Vascular endothelial growth factor antisense oligodeoxynucleotides
with lipiodol in arterial embolization of liver cancer in rats

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AIM: Transcatheter arterial embolization (TAE) of the hepatic artery has been accepted as an effective treatment for unresectable hepatocellular carcinoma (HCC). However, embolized vessel recanalization and collateral circulation formation are the main factors of HCC growth and recurrence and metastasis after TAE. Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis. This study was to explore the inhibitory effect of VEGF antisense oligodeoxynucleotides (ODNs) on VEGF expression in cultured Walker-256 cells and to observe the anti-tumor effect of intra-arterial infusion of antisense ODNs mixed with lipiodol on rat liver cancer.

METHODS: VEGF antisense ODNs and sense ODNs were added to the media of non-serum cultured Walker-256 cells. Forty-eight hours later, VEGF concentrations of supernatants were detected by ELISA. Endothelial cell line ECV-304 cells were cultured in the supernatants. Seventy-two hours later, growth of ECV-304 cells was analyzed by MTT method. Thirty Walker-256 cell implanted rat liver tumor models were divided into 3 groups. 0.2 mL lipiodol (LP group, n=10), 3OD antisense ODNs mixed with 0.2 mL lipiodol (LP+ODNs group, n=10) and 0.2 mL normal saline (control group, n=10) were infused into the hepatic artery. Volumes of tumors were measured by MRI before and 7 d after the treatment. VEGF mRNA in cancerous and peri-cancerous tissues was detected by RT-PCR. Microvessel density (MVD) and VEGF expression were observed by immunohistochemistry.

RESULTS: Antisense ODNs inhibited Walker-256 cells’ VEGF expression. The tumor growth rate was significantly lower in LP+ODNs group than that in LP and control groups (140.1±33.8% vs. 177.9±64.9% and 403.9±69.4% respectively, F=60.019, P<0.01). VEGF mRNAs in cancerous and peri-cancerous tissues were expressed highest in LP group and lowest in LP+ODNs group. The VEGF positive rates showed no significant difference among LP, control and LP+ODNs groups (90%, 70% and 50%, H=3.731, P>0.05). The MVD in LP+ODNs group (53.1±18.4) was significantly less than that in control group (73.2±20.4) and LP group (80.3±18.5) (F=5.44, P<0.05).

CONCLUSION: VEGF antisense ODNs can inhibit VEGF expression of Walker-256 cells. It may be an antiangiogenesis therapy agent for malignant tumors. VEGF antisense ODNs mixed with lipiodol embolizing liver cancer is better in inhibiting liver cancer growth, VEGF expression and microvessel density than lipiodol alone.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in human beings[1,2]. In China, HCC is responsible for 130 000 deaths every year and the second cause of cancer deaths[3,5]. Surgical resection is still the only potentially curative treatment for HCC, particularly for small HCC[4,6]. To date, the resection rate for HCC is unfortunately less than 30%-35%. Since HCC’s blood supply is derived almost exclusively from hepatic arteries, transcatheter arterial embolization (TAE) of the hepatic artery has been accepted as an effective treatment for unresectable HCC[7-10]. Although embolic materials and technique have been improved during the last decade, the outcome of TAE is not satisfactory. It hardly leads to tumor necrosis totally. The 3-year survival rate is about 14-35%[10,14]. Recanalization of the embolized vessel and collateral circulation formation, which are related with tumor angiogenesis, are the main factors of HCC growth, recurrence and metastasis after TAE[15,16]. In TAE, HCC cells undergo coagulative necrosis, a pathologic feature of anoxia. Anoxia and hypoxic liver injury are caused by the absolute and relative deficiency of oxygen, respectively. It is well known that hypoxia tension is a key factor of the gene expression of angiogenic factors such as VEGF, acidic and basic fibroblast growth factors (FGF), platelet derived growth factor (PDGF). These factors could promote tumor angiogenesis, growth, recurrence and metastasis[17,18]. It is possible that in addition to elimination of cancer cells, TAE may play a role in enhancing some cells’ malignant potency and ability to escape anoxia and ischemia after treatment.

VEGF is a key mediator of pathological angiogenesis. Many researchers found that it overexpressed in many solid tumor tissues including HCC, and was associated with tumor growth, recurrence, metastasis and patient’s prognosis[19,20]. It has been reported that preoperative TAE enhanced VEGF expression in both HCC cells and non-carcinoma liver cells. Antisense RNA could inhibit and block gene expression effectively[24,25]. In this study, we devised antisense ODNs specific to VEGF mRNA, and observed their inhibitory effects on VEGF expression in Walker-256 cell lines in vitro, and the anti-tumor effect of them mixed with lipiodol arterial embolization on Walker-256 cell transplanted rat liver cancer models.

MATERIALS AND METHODS

Cell culture

Walker 256 carcinosarcoma cells were purchased from China
Center for Type Culture Collection. After recovery, the cells were inoculated in the abdominal cavity of male pathogen-free Wistar rats, weighing 100-120 g (supplied by Department of Experimental Animal, Tongji Medical College). Three days later, cancerous ascites was aspirated and cultured in RPMI-1640 containing 50 mL/L fetal calf serum (FCS) (Gibco, Grand Island, NY) and equilibrated with 950 mL/L air and 50 mL/L CO₂. Cells were passaged every 2 d. The cells at passage 3 were used for experiments.

**Oligodeoxynucleotides (ODNs)**

Phosphorothioate ODNs were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., LTD. The sequences of ODNs were designed as follows: antisense ODNs: 5'-GCA GTA GCT GCC CTG ATA GCG C-3', complementary to the linkage area of VEGF exon 2 and exon 3; sense ODNs: 5'-GCA GTA CTA TCA GCC CAG CTA CTG C-3', equivalent to the linkage area