Cancer Chemother Pharmacol (2006) 57: 248–256
DOI 10.1007/s00280-005-0046-z

ORIGINAL ARTICLE

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Experimental study of the inhibition of human hepatocarcinoma Bel7402 cells by the tripeptide tyroserleutide(YSL)

Received: 2 January 2005 / Accepted: 6 March 2005 / Published online: 19 July 2005

Abstract *Purpose*: To investigate the antitumor effects of tyroserleutide (tyrosyl-seryl-leucine, YSL) on human Bel7402 hepatocarcinoma in vitro and in vivo, with preliminary exploration of its antitumor mechanism. *Methods*: MTT was used to observe the anticarcinogenic effects of YSL on human hepatocarcinoma Bel7402 cells in vitro. The ultrastructure of tumor cells was observed by electron microscopy. Nude mice bearing xenografts of human hepatocarcinoma Bel7402 were given daily i.p. injections of YSL or saline and an admixture of amino acids as controls, after tumor implantation. The inhibition of xenografts was determined by calculating the tumor volume and measuring tumor weight. The effects of YSL on the cell cycle and apoptosis of Bel7402 cells were determined by flow cytometry, and the effects on the ultrastructure of the cells by electron microscopy. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and proliferating-cell nuclear antigen (PCNA) immunohistochemical staining were used to investigate apoptosis in tumor tissue in nude mice. *Results*: In vitro YSL inhibited the proliferation of human Bel7402 tumor cells and changed their ultrastructure, resulting in the necrosis and apoptosis of the tumor cells. YSL at 80, 160 or 320 μg/kg/d inhibited tumor growth in nude mice by 40.26, 64.17, and 59.19%, respectively, which are significantly lower than the inhibition exerted by saline and an admixture of YSL amino acids ($P < 0.05$). The ultrastructure and cell cycle of human hepatocarcinoma Bel7402 cells were changed by treatment with YSL, with a rate of apoptosis higher than that of the control group. TUNEL and PCNA analysis showed that YSL inhibited the proliferation of tumor cells and induced apoptosis at the level of the cell. *Conclusions*: YSL significantly inhibited human hepatocarcinoma Bel7402 growth in vitro and in vivo. The growth inhibition of the tumor may involve necrosis and apoptosis of the tumor induced by YSL.

Keywords Tyroserleutide · YSL · MTT · Hepatocarcinoma Bel7402 · Apoptosis · Antitumor

Abbreviations YSL: Tyroserleutide · MTT: 3-(4, 5-diemethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide · TUNEL: Terminal transferase uridyl nick end labeling · PCNA: Proliferating-cell nuclear antigen · HCC: Hepatocellular carcinoma · FDA: Food and Drug Administration · SPF: Specific-pathogen free · DAB 3: 3′-diaminobenzidine tetrahydrochloride · FBS: Fetal bovine Serum · EDTA : Ethylenediaminetetraacetic acid · ELISA: Enzyme-linked immunosorbent assay

This study was supported by grants for the National High Technology Research and Development Program of China (2004AA2Z3170) (863 Program), The National Basic Research Program(2003CCA04300)(973 Program) and a project grant (03007) from the Department of Education of China.

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Introduction

The spleen contains many immune cells such as macrophages, reticuloendothelial cells, lymphocytes, and dentritic cells. These cells, as well as the molecules they

produce may modify tumor cell signal transduction and tumor cell killing. Many different peptides with anticancer or immune- activating activity have been identified from splenic tissues [1–2]. This provide a rationale to explore the spleen for peptides that target cancer. Accordingly, we explored the biological character of 132 small molecule peptides, which were extracted from the spleen of pigs. One peptide, tyroserleutide (YSL), showed some antitumor effects in the early studies [3–5].

Tyroserleutide (YSL) is a tripeptide compound that consists of three natural amino acids, L-tyrosine, L-serine, and L-leucine. YSL was entered into stages I and II of clinical trials as a type I clinical drug (clinical file 2003L03492) in September 2003. In September 2004, the Food and Drug Administration (FDA, USA) approved the request for orphan-drug designation for the ''treatment of hepatocellular carcinoma (HCC)''. In this study, we systemically tested the antitumor properties of YSL in both in vitro and in vivo models and undertook preliminary investigation of its anticancer mechanism to provide a theoretical basis for its clinical application.

Materials and methods

Drugs

Tyroserleutide and an admixture of its constituent amino acids, used in this study, were manufactured by Shenzhen Kangzhe Pharmaceutical Co. Ltd., China. Saline was purchased from China Otsuka Pharmaceutical Co. Ltd., China.

Cell culture

Human hepatocarcinoma Bel7402 cells were purchased from the Cancer Research Department, China Medical Science Institute, and were routinely examined and found to be free of mycoplasma contamination. The cells were cultured in RPMI 1640 medium (Gibco Invitrogen Corp., USA, 1190220) supplemented with 10% fetal bovine serum (FBS; Hyclone, AMC15819) in a humidified atmosphere of 5% CO_2 at 37°C.

Animals

We purchased 50 male nude mice (BALB/c nu/nu; 4–5 week-old; 18–22 g) and two nude mice bearing human hepatocarcinoma Bel7402 from the China Medical Academy of Science (Beijing, China). The animals were maintained under specific-pathogen-free (SPF) conditions using a laminar air-flow rack, with continuous free access to sterilized food and autoclaved water under controlled light/dark cycle, temperature, and humidity conditions at our university. Experiments were commenced after 1 week of acclimatization.

