Octreotide inhibits proliferation and induces apoptosis of hepatocellular carcinoma cells

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KEY WORDS hepatocellular carcinoma; octreotide; apoptosis; somatostatin receptors

ABSTRACT

AIM: To study the effect of octreotide on cell proliferation and apoptosis in different hepatocellular carcinoma (HCC) cells and hepatocytes. **METHODS:** The proliferation of HCC cells (HepG2, SMMC-7721) and hepatocytes (L-02) was determined by MTT assay. Apoptosis was detected either by fluorescent staining, transmission electron microscopy or flow cytometry. The content of AFP in the supernatant of cultured HCC cells was determined by electrochemiluminescence immunoassay. The expression of SSTR subtypes was identified by RT-PCR. **RESULTS:** The proliferation of HCC cells and L-02 cells was inhibited significantly by octreotide (0.25, 0.5, 1.0, 2.0 and 4.0 mg/L). However, the apoptosis of HCC cells markedly increased in a concentration-dependent manner. Both the apoptosis index and the percentage of apoptotic cells in L-02 cells were significantly lower than those of HepG2 and SMMC-7721 cells. The content of AFP in the supernatant of cultured HepG2 cells treated with octreotide was also statistically reduced. Furthermore, SSTR2 and SSTR4 were positive in both the hepatocellular carcinoma cells and in the L-02 cells. SSTR3 was only expressed in the two heptatocellular carcinoma cells, and SSTR5 was found in the SMMC-7721 cells. No SSTR1 was detected either in HCC cells or L-02 cells. **CONCLUSIONS:** Apoptosis induction is a major mechanism of octreotide inhibition on hepatocellular cells. SSTR3 is expressed in the HCC cells, but not in the L-02 cells, which suggests a molecular basis for the HCC-selective effects of octreotide.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in China, and the treatment of inoperable HCC remains unsatisfactory. To improve the prognosis and the quality of life in those patients with inoperable HCC, it is necessary to search for effective drugs. Studies have shown that somatostatin and its analogue, octreotide, exert potent antitumor activity not only on hormone producing tumors, but also on non-hormone tumors[1-4]. Recently, in several randomized controlled trials using octreotide, the survival rate was significantly improved in those patients with advanced HCC[5-7]. However, the mechanism by which octreotide works has not been completely elucidated. Although it has been reported that octreotide inhibits the proliferation and induces apoptosis of HCC cells *in vitro*[7-10], the expression of somatostatin receptors (SSTRs) and the influences of octreotide on hepatocytes are not well known. In the present study, we investigated the effect of octreotide on the human hepatocyte (L-02 cells) as well as on different HCC cells (HepG2, SMMC-7721). In addition, we identified the expression of SSTRs in these cells.

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MATERIALS AND METHODS

Reagents Cell lines of human hepatocellular carcinomas (HepG2, SMMC-7721) and human hepatocytes (L-02) were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS) and Fluorochrome Hoechst 33258 were purchased from Gibco Co (Tulsa, Oklahoma,USA). Octreotide (lot 01900) was provided by Novartis Pharma AG (Basel, Switzerland), and methyl thiazolyl tetrazoliun (MTT) was obtained from Fluka Co (Buchs, Switzerland). Alpha-fetoprotein (AFP) immunoassay kits Elyecsys were purchased from Roche Co (Mannheim, Germany). Trizol was purchased from Sigma (St. Louis, Missouri, USA), and the commercial RT-PCR kit was purchased from Takara Co (Kyoto, Japan).

MTT chromometry[11] HepG2, SMMC-7721 and L-02 cells were placed in 96-well culture dishes (Corning-Coster Corp, US) at a density of 2×10^4 cells/well in DMEM culture medium containing 10 % FCS at 37 ºC, 5 % CO_2. After 24 h, the cells were washed and placed in culture medium with different concentrations of octreotide (0.25, 0.5, 1.0, 2.0, and 4.0 mg/L) for another 24 h. For controls, cells were placed in medium only. Following removal of the supernatant, the cells were treated with 0.5 % MTT for 4 h, the purple-blue sediment dissolved in 100 mL Me_2SO, and the absorbance at 490 nm was determined by a multi-well scanning spectrophotometer (Multiskan Ascent, Finland). The inhibition rate of cell growth was calculated by the following formula: mean value of (control group–treated group)/control group×100 %. Triplicate wells were analyzed per each concentration.

