Effects of octreotide on necrosis of hepatocellular carcinoma xenografts in nude mice

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[Abstract] Background and Objective: Octreotide, a kind of somatostatin analogue, may inhibit the growth of hepatocellular carcinoma (HCC). This study was to investigate the mechanism of inducing necrosis of HCC xenografts in nude mice by octreotide. Methods: The proliferation of HepG2 cells was determined by MTT assay. Nude mice bearing HepG2 xenografts were treated with octreotide [100 μg·(kg·d)⁻¹] or normal saline (as control) for eight weeks. The necrosis of HCC was estimated by histology. Vascular endothelial growth factor (VEGF) was detected by immunohistochemistry. Somatostatin receptor 2 (SSTR2) was quantified by Western blot and located with immunohistochemistry. Results: The proliferation of HepG2 cells was not obviously affected by 24-hour treatment of octreotide (0.1–1000 nmol/L) in vitro. The tumor weight was significantly heavier in octreotide group than in control group [(7.15±2.96) g vs. (4.21±3.11) g, P<0.05], while the proportion of necrotic volume was significantly higher in octreotide group than in control group [(81.86±0.05)% vs. (43.75±0.06)%]. In contrast with control group, VEGF was undetected in the xenografts in octreotide group. SSTR2 expression in xenograft sinusoids was similar in both groups. Conclusion: With active proliferation of HCC cells, octreotide can induce necrosis in HCC xenografts only through the inhibition of angiogenesis mediated by SSTR2 in the tumor. Key words: octreotide, liver neoplasm, HepG2 cell, xenografts, necrosis, vascular endothelial growth factor

Hepatocellular carcinoma (HCC) is a common malignant tumor in the world, and the prognosis of HCC is extremely poor due to lack of effective drugs. In 1998, Greek researchers firstly reported that octreotide, a kind of somatostatin analogue (SSTA), could improve life quality of advanced HCC patients and prolong their survival.¹ From then on, many experimental studies on anti-HCC effect of SSTA have been reported, however, its efficacy and mechanism are controversial, and various clinical results make its pharmacodynamics more complicated and confusing.²,³

We have previously reported that octreotide could inhibit the growth of HCC SMMC-7721 cells in vitro and in vivo.⁴⁻⁵ If octreotide can induce the necrosis of HCC with the efficacy similar to that of tumor resection, it will help to alleviate tumor burden. Our previous experimental results have shown that octreotide has no significant effect on HepG2 cells in vitro,⁶ and other SSTAs also have no inhibitory effect on the growth of HepG2 cells.⁷ Although...
Reynaert et al.⁷ has detected five kinds of somatostatin receptors (SSTRs) in human HCC tissues and HepG2 cells, no related studies in vivo have been reported thereafter due to the negative results of in vitro studies on the effects of SSTA on HepG2 cells.

It was often found that certain drugs, in studies on anticancer pharmacodynamics, have very high anticancer activities in vitro. However, they have no effectiveness in vivo. Generally, chemotherapeutic agents have strong efficacy on the tumors that proliferate actively in vivo. If octreotide can inhibit the angiogenesis in HepG2 tumor, the cut-off of nutrition supply will lead to the necrosis of HepG2 cells which proliferate rapidly in spite of octreotide interference. In present study, the anticancer effects of octreotide on HepG2 cells in vitro and in vivo were investigated, and the mechanism of octreotide induced necrosis was also explored.

Materials and Methods

Materials and reagents. Human HCC cell line HepG2 was preserved at our lab; BALB/c nu/nu nude mice (male, SPF grade, 6-8 weeks old, 20-30 g) were purchased from Animal Center at West China Center of Medical Sciences in Sichuan University [certificate number: SCXK (CHUAN) -09-2006]. Octreotide (Sandos, Switzerland), MTT reagent and DNA terminal in situ labeling and staining (TUNEL) kit were purchased from Roche Company; Annexin-V-FITC kit was purchased from and Nucleoprotein and Cytoplasmic Protein Extraction Kit were provided by Nanjing KeyGen Biotech Company; RPMI-1640 powder medium was produced by Hyclone Company; BCA Protein Assay Kit and SuperSignal West Pico Chemiluminescent Substrate Kit were purchased from Pierce Company. calf serum was from Chengdu Harris Biological Engineering Company; polyvinylidene difluoride membranes were purchased from Millipore Company; β-actin and SSTR-2 goat anti-human monoclonal antibodies were produced by Santa Cruz Company; VEGF rabbit anti-human polyclonal antibody, SP9001 and SP9003 immunohistochemistry kits were purchased from Beijing Zhongshan Goldenbridge Biotechnology Company; SSTR2 primary antibody was the product of Novus Company; Horseradish peroxidase-labeled goat anti-rabbit secondary antibody was purchased from Cell Signaling Company.