Inhibition of human hepatocellular carcinoma Bel7402 cells in vitro assayed with MTT [6]

Bel7402 cells growing in log phase were detached with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) for 2–3 min. The cells were then examined by dark-ground microscopy, removing the suspension after cytoplasmic pyknosis and the dilatation of the cell compartment were observed. A little RPMI 1640 medium with 10% FBS was added to stop the effects of trypsin–EDTA. To harvest the homogenized cell suspension, cells were eluted from the inner wall of glass bottles by blowing with a tapered pipette and repeated washing. The homogenate was centrifuged at 150×*g* for 10 min and the supernatant was discarded. Cells were then washed twice with Hank's solution by centrifugation at 150×*g* for 5 min. The cell pellets were re-suspended in RPMI 1640 medium with 10% FBS. The cell concentration was adjusted to 5×10^4/ml. Bel7402 cells were placed into a 96-well cell-culture plate at 100 μl/well. The cells were incubated for 24 h at 37°C under 5% CO_2. The experiment included four groups treated with different doses of YSL, an admixture group, and a negative control group. Each group consisted of seven parallel wells. YSL (100 μl/well) at different concentrations was added to the wells of the treatment group, and 100 μl/well admixture and 100 μl/well plain RPMI 1640 to those of the admixture group and the negative control, respectively. The cells were incubated for 48 h at 37°C under 5% CO_2, then pelleted by centrifugation at 150×*g* for 10 min. The supernatants were discarded, 100 μl/well MTT (0.5 mg/ml in RPMI 1640) was added to the cell pellets, and the cells were re-suspended by shaking for 2 min. Incubation was continued for 4 h. The supernatants were discarded after centrifugation at 150×*g* for 10 min. After the plate was dried on filter paper, 40 mM HCl-isopropanol (100 μl/well) was added to the cell pellets, which were shaken for 3 min. An enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad, Model 550) was used to measure the OD_{570} (A value) of each well referenced at 630 nm. The following formula was used to calculate the inhibition of cell growth: growth inhibition (%) = [(mean A value of control − mean of A value of treatment group)/ mean A value of control]×100%.

Observation of human hepatocarcinoma Bel7402 cells in vitro by electron microscopy

The concentration of Bel7402 cells growing in log phase was adjusted to 5×10^4/ml in RPMI 1640 medium with 10% FBS. The cells were placed in 60 cm^2 cell-culture dishes at 4 ml/dish. The cells were incubated for 24 h at 37°C under 5% CO_2. The experiment included four groups treated with different doses of YSL and a negative control group. Each group contained three parallel dishes treated similarly. YSL (4 ml/dish) of different

concentrations was added to the treatment group dishes; plain RPMI 1640 (4 ml/dish) was added to the negative control. The cells were incubated for 48 h at 37°C under 5% CO_2. The cells were then immediately fixed in 2.5% glutaraldehyde solution. The ultrastructures of the cells were observed with a JEOL-100CX-type transmission electron microscope.

Human hepatocarcinoma Bel7402 implantation [7]

Nude mouse bearing a tumor with diameter < 1 cm was selected for withdrawal of tumor cells. The tumor was removed aseptically and cut into 2–4 mm^3 pieces in RPMI 1640 culture medium. A piece was implanted subcutaneously with a trochar on the right flank of each nude mouse.

Mice bearing human Bel7402 hepatocarcinoma were randomized into the YSL groups (80, 160, or 320 μg/kg/day), the admixture of amino acids group (160 μg/kg/day), or the saline group (0.2 ml/day). Solutions were administered from the day after tumor implantation by intraperitoneal injection, once per day for 60 days. Tumor sizes and mouse weights were measured by caliper and balance, respectively, every 3 days until the experiment was terminated after 12 days. Tumor sizes were calculated as $1/6\times\pi\times\text{length}\times\text{width}^2$. On the day after the last dose, the tumor of each mouse was dissected and the weight of the tumor recorded. Three diameters of the tumor (A, B, C) were measured by caliper, and the tumor volume (V) was calculated by the formula: $V = 1/6\times\pi\times A\times B\times C$. The tumor growth inhibition index was calculated according to the following formula: tumor growth inhibition index (%) = [(mean tumor weight of control group − mean tumor weight of treatment group)/ mean tumor weight of control group]×100.

Electron microscopy

Pieces (1–2 mm^3) were cut from the hard parts of the tumors where there was no obvious necrosis or ulceration, and dipped immediately into 2.5% glutaraldehyde solution. The samples were maintained at 4°C for analysis by the transmission electron microscopy. The tissue was then removed from the applicator sticks with citric acid and stained with uranyl acetate. The ultrastructure of the tumors was observed with a JEOL-100CX-type transmission electron microscope.

Flow cytometry

Pieces (1–2 mm^3) were cut from the hard parts of the tumors where growth was good, with no obvious necrosis or ulceration, and dipped into the cold 70% ethanol for flow cytometric detection.

Immunohistology of proliferating-cell nuclear antigen (PCNA)

Immunohistology was performed on 4–5 postmortem serial sections of the routinely processed tissues using a standard protocol. We used monoclonal antibodies specific for PCNA (Santa Cruz Biotechnology, Inc., USA) applied to formalin-fixed paraffin-embedded human Bel7402 hepatocarcinoma tissue, and horseradish peroxidase (HRP)-conjugated goat antimouse polyclonal antibody (KPL Ltd., UK). 3,3′-diaminobenzidine tetrahydrochloride (DAB; 20%) in 0.1 M imidazole (pH 7.1) containing 0.01% H_2O_2 was used as chromogen. The sections were counterstained with Meyer's hematoxylin.