Assessment of apoptosis Cell apoptosis was quantified by counting the number of apoptotic cells per 100 cells under fluorescenct microscopy (Olympus) after the cells had been cultured with octreotide (0.25, 1.0, and 4.0 mg/L) for 48 h in 24-well culture dishes. After staining with Hoechst 33258, the apoptotic cells could be identified because of their enhanced fluorescence and characteristic nuclear morphology. One hundred cell samples were determined for each test triplicately.

The apoptosis index was determined by flow cytometry (FACscan, Becton-Dickonson Corp, USA). Cells treated with octreotide (0.25, 1.0, and 4.0 mg/L) for 48 h were fixed in 60 % alcohol at 4 ºC for 12 h, digested with 1 % RNAse, and stained with 100 mL propidium iodide at 37 ºC for 20 min before testing.

The apoptosis of HepG2 and L-02 cells was also detected using transmission electron microscope (Hitachi, Japan). After incubation with octreotide (0.25, 1.0, and 4.0 mg/L) for 48 h, the cells were fixed with 2 % glutaraldehyde for 2 h and then treated with 1 % osmium teroxid. Ultrathin sections were then prepared.

Assay of AFP synthesis HCC cells (HepG2, SMMC-7721) were placed in 24-well culture dishes (Corning-Coster Co, US) at a density of 9×10^4 cells/well in DMEM culture medium containing 10 % FCS at 37 ºC in 5 % CO_2. After 24 h, the cells were washed and incubated with different concentrations of octreotide (0.25, 0.5, 1.0, 2.0, and 4.0 mg/L). For controls, cells were placed in medium only. Each concentration was performed in triplicate wells. The amount of AFP in the culture supernatant was analyzed by electrochemiluminescencemetry (Elecsys systems, Roche Co) at 24, 48, 72, and 96 h.

RT-PCR for SSTRs Total RNA of cultured cells was extracted with Trizol, and reversely transcribed for 10 min at 30 ºC, followed by 1 h at 42 ºC, 5 min at 99 ºC, and 5 min at 5 ºC, in a 20 mL reaction mixture. To improve the specificity and sensitivity of amplimers, touch-down PCR was used in the amplification of cDNA. The annealing temperature was gradually decreased by one degree from 68 ºC to 54 ºC with 2 cycles at each temperature. The final 15 cycles was at 53 ºC. The components of the touch-down PCR included gene-specific primers (Tab 1), $MgCl_2$ 1.5 mmol/L, dNTPs 0.2 mmol/L, 1.25 units of *Taq*-DNA polymerase, and 2 μL of 10×PCR buffer in a total volume of 20 μL. The reactions were pre-denatured at 94 ºC for 5 min, denatured at 94 ºC for 1 min, annealed for 1 min, elongated at 72 ºC for 1 min, and finally elongated at 72 ºC for 10 min. Negative control was performed with the cDNA omitted. Electrophoresis of all amplimers was performed with 1.5 % agarose gels containing 0.005 % ethidium bromide.

Analysis of results and statistics Results were expressed as mean±SD, and the significance of the difference between the groups was determined by the *t*-test using the SAS system.

RESULTS

Cell proliferation The proliferation of HCC cells and L-02 cells was inhibited by different concentrations of octreotide as determined by MTT chromometry (Fig 1).

Effects of octreotide on apoptosis The HCC cells

Tab 1. The sequences of the primers for different SSTR subtypes.

SSTR subtype	Sequence	Product size
SSTR1	UPPER: 5'-TCAGCTGGATGGACAACGCC-3'	221 bp
	LOWER: 5'-CTCCCCCTCTTTTGGCTCAG-3'	
SSTR2	UPPER: 5'-TGGTCAAGGTGAGCGGCACA-3'	213 bp
	LOWER 5'-AGGGGTGGGAGGCAGGAAG-3'	
SSTR3	UPPER: 5'-GGAGAAGACTGAGGAGGAGG-3'	235 bp
	LOWER: 5'-TTTCCCCAGGCCCCTACAG-3'	
SSTR4	UPPER: 5'-GAAGGTGCTGGAGGTGCTGA-3'	176 bp
	LOWER: 5'-AAGGGCTCCTCAGAAGGTGG-3'	
SSTR5	UPPER: 5'-AAGGACGCTGACGCCACGG-3'	191 bp
	LOWER: 5'-GGTCATGGGTGGGGGTCAC-3'	