Cell culture. HepG2 cells were cultured with RPMI-1640 medium containing 10% inactivated calf serum at 37°C in an incubator with 5% CO₂ and saturated humidity, and passed every 2-3 days through trypsinization by 0.25% trypsin.

MTT assay. HepG2 cells in logarithmic phase were collected through trypsinization by 0.25% trypsin, and then seeded into a 96-well plate at a density of 1.2 × 10⁴ cells/well. After 24-hour cell culture, original medium was replaced with RPMI-1640 medium containing 1% calf serum for another 24-hour cell culture. Control group and octreotide groups in triplicate wells were set up in the experiment. In control group, no octreotide was added. In octreotide groups, octreotide at various concentrations of 0.1-1000 nmol/L were added into wells, respectively. The total volume per well was 200 µ L. After further 24-hour cell culture, MTT reagent (20 L) was added into each well, and the cells were further cultured for 4 h at 37°C in an incubator with 5% CO₂ and saturated humidity. Then, the supernatant was removed, and DMSO (150 L) was added into each well. After gently shaking for 10 min, the absorbance at 490 nm was measured on an enzyme-link immunoassay meter. Finally, cell proliferation curves were plotted according to the absorbance values at various concentrations of octreotide.

TUNEL method. HepG2 cells were seeded in a 24-well plate containing a small glass slide in each well at a density of 2.5 × 10⁴/well. After 24-hour cell culture, original medium was replaced with fresh culture medium. In octreotide groups, 0.1, 10 and 1000 nmol/L of octreotide were added, respectively. The same volume of culture medium was added in control group. The total volume in each well was 1 mL. After further 24-hour culture, HepG2 cells were fixed for 30 min using 4% paraformaldehyde.
Then, TUNEL assay was performed according to the protocols. Five visual fields (× 400) were randomly selected for cell counting by microscopy. The cells, in which nuclei were stained in brownish yellow, were positive cells. Apoptosis index = number of apoptosis cells / number of total cells × 100%.

Detection of early apoptotic cells by Annexin-V fluorescent labeling. HepG2 cells in logarithmic phase were collected and seeded into 6-well plates at a density of 2.5 × 10^6 cells/well, cultured in serum-free medium for 24 h, then added with 10 and 1000 nmol/L of octreotide, respectively. After 24-hour culture, the supernatant was collected. The cells were collected through trypsinization, rinsed with PBS buffer and centrifuged at 250 × g for 5 min for three times. According to the Annexin- V -FITC kit protocols, early apoptotic cells were detected by flow cytometry.

Establishment of orthotopic implantation HCC model. A total of 2 × 10^6 HepG2 cells in logarithmic phase were implanted in the anterior axillary region of BALB/c nu/nu nude mice to induce tumor formation. Then, tumor-bearing mice were killed, and the tumor masses were excised, and immediately immersed in normal saline (NS) solution containing 100 u/mL penicillin and streptomycin. The peritumorous connective tissues were removed. Then, the tumor tissue at tumor edge was cut into blocks with a diameter of 2 mm. Nude mice were randomly divided into two groups (control and octreotide group), and each group included eight nude mice. After intraperitoneal anesthesia by injection of pentobarbital sodium at a dose of 50 mg/kg, the livers of nude mice were exposed through the median incision of lower abdomen. Then, tumor blocks were implanted in the right lobe of liver through a slanting incision. Finally, the abdominal cavity was sutured layer by layer after stanching by gelatin sponge.

Animal experiment in vivo. At 24 h after implantation of tumor blocks, each nude mouse in octreotide group received subcutaneous injection of octreotide at a dose of 100 μg·kg⁻¹·d⁻¹; each mouse in control group received injection of the same volume of NS. After continuous treatment for eight weeks, nude mice were killed, and the tumor masses were excised, weighed, and fixed using 4% paraformaldehyde. Continuous paraffin sections were made for histological observation. Tissue necrosis area in each slice was measured to calculate the percentage of tumor necrosis volume.

Detection of SSTR2 expression in tumor tissues by Western blot. Total proteins were respectively extracted from 100-200 mg tumor tissue in each sample using Nucleoprotein and Cytoplasmic Protein Extraction Kit according to manufacturers instructions, and quantified using the BCA Protein Assay Kit. Each sample of total proteins was vortexed with the same volume of 2 × loading buffer, then heated at 100 °C for 5 min and kept at -20 °C for further Western blot assay.