TUNEL detection

An in-situ apoptosis detection kit (TACS TdT Kit, R &D Systems, Inc., USA) was used to detect apoptosis in the tumors of the nude mice.

Statistical methods

All experimental data are expressed as means ± standard deviations. The experimental data on the growth of human hepatocarcinoma Bel7402 cells in vitro and in vivo were analyzed by ANOVA and the significant differences between two groups were assessed by Student Newman–Keuls (SNK) with SPSS statistical software.

Results

Inhibitory effects of YSL on human hepatocarcinoma Bel7402 cells in vitro

Table 1 show that 20 and 40 μg/ml YSL significantly inhibited the growth of human hepatocarcinoma Bel7402 cells grow in vitro compared with the negative control ($P < 0.05$).

Electron microscopic analysis of the effects of YSL on the ultrastructure of human hepatocarcinoma Bel7402 cells

In the control groups, hepatocarcinoma cells had large nucleoli and many fenestrae. (Fig. 1) Cell organelles (chondriosomes and endoplasmic reticulum) were concentrated in the local cytoplasm in few cancer cells. All cells had uneven distribution of the organelles compared to normal cells. The characteristics of apoptosis were not obvious compared with the ultrastructures of the YSL-treated cells.

Table 1 Inhibition by YSL of human hepatocarcinoma Bel7402 cells in vitro

Group	Concentration (μg/ml)	*N*	OD_{570}	Tumor inhibition index (%)
YSL	40	7	0.2512 ± 0.0120*	36.29
YSL	20	7	0.3043 ± 0.0084*	22.84
YSL	10	7	0.3814 ± 0.0092	–
YSL	5	7	0.3864 ± 0.0103	–
Admixture	40	7	0.3982 ± 0.0356	–
Control		7	0.3943 ± 0.0033	–

In the YSL-treated groups, almost half the hepatic carcinoma cells were necrotic, and most of the cytoplasm had disintegrated. Cytomembranes had dissolved almost totally and could not be identified. Chondriosomes and rough endoplasmic reticulum in the cells had totally disintegrated and dissolved denaturalized, and the membranous structures seemed to be vesicular. Some euchromosomes in the centers of the nuclei were extensively dissolved, some had disappeared, and some formed a loose net. Lowered electron density was apparent. The endomembranes adjacent to nuclei and the nucleoli and chromatin at the centers of nuclei were concentrated and contracted, the nucleoli were slightly degraded, most fenestrae had disappeared, and there were many vesicles in the bodies of nucleoli that had interfused to form patches. The apoptosis of some of hepatic carcinoma cells was uncharacteristic: all the cell was crumpled, the cisternae of the dark endoplasmic reticulum were expanded and cystic, and the cell nucleus lacked the typical signs of apoptosis. Chondriosomes were darkened, and the intervals of the cisternae highly expanded.

Effects of YSL on the growth of human hepatocarcinoma Bel7402 transplanted into nude mice

Tyroserleutide significantly inhibited the growth of Bel7402 hepatocarcinoma transplanted into nude mice (Table 2). At doses of 80, 160, or 320 μg/kg/day, inhibition was 40.26, 64.17, or 59.19%, respectively, which differ statistically significantly from the inhibition in the saline group or the admixture of amino acids group ($P < 0.05$). The trend in tumor volumes (Fig. 2) indicates that YSL slowed the growth of the tumor during a later period of growth. In the period of administration, nude mice in the YSL groups were in better condition than control mice, showing increasing weights. YSL had no obvious effect on the weight of the spleen, and the spleen coefficient (%) of the YSL group [= (weight of spleen/body weight)×100] was not significantly different from that of the saline group (Table 3).

Electron microscopic analysis of the effects of YSL on the ultrastructure of human hepatocarcinoma Bel7402 cells (Fig. 3)

The chondriosomes and rough endoplasmic reticulum in cells disintegrated totally and dissolved denaturalized,