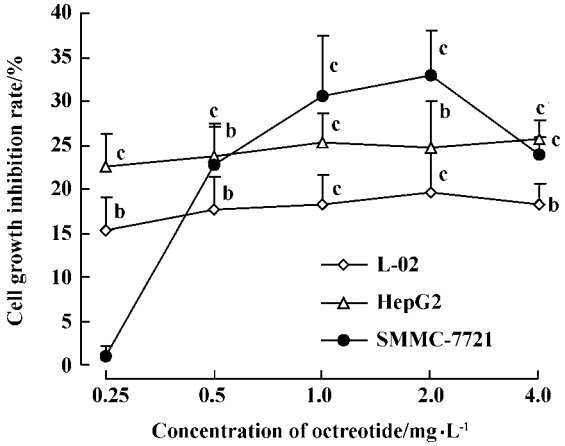

Fig 1. The inhibition rate of octreotide on HepG2, SMMC-7721 and L-02 cell proliferation after 24 h. *n*=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

when cultured with different concentrations of octreotide for 48 h, showed condensed nuclear morphology, increased fluorescence of the chromatin, and the presence of apoptotic bodies (Fig 2A). These results indicated that the cells were undergoing apoptosis. Simultaneously, the features of apoptosis, such as condensed, crescentic, and blebbed chromatin were demonstrated using transmission electron microscope in HepG2 cells (Fig 3B). In comparison with the controls, the percentage of apoptotic cells was significantly higher in the above three kinds of cells incubated with octreotide. The percentage of apoptotic cells incubated with octreotide increased in a concentration-dependent manner. However, the apoptotic rate of the L-02 cells was much lower than that of the HCC cells (Fig 2B).

A typical apoptotic peak could be detected by flow cytometry in HCC cells treated with octreotide (Fig 4), and the apoptosis index was associated with the concentration of octreotide. The apoptosis index of the L-02 cells was statistically lower than that of HCC cells (Fig 5).

AFP content in supernatant The content of AFP in the supernatant of HepG2 cells cultured with octreotide was significantly reduced in a concentration-dependent manner (Tab 2). AFP was undetectable in the culture supernatant of SMMC-7721 cells.

Tab 2. Effects of Octreotide on AFP Synthesis of HepG2. *n*=3. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control.

Octreotide /μg·L^{-1}	AFP/μg·L^{-1} 24 h	48 h	72 h	96 h
0	96±4	261±6	421±11	489±17
0.25	95±7	257±5	344±9^{b}	423±15^{c}
0.5	91±5	255±7^{b}	322±7^{c}	395±17^{c}
1.0	91±5^{b}	244±8^{b}	322±10^{c}	357±11^{c}
2.0	89±5^{b}	238±6^{c}	302±11^{c}	353±12^{c}
4.0	88±5^{c}	222±6^{c}	284±6^{c}	347±15^{c}

SSTRs expression Amplification of cDNA showed that SSTR2 and SSTR4 were positive in two HCC cells and in the L-02 cells. In contrast, SSTR3 was expressed only in the two HCC cells and not in the L-02 cells, SSTR5 was found in SMMC-7721 cells (Fig 6). No SSTR1 was detected in HCC cells or L-02 cells.

