mm^3)	Dn-Do (mm^3)	Inhibitoryrate (%)
High dose of S4	2.0	2.19±0.24	2.81±0.49	0.61±0.38	59[b]
Low dose of S4	1.0	2.00±0.12	2.55±0.22	0.56±0.26	63[b]
Doxorubicin	1.0	2.26±0.12	2.16±0.32	0.10±0.29	107[b]
Control	0.0	2.17±0.23	3.67±0.52	1.50±0.55	

Do: Dimension of tumors at onset; Dn: Dimension of tumors at necropsy. [b]P<0.01, *vs* control.

Table 6 Apoptosis of hepatoma cells in nude mice induced by S4 (LI, $\bar{x}\pm s$, n=6)

	Dose (mg/kg)	LI (%)
High dose of S4	2.0	18.70±4.92[b]
Low dose of S4	1.0	13.22±1.74[b]
Doxorubincin	1.0	14.18±2.46[b]
Control	0.0	8.40±2.81

[b]P<0.01, *vs* control. LI: lable index.

DISCUSSION

There are lots of substances in the human body which can stimulate the proliferation and regeneration of liver, e.g. insulin, platelet derived growth factor (PDGF), transfer growth factor β (TGF-β), epithelial growth factor (FGF), etc, but their effects are not specific. It has been shown that the hepatocytes of young animal livers might produce network self-regulators to modu late their growth, differentiation and apoptosis. Because of the differences in extracting and preparing the liver derived growth stimulating factors and inhibitors, it is difficult to enunciate whether these stimulating or inhibitory effects are caused by the double or poly-functional effects of one factor or by the specific effects o f two factors with different functions.

It was reported by Labrecque[1] that a series of substances extracted from the regenerating livers of young rats, dogs and rabbits with M_r12 000-21 000 are called hepatic stimulating substance (HSS) that can stimulate the DNA synthesis of the primarily cultured hepatocytes and the hepatocytes after partial hepatectomy (2/3)[1]. HSS can raise the survival rate of rats with fulminant hepatic failure and inhibit the growth of some neoplasm cell lines.

A series of polypeptides which can raise the survival rate of human fulminant he patitis, reduce sALT, eliminate jaundice and ameliorate liver functions are called promoting hepatocyte growth factors (pHGF). The monoclonal

immunohistochemical study showed that pHGF is located in the hepatocytes, not in mesenchymal cells such as Kupffer cells, endotheliocytes and fibroblasts[6].

In our previous papers[4], we found that pHGF contained not only hepatic regeneration stimulating substances, but also growth inhibitory factors which can inhibit the growth of hepatoma cell lines. These inhibitory factors can not inhibit the growth of normal primarily cultured hepatocytes. Homogeneous fraction S4 extracted from sucking pig liver by HPLC and FPLC in this report is a small peptide with M_r4020. Its inhibitory activities are 100 times as that of pHGF and can be destroyed by pronase K, but resist to acids, alkalis, RNAase and DNAase.

We selected 8 neoplasm cell lines to study the biological activities of S4 in the growth inhibition, apoptotic induction and oncogene expression related with apoptosis of these cell lines *in vitro*, and studied the effects of S4 in the hepatoma cells transplanted in nude mice *in vivo*. The results showed that S4 inhibit the proliferation of neoplasm cell lines *in vitro* by inducing apopto sis in a clear dose and time dependent manner. The apoptotic effect of S4 on the se cells is exerted via regulating the expression of oncogenes related to apoptosis, especially by affecting Bcl-2 gene expression. It was testified by *in vitro* and *in vivo* tests that S4 is an effective apoptotic inducer of neoplas mcell lines.

Modern molecular biology investigations have indicated that proliferation inhibition of some neoplasm cells is related to apoptosis induction regulated by the oncogene expression of these cells. The different growth inhibitions of S4 were found in 8 neoplasm cell lines with the different expressions of p53, Fas, Bcl-2 and c-myc oncogenes. S4 can apparently promote the oncoprotein expression of p53, Fas and depress the Bcl-2 and c-myc oncogenes while inducing hepatoma cells to die. It was reported that p53 induced apoptosis via stimulating apoptosis inducing genes, and inhibiting the survival necessitating genes, or participating in the enzyme cleavage of DNA. The apoptotic induction of Fas is caused by its ligands and receptor reaction, and the apoptotic induction of c-myc is related to the apoptotic mechanism controlled by Bcl-2 gene, which depends on the circumstantial factors, leading to either death or proliferation[7,8].

It is generally considered that Bcl-2 is an important gene for cells to survive, we have further confirmed that the Bcl-2 gene expression of BEL-7402 hepatoma cells is inhibited by S4 at m-RNA transcript level by using the techniques of blot hybridization. This may be the initial factor for the cell apoptotic induction, because Bcl-2 gene can inhibit the apoptosis by blocking the last tunnel of apoptotic signal transmitted system.

S4 inhibited the growth of hepatoma *in vivo* by inducing apoptosis, and had no apparent effect on the mitosis and differentiation of hepatoma cells. The results showed that the apoptotic induction of S4 is directly working against the target cells.

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