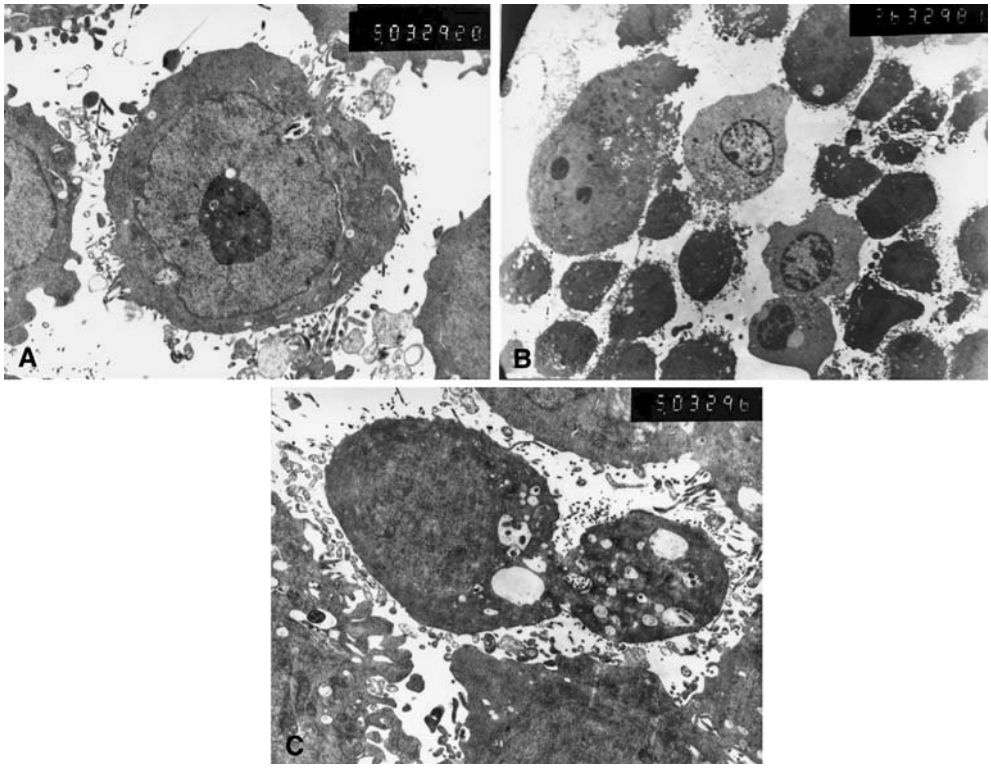

Fig. 1 Effects of YSL on human hepatic carcinomas Bel7402 in vitro under electron microscopy. **a** Control group. Hepatic carcinoma cells have the characteristics of tumor cells: large nucleolus, nuclear chromatin is widely distributed, and microvilli on the cell surface are not evenly distributed (×7,500). **b** YSL (160 μg/kg/day). Some of the cancer cells have swollen and died (×4,950). **c** YSL (160 μg/kg/day).Cancer cell apoptosis is uncharacteristic in the (×7,500)

Table 2 Inhibitory effects of YSL on human hepatocarcinoma Bel7402 transplanted into nude mice

Group	Dose (μg/kg/day)	*N*	Tumor volume (cm^3)	Tumor weight (g)	Tumor inhibition index (%)
YSL	320	10	0.8950 ± 0.2528*	0.8267 ± 0.2131*	59.19
YSL	160	10	0.6485 ± 0.4556*	0.5627 ± 0.3230*	64.17
YSL	80	10	1.0013 ± 0.4230	0.9381 ± 0.3887	40.26
Admixture	160	10	1.5217 ± 0.9669	1.4860 ± 0.9897	–
Saline	–	10	1.9019 ± 0.8116	2.0258 ± 0.9147	–

*Compared to saline, $P < 0.05$, Student Newman–Keuls (*SN-K*), one-way ANOVA

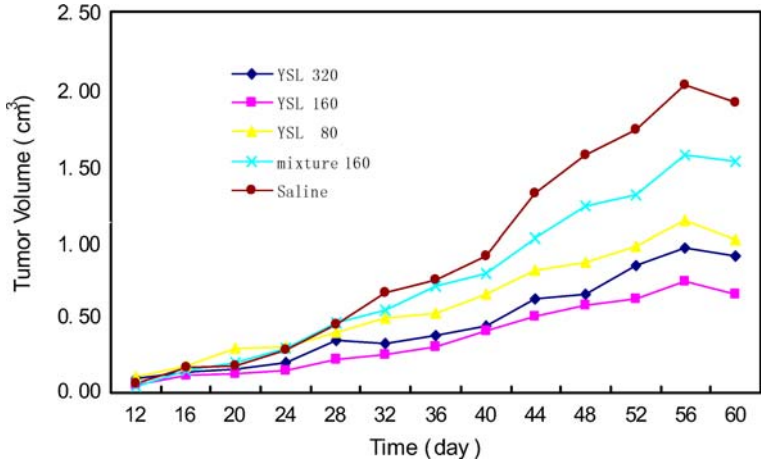

Fig. 2 Inhibitory effects of YSL on human hepatocarcinomas Bel7402 transplanted into nude mice. YSL slowed the growth of the tumor during a later period of growth

and the membranous structures seemed to be vesicular. Some euchromosomes in the centers of nuclei had dissolved extensively, some had disappeared, and some formed loose nets. Lowered electron density was apparent. The endomembranes adjacent to nuclei and the nucleoli and chromatin at the centers of nuclei were concentrated and contracted, the nucleoli were slightly degraded, most fenestrae had disappeared, and there were many vesicles in the bodies of the nucleoli that interfused to form patches. The apoptosis of some hepatic carcinoma cells was uncharacteristic: the entire cell was crumpled, the cisternae of the darkened endoplasmic reticulum were expanded and cystic, and the cell nuclei lacked the typical signs of apoptosis. Chondriosomes were darkened, and the intervals of the cisternae highly expanded, with a tendency to be densely interfused.

Inhibitory effects of YSL on human hepocarcinoma Bel7402 transplanted into nude mice analyzed by flow cytometry (Table 4, Fig. 4)

Flow-cytometric analysis showed that YSL markedly increased the proportion of G_0/G_1(DNA-synthesis resting phase) cells, decreased the proportion of S-phase (proliferative phase) cells, and increased the apoptosis rate statistically significantly relative to the corresponding parameters of the saline group ($P < 0.05$).

Immunohistology of PCNA (Fig. 5)

Tyroserleutide obviously decreased the expression of PCNA compared with that in the saline group.