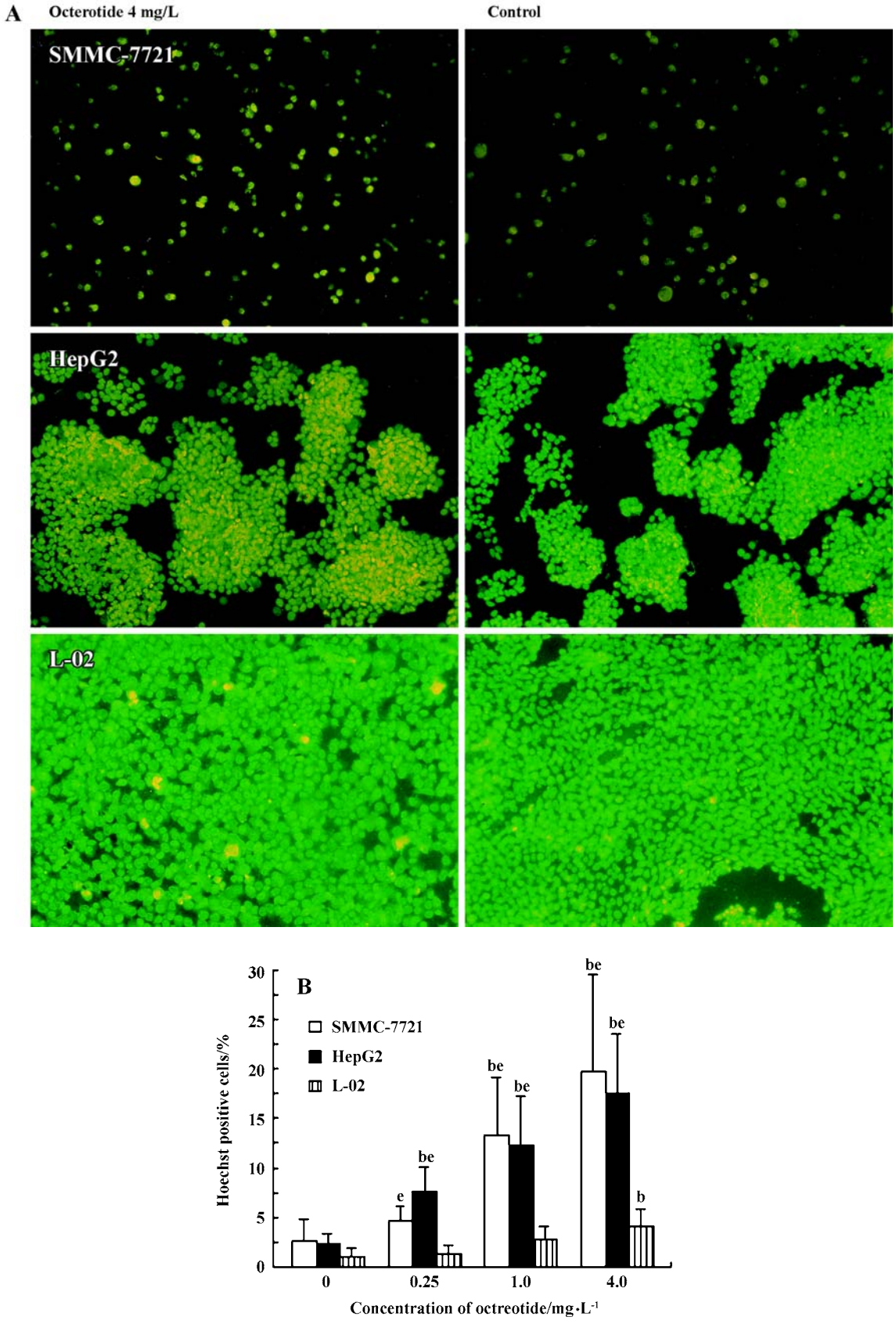

Fig 2. Effect of octreotide on apoptosis after 48 h detected by fluorescence staining. A) Hoechst 33258 staining of HepG2 and SMMC-7221 cell lines and hepatocyte L-02 cells (×200). B) The percentage of apoptotic cells. *n*=3. Mean±SD. $^{b}P< 0.05$ *vs* control; $^{e}P< 0.05$ *vs* L-02 cell.