Western blot was performed according to the following procedures. Firstly, 12% separation gel and 4% stacking gel was prepared. For each sample, total proteins (40 μg) was respectively loaded into sample well in the gel, and then was separated through 4% stacking gel electrophoresis at a constant voltage of 80 V and subsequent 12% separation gel electrophoresis at a constant voltage of 120 V. After electrophoresis, the separated protein in the gel was transferred onto the polyvinylidene difluoride membrane (PVDF membrane) by the semi-dry process at a constant current for 1 h according to the calculation by the value of 1.2 mA/cm². PVDF membrane was blocked with TBST (Tris-buffered saline containing 0.1% Tween-20) containing 5% fat-free milk at 4 °C overnight, then rinsed with TBST for 5 min for three times, incubated respectively with β -actin (1:1000) and SSTR2 primary antibody (1:750) at room temperature for 1 h, then rinsed again with TBST for 5 min for three times, and incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:2000) at room temperature for 1 h. Finally, the PVDF membrane was rinsed with TBST for 5 min for three times and with TBS for 5 min once, then exposed to develop using SuperSignal West Pico Chemiluminescent Substrate Kit. Xray films were scanned to assess the bans of proteins.
Detection of VEGF and SSTR2 expression in tumor tissues by immunohistochemistry. The paraformaldehyde-fixed tumor tissues were conventionally embedded in paraffin. Serial 5 μm sections of paraffin-embedded tumor tissues were dewaxed, hydrated, then reared at high pressure using 0.01 mol/L citric acid for 5 min. Immunohistochemical staining was performed with VEGF rabbit anti-human polyclonal antibody (1:200) and SSTR2 primary antibody (1:200) at 4°C overnight using SP9001 and SP9003 immunohistochemistry kits (three-step method) according to manufacturers instructions.

VEGF was expressed in cytoplasm in chocolate brown. Five high power fields were randomly selected to count more than 500 cells using the new CAST system. According to staining intensity, no staining was scored 0; weak staining was scored 1; strong staining was scored 2. According to the positive rate of cells, ≤ 5% was scored 0; 6%-25% was scored 1; 26%-50% was scored 2; 51% was scored 3. The final score of each section was the product of the score of staining intensity and score of positive rate. The staining was classified as follows: a score of 0-1 was classified as negative (-); 2-3 as weak positive (+); 4-6 as moderate positive (++); >6 as strong positive (+++).

SSTR2 was expressed in cytoplasm and on membrane in chocolate brown.

Statistical analysis. All data were presented as mean ± SD, and analyzed using SPSS 13.0 statistical software. Enumeration data were compared by the t test; measurement data were compared by the chi-square test.

Results

Effect of octreotide on proliferation of HepG2 cells. At 24 h after treatment, no significant difference in the proliferation rate of HepG2 cells was observed between octreotide group and control group, no concentration-associated inhibitory effect of octreotide on proliferation was found (Fig. 1).

Effect of octreotide on apoptosis of HepG2 cells. A few scattered apoptotic HepG2 cells, with nuclei stained in brownish yellow, were observed under optical microscope after 24-hour treatment of various concentrations of octreotide (0.1, 10, 1000 nmol/L). However, compared with the apoptosis rate [(1.14 ± 0.9)%] in control group, 1000 nmol/L octreotide did not induce significant apoptosis of HepG2 cells [(1.66% ± 1.23)%], (P >0.05). The early apoptosis rates of HepG2 cells were similar in 1000 nmol/L octreotide group and control group [(3.8 ± 2.2)% vs. (3.5 ± 1.2)%], (P >0.05) (Fig. 2).

Effect of octreotide on necrosis of HCC xenografts in nude mice. HCC orthotopic implantation tumors formed in all nude mice and grew rapidly. During the eight weeks treatment, four (50%) nude mice in control group died, none in octreotide group died. HCC xenografts were round or ellipse, with nodules on the surface of some tumors. The mean tumor weight was significantly heavier in octreotide group than in control group [(7.15 ± 2.96) g vs. (4.21 ± 3.11) g, P<0.05].

Figure 1 Effect of octreotide on proliferation of HepG2 cells

Figure 2 Effect of octreotide on early apoptosis of HepG2 cells measured by flow cytometry
Only necrosis in small scale [(43.75 ± 0.06)\%] was found in central region of tumor in control group, and a large part of tumor was fish meat-like tissue. Necrosis area [(81.86\% ± 0.05)\%] octreotide group was significantly bigger than that in control group (P<0.05). In octreotide group, cavities were found in the necrosis area, and small amount of fish meat-like tissue was found at the edge of xenografts (Fig. 3).