Table 3 Effects of YSL on body weight and spleen weight of nude mice bearing human hepatocarcinomas Bel7402

Group	Dose (μg/kg/day)	Animal		Weight (g)		Increase in weight (g)	Spleen index
		Initial	Final	Initial	Final		
YSL	320	10	10	17.27 ± 1.34	23.31 ± 3.51	6.04 ± 4.08	0.8728 ± 0.1901
YSL	160	10	10	18.27 ± 2.02	22.84 ± 2.12	4.57 ± 1.56	0.7858 ± 0.0922
YSL	80	10	10	18.34 ± 1.37	22.01 ± 2.57	3.67 ± 2.55	0.8320 ± 0.1896
Admixture	160	10	10	18.64 ± 2.92	22.93 ± 2.54	4.29 ± 2.49	0.8467 ± 0.1787
Saline	–	10	10	18.82 ± 1.54	23.62 ± 3.09	4.80 ± 2.90	0.9524 ± 0.1773

*Compared to saline, $P < 0.05$, Student Newman–Keuls (*SNK*), one-way ANOVA

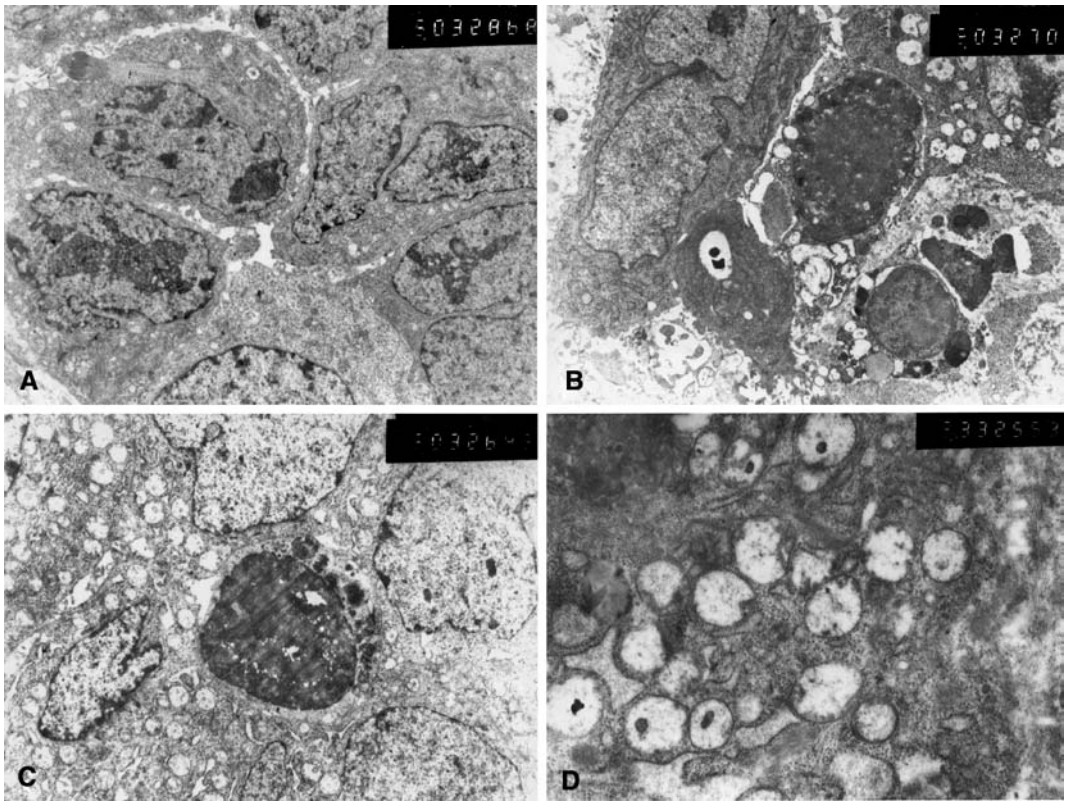

Fig. 3 Effects of YSL on human hepatocarcinomas Bel7402 transplanted into nude mice **a** Saline group. Cancer cells grew very well (×7,500). **b** YSL (160 μg/kg/day). An apoptotic body (×7,500). **c** YSL (160 μg/kg/day). Cell of apoptosis; nuclei of cancer cells have dissolved (×7,500). **d** YSL (160 μg/kg per day). Bubbles of chondriosomes and endoplasmic reticulum (×12,450)

TUNEL detection (Fig. 6)

Tyroserleutide obviously increased the apoptosis of tumor cells relative to that of the saline group. A few apoptotic cells were detected in the saline group.

Discussion

Uncontrolled growth is one of the most important characteristic of cancer cells. Cancer cells in deregulated proliferation undergo more cycles before the death of the cell than do normal cells, and this constitutes one of their most important differences from normal cells. Loss of the mechanism that limits cell growth allows neoplastic progression [8]. Therefore, targeting these critical events should have potent and specific therapeutic consequences for cancer. Apoptosis and necrosis are two forms of cell death, defined based on the distinguishable morphological criteria [9–10]. Apoptosis is an important mechanism in the regulation of tissue homeostasis, and is an important feature of various pathological conditions. The malfunctioning of apoptosis will lead to the abortion or abnormalities in the organism, and is one of the reasons for tumor formation. It is critical to the occurrence, development, and treatment of tumors [11]. In one treatment approach, current antitumor drugs induce the apoptosis of tumor cells, and their antitumor effects relate to their capacity to induce apoptosis [12–13].