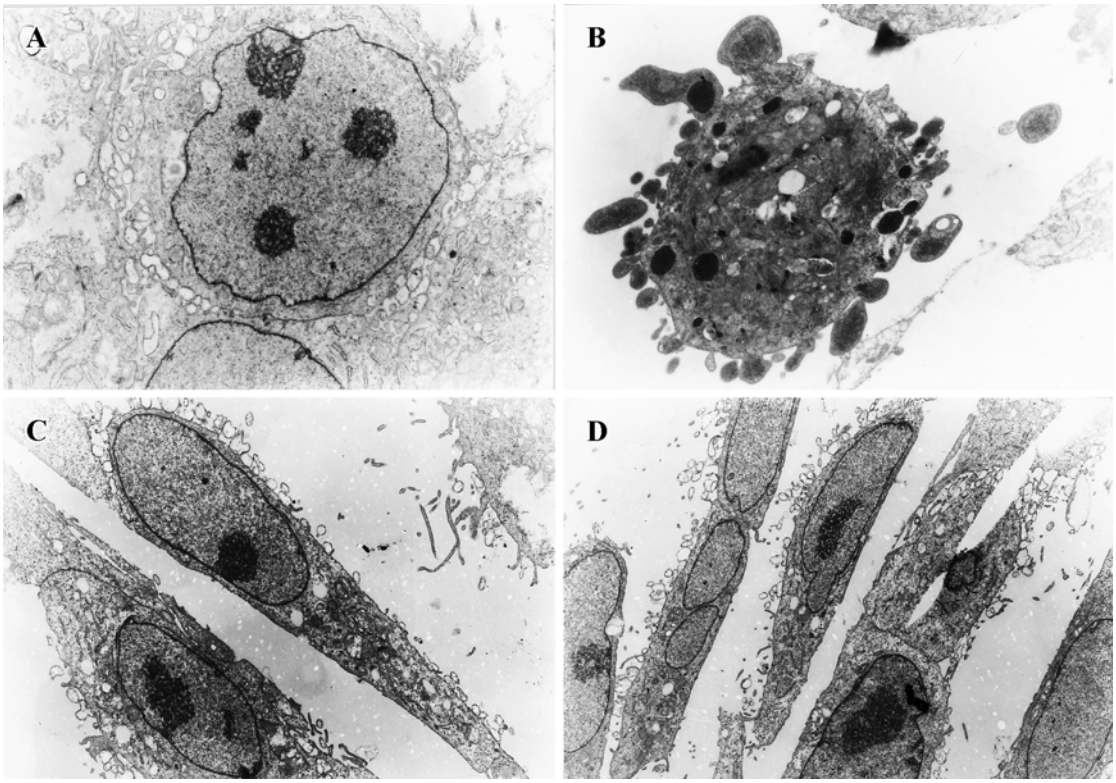

Fig 3. Apoptosis under transmission electron microscope after 48 h (×4000). A) control of HepG2. B) HepG2 cells treated with octreotide 4.0 mg/L. C) control of L-02 cells. D) L-02 cells treated with octreotide 4.0 mg/L.

DISCUSSION

Octreotide is an 8 peptide long analogue of somatostatin, which possesses a much longer duration of action. It has been demonstrated that after administering octreotide to nude mice bearing xenografts of a HCC cell line, the size and quantity of the neoplasm significantly decreased[8]. In addition, octreotide can reduce the post-operative recurrence of HCC[12-14].

Apoptosis is a crucial factor in carcinogenesis and cancer development. In this study, cells of two HCC strains incubated with octreotide were seen to have morphological changes of karyopyknosis, karyorrhexis and apoptotic body formation when observed under fluorescenct microscopy. Using flow cytometry, a typical apoptotic peak was observed. Both the percentage of apoptotic cells and the apoptosis index were increased statistically in a concentration-dependent manner. These results indicated that octreotide could stimulate apoptosis of HCC cells directly.

The actions of somatostatin and its analogues are mediated by interaction with five different subtypes of SSTR (SSTR1-SSTR5), which belong to the guanine nucleotide binding protein-linked receptor family. Octreotide can bind to SSTR2, SSTR3 and SSTR5 with high affinity[15-17]. Although the activation of SSTR2 may promote cell apoptosis through a p53-independent pathway[18], it was demonstrated that SSTR3 activation played a major role in somatostatin-induced apoptosis by accumulation of p53[19]. We identified SSTR subtype expression in HCC cells (HepaG2, SMMC-7721) and hepatocytes (L-02) using RT-PCR. SSTR3 was expressed in two HCC cells but not in L-02 cells. This finding suggested that SSTR3 activation may be responsible for the differences in the apoptotic rate between HCC cells and L-02 cells.

Besides the anti-proliferative effects on HCC cells, it must be concerned as well what influences of octreotide on hepatocytes. L-02 cell strain was established in 1979 from human liver. Both the percentage of apoptotic cells and apoptosis index induced by octreotide in L-02 cells were markedly lower than those of HCC cells. These differences, ie, HCC cell-selective effects to a certain extent, were quite important for long term use of octreotide in clinical treatment.

AFP is synthesized by hepatocellular carcinoma cells. Determination of the serum level of AFP is often used to determine whether a tumor has been completely