Effects of octreotide on blood supply in tumor tissue and expression of VEGF. In control group, a lot of blood sinuses with red blood cells were observed among tumor cells in tumor tissues, but no apoptotic and necrotic tumor cells were found in blood sinuses. However, in octreotide group, no blood sinus-like histological structure was observed in the fish meat-like tumor tissue, but a lot of necrotic tumor cells with homogeneous red staining were found. In four tumors in control group, VEGF-positive cells with brown cytoplasmic staining were found in three tumors. However, in octreotide group, no VEGF expression was found in all eight tumors (Fig. 4).

Expression of SSTR2 in tumor tissue. The expression of SSTR2 was detected by Western blot in tumor tissues in both groups, with similar expression levels. No expression of SSTR2 on the surface of liver cells was detected by immunohistochemistry, but positive granules were scattered in peripheral space of liver cells (Fig. 5).

![Figure 3](image1.png)

**Figure 3** The necrotic lesions in HepG2 cell xenografts in nude mice after treatment of octreotide

Green arrow indicates the fish-like tumor tissue; black arrow indicates tumor necrosis.

![Figure 4](image2.png)

**Figure 4** Blood accommodation in the xenografts of two groups

Upper panel: Green arrow indicates the sinusoids between tumor cells; no sinusoid is seen in tumor tissues of octreotide group (HE ×400).

Lower panel: The positive staining of vascular endothelial growth factor (VEGF) is visualized in xenografts of control group but not in octreotide group (IHC ×400).
octreotide group were significantly heavier than those in control group. However, the percentages of necrotic tumor volume were 82% in octreotide group, and 44% in control group. Because the volume and weight of necrotic foci were increased by the inflammatory exudation and swelling induced by necrotic tissue, the tumor weight in octreotide group was significantly heavier than that in control group. Therefore, anticancer effectiveness with tumor necrosis should be evaluated by tumor necrosis volume rather than tumor weight and volume.

HCC is a tumor with high vascularization, and blood vessels play an important role in tumor growth and metastasis. However, the structure and functions of blood vessels in tumor tissues are different from those in normal tissues. Generally, blood vessels in tumor tissues have irregular inner diameter and abnormal branched structure, lack endothelial cells and intact basement membranes. Tumor angiogenesis is generally associated with VEGF induction. It has been reported that the monoclonal antibody of VEGF could inhibit the growth of HCC in vivo. In present study, no normal structures of blood vessels were observed in HepG2 xenografts, but blood sinuses intensively grew around cells with SSTR2 expression mainly found in these areas. After treatment of octreotide, the expression of VEGF disappeared in HepG2 xenografts. This could be explained that octreotide could bind to SSTR2 in blood sinus to initiate a series of signal transduction pathways in stromal cells and inhibit VEGF synthesis, which reduces angiogenesis in HCC xenografts, blocks essential nutrition supply for tumor growth and finally induces tumors necrosis in large scale. In addition, other groups have also reported that octreotide could inhibit tumor angiogenesis, which was concluded in our present study. Jia et al. found that octreotide could inhibit VEGF-stimulated growth of vascular endothelial cells via the mediation of SSTR3, and inhibit angiogenesis.

In present study, the mean tumor weight of HepG2 xenografts was 4.21 g, which was about five times of that of SMMC-7721 liver xenografts in our previous study. Rapid growth
of HepG2 xenografts led to a mortality of 50% in control group in eight weeks, and led to tumor necrosis due to lack of blood supply. Octreotide could further reduce the blood supply and induce tumor necrosis in larger scale. Octreotide showed remarkable anti-HCC effect in nude mice, and the survival rate of mice in octreotide group was one time than that in control group. In other words, good anticancer effectiveness is also obtained through effective block of tumor blood supply when rapid growth of cancer cells is not directly inhibited.

Reviewing the effect of octreotide on HCC xenografts, we can classify the antitumor effects of octreotide as the following two categories according to types of HCC cell line. 1) Octreotide has direct inhibitory effect on the growth of HCC cells, such as SMMC-7721. This type of tumor cell line can not vigorously grow. Although inhibiting tumor angiogenesis, octreotide can only slow down tumor growth. 2) Octreotide has no direct inhibitory effect on the growth of HCC cells, such as HepG2 in present study. This type of tumor cell line can vigorously grow. Inhibiting angiogenesis can lead to tumor necrosis, and this kind of effect is similar to cytotoxic effect. In summary, the expression of SSTR in HCC cells is not essential for candidates of octreotide treatment, however, the expression of SSTR in blood sinuses of HCC tissue is very important.

References