In this study, we established a human hepatocarcinoma Bel7402 model in vitro and in vivo to verify the antitumor effects of YSL. The human hepatocarcinoma Bel7402 cell line was established in 1975 by Chen-ruiming [14] in China, and originated from the hepocarcinoma of a 75-year-old male patient. The tumor tissue was cut into pieces and cultured from round tubes to monolayer

Table 4 Effects of YSL on the cell cycle and apoptosis index of human hepatocarcinoma Bel7402

Group	Dosage	*N*	G_0/G_1	S	G_2/M	Ratio of apoptosis (%)
YSL	160 μg/kg/day	10	75.72 ± 3.64*	12.51 ± 1.65*	11.77 ± 2.76	9.79 ± 1.98*
YSL	80 μg/kg/day	10	76.20 ± 5.00*	10.96 ± 2.77*	12.84 ± 3.79	12.62 ± 2.09*
Saline	0.2 ml/day	11	67.28 ± 6.15	17.63 ± 3.41	15.10 ± 3.34	2.09 ± 0.46

*Compared to negative group, $P < 0.05$, Student Newman–Keuls (SNK), one-way ANOVA

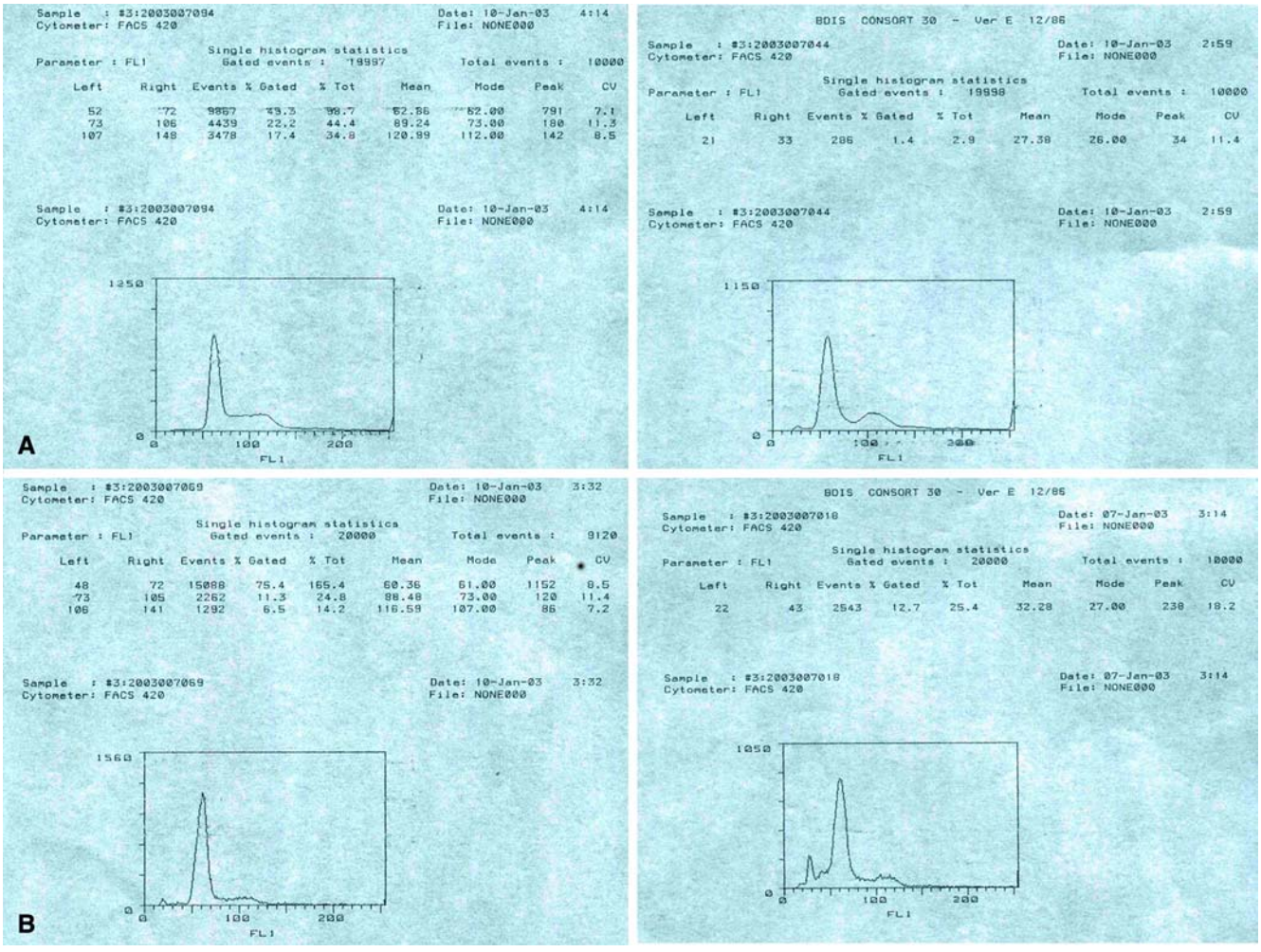

Fig. 4 Inhibitory effects of YSL on human hepatocarcinoma Bel7402 transplanted into nude mice **a** Saline: G_0/G_1, 55.48%; S, 24.96%; G_2/M, 19.56%. Ratio of apoptosis (%): 1.4. **b** YSL (160 μg/kg per day): G_0/G_1, 80.94%; S, 12.13%; G_2/M, 6.93%. Ratio of apoptosis (%): 12.7

dishes in vitro, maintaining the characteristics of human hepatocarcinoma and their malignant character. The characteristics of the human hepatocarcinoma Bel7402 cell line are similar to those of clinical human hepatocarcinoma, and inherent traits are preserved. The effects of YSL on cell growth were analyzed using the MTT method. YSL significantly inhibited Bel7402 cell growth, up to approximately 40% inhibition at 40 μg/ml, but below 50%. At the cellular level, electron microscopy showed that YSL changes the ultrastructure of tumor cells and induces necrosis and apoptosis. In this study, we established the human hepocarcinoma Bel7402 in nude mice. Doses of 80–320 μg/kg/day YSL inhibited the growth of these tumors. The results were consistent through five repetitions, with a tumor inhibitory rate of 40–60%. An admixture of amino acids had no significant effect compared with the control group, suggesting that the chemical structure of YSL is the basis of its antitumor