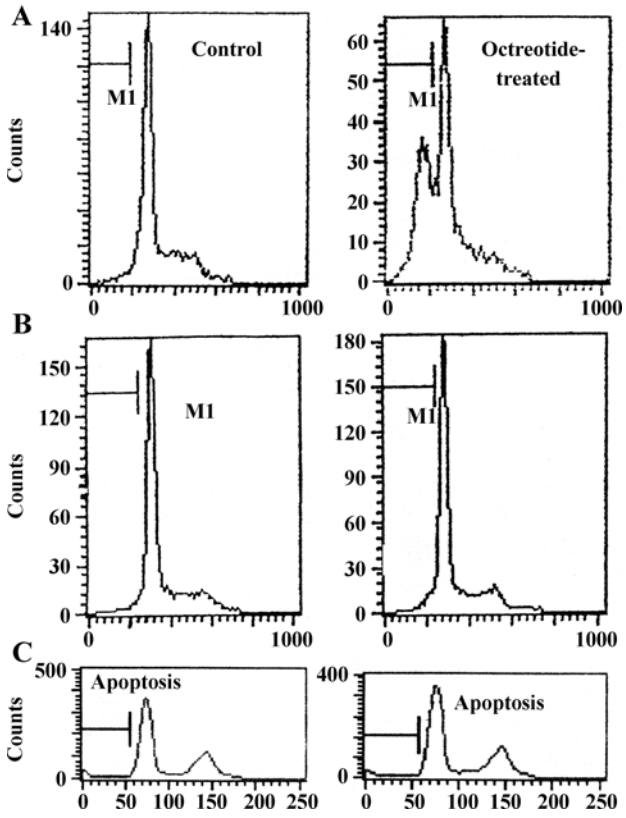

Fig 4. The flow cytometry: showing typical apoptotic peak in HepG2 and SMMC-7721 cell lines at 48 h after treatment with octreotide 4 mg/L. A) HepG2 cell; B) L-02 cell; C) SMMC-7721 cell.

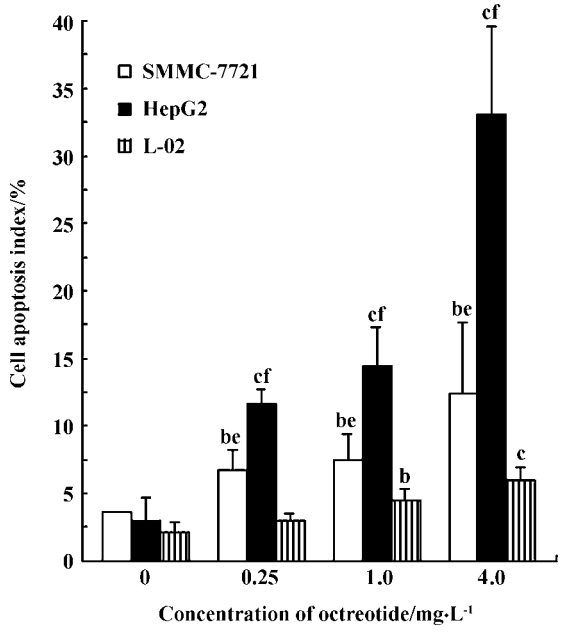

Fig 5. The apoptosis index of SMMC-7721 cells cocultured with octreotide as assessed by flow cytometry. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* L-02 cell.

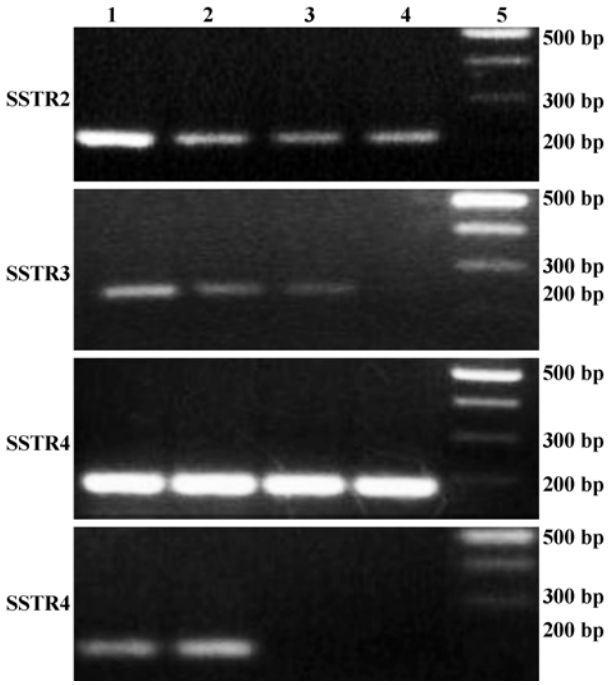

Fig 6. SSTR subtypes amplified with RT-PCR. Lane 1: SMMC-7721 cell; lane 2: HHC-98 cell; lane 3: HepG2 cell; lane 4: L-02 cell; lane 5: DNA markers.

resected to evaluate the efficacy of therapy, and to monitor for the recurrence of cancer[20-22]. The content of AFP in the supernatant of cultured HepG2 cells was significantly reduced by octerotide. These results indicated that, it may be a result of growth inhibition and apoptosis of the HepG2 cells.

In conclusion, octreotide can directly inhibit HCC cell proliferation, reduce AFP synthesis, and induce HCC cell apoptosis. Moreover, the apoptosis of hepatocytes by octreotide are negligible. SSTR3 is expressed uniquely in HCC cells, which may be a major reason for these different effects.

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