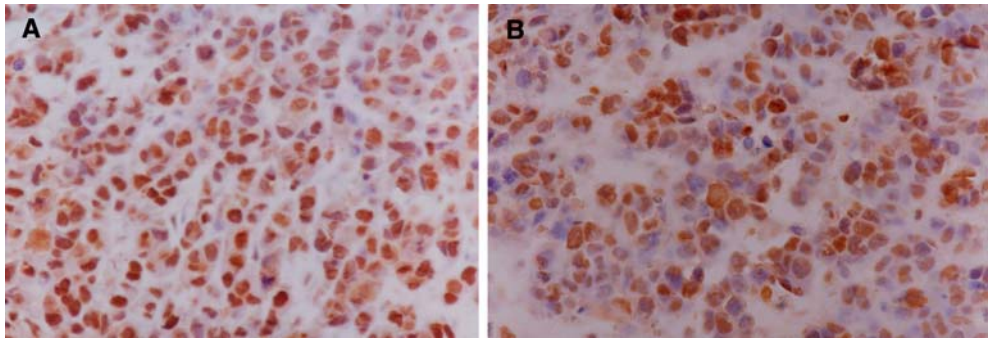

Fig. 5 Immunohistological staining for PCNA expression in tumor tissues treated and untreated with YSL from human hepatocarcinoma Bel7402 in nude mouse. A Saline group (× 400), B YSL (160 μg/kg/d) (× 400). The cell whose nucleus appears a chocolate brown color is positive cells

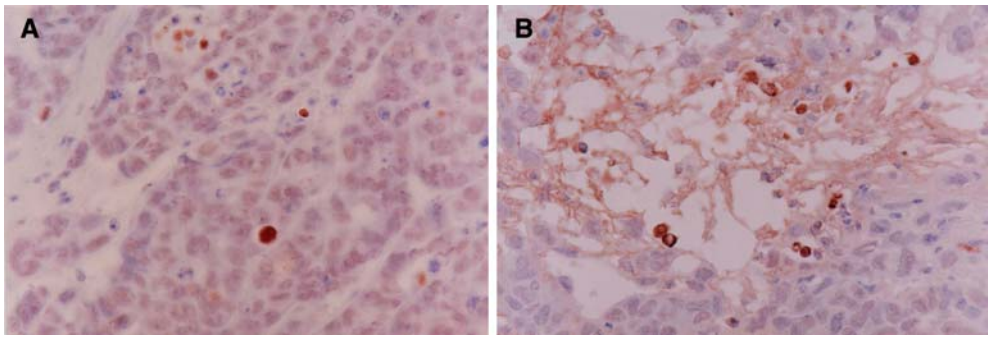

Fig. 6 TUNEL detection of apoptotic cells in tumor tissues of nude mice bearing human hepatocarcinoma Bel7402 **a** Saline group (×400), B YSL (160 μg/kg/day) (×400). The cell whose nucleus appears a *chocolate brown color* is positive cells.

effect. Direct evidence that YSL inhibits the growth of tumors has been provided by in vitro and in vivo experiments. We will proceed to explore the anticancer mechanisms of YSL further.

The metabolic activity of cells is usually measured in cell populations by incubation with a tetrazolium salt (e.g., MTT) that is cleaved to a colored formazan product by the metabolically active cells. The OD value of the product measured by an absorbance reader reflects cell viability [15]. YSL decreased the tumor's capacity for metabolism, thus inhibiting the growth of human hepatocarcinoma Bel7402.

Our study demonstrates the antiproliferation action of YSL on the growth of tumors in nude mice by demonstrating a decrease in PCNA. In vitro and in vivo studies have suggested that the inhibition of tumor cell proliferation is an effective approach to anticancer therapy. PCNA [16] was discovered with auto-antibodies from patients with systemic lupus erythematosus and identified as an evolutionarily highly conserved 36-kD auxiliary protein of DNA polymerase-δ. This enzyme is probably involved in the catalysis of eukaryotic leading-strand synthesis and in DNA repair during the G_0 or G_1 phase of the cell cycle. It is easy to assess the proliferation activity of human malignant tumors by estimating the expression of cell proliferation markers such as PCNA.

Current methods for studying the apoptosis mainly involve the assessment of morphological changes at the cellular level by light and electron microscopy and biochemical assays to detect DNA fragmentation by agarose gel electrophoresis or by TUNEL and assaying the marker in apoptosis binding dye by flow cytometry. Under electron microscopy, cells undergoing apoptosis display cell shrinkage, apoptotic body formation, and chromatin condensation [17]. A number of biochemical methods have been developed with which to study apoptosis but there is no diagnostic change that only occurs in apoptosis. The apoptotic process is characterized by fragmentation of the DNA into oligonucleosomal fragments. Although fragmentation of chromosomal DNA is not the direct cause of cell death, it is the biological hallmark of apoptosis [18]. An important method for visualizing the DNA fragments of apoptotic cells is the TUNEL method [19]. During apoptosis, specific calcium-dependent endonucleases degrade genomic DNA, creating fragments with double-stranded breaks. TUNEL detection identifies apoptotic cells by identifying DNA fragmentation through a combination of enzymological and immunohistochemical techniques. It permits in-situ visualization of DNA cleavage by inserting a marker at the 3′ nicked end. To identify apoptotic cells in a tumor, flow cytometric techniques are used to evaluate the presence of phosphatidylserine on the cell surface and the extent of DNA fragmentation. The use of flow cytometric techniques has significantly augmented the rapid advancement of our understanding of the process of apoptosis. Using flow cytometry to detect apoptosis in cell cultures and to measure intracellular changes that occur during apoptosis has be a critical factor in basal research into tumors. Observing the ultrastructure of tumor cells in vitro and in vivo showed that apoptotic tumor cells were more prevalent in the YSL treatment groups than in the saline group. The tumor cells in the YSL groups had characteristics typical of apoptosis, such as karyopyknosis, darker cells, and high electron density. Besides typical apoptosis, characteristics of necrosis were also observed, including karyolysis, karyorrhexis, and dilated endoplasmic reticulum. TUNEL analysis and flow cytometry also suggested that YSL decreases the ratio of cells in S phase and increases those in G_0/G_1, so it prevents cells in G_0/G_1 phase progressing to S phase, thus inhibiting tumor cell proliferation. Electron microscopic observations further confirmed that YSL induces tumor apoptosis or necrosis. Overall, we deduced that the anticancer effects of YSL may result from its inhibition of tumor cell proliferation and the induction of apoptosis.

The apoptosis and proliferation of cells are the main factors in the adjustment of the development and stabilization of organisms. Their dynamic balance can determine the status of some organs, between homeostasis and morbidity. The evaluation of antitumor drugs lies not only in their ability to kill tumor cells but also in their ability to induce apoptosis in tumor cells [20]. We infer that inducing cell apoptosis may be the main anticancer mechanism of YSL.

Tyroserleutide is a new kind of small polypeptide medicament with significant anticancer effects. Our study provides an experimental justification for the development of new effective anticancer medicament.

References

1. Dwyer JM (1996) Transfer factor in the age of molecular biology: a review. Biotherapy 9:7–11
2. Berressem Peter (1991) Application of glycopeptide fractions in the treatment of tumor patients suffering from immunodeficiency. Therapeutikon 5:264–270
3. Wang C, Ding W, Zhao M, Lin G, Peng S (2003) Studies on the large scale synthesis and anti-tumor activity of YSL. Prep Biochem Biotechnol 33:189–195
4. Ding W, Zhang J, Yao Z, Lu R, Wu D, Li G et al (2004) The synthesis, distribution, and anti-hepatic cancer activity of YSL. Bioorg Med Chem 12:4989–4994
5. Li Y, Tang ZY, Wang L, Yao Z, Lu R, Xue Q et al (2004) The inhibitory of tripeptide CMS024 on the metastasis of human hepatocarcinoma in nude mice. J Chin Med 84:675–679
6. Xu SY, Bian RL, Chen X (2002) Methodology of pharmacological experiment (3rd edn). People's Health Publishing House pp 1785–1786
7. Han Y (1997) The research and experiment techniques of anticarcinoma drugs (1st edn). Beijing medical university and China Xiehe medical university united Publishing House p 299
8. Roger JB (1996) Cancer Biology (2nd edn). King Pearson Education Limited:199
9. Schwartzman RA, Cidlowski JA (1993) Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine Rev 14:133
10. Vermes I, Haanen C (1994) Apoptosis and programmed cell death in health and disease. Adv Clin Chem 31:177
11. Xu KL (1997) The review of the abnormal modulation of apoptosis gene in the occurrence and development of tumor and lung cancer. J Cancer Chin 7:4
12. Lowe SW, Lin AW (2000) Apotosis in cancer. Carcinogenesis 21:485–495
13. Chabner BA (1982) The role of drugs in cancer treatment. In: Chabner B (ed) Pharmacologic principles of cancer treatment. Saunders, Philadelphia, pp 1–14
14. Chen ruiming, Zhu dehou, Ye xiuzhen (1975) The establishment and character of human hepacarcimoma cell line (BEL-7402) in vitro. Comm Sci 20:434–436
15. Wright WE, Shay JW (2001) Cellular senescence as a tumor-protection mechanism: the essential role of counting. Curr Opin Genet Dev 11:98–103
16. Roninson IB, Broude EV, Chang BD (2001) If not apoptosis, then what treatment-induced senescence and mitotic catastrophe in tumor cells. Drug Resist Updat 4:303–313
17. Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–247
18. Marcel L, Simone K, Barbara S (1998) Differentiation between apoptotic and necrotic cell death by means of the bm cell death detection elisa or annexin V staining. Biochemicano 2
19. Darzynkiewicz Z, Li X, Gong J (1994) Assays of cell viability: discrimination of cells dying by apoptosis. Methods Cell Biol 41:15–38
20. McDonnell TJ, Meyn RE, Robertson LE (1995) Implications of apoptotic cell death regulation in cancer therapy. Semin Cancer Biol 6:53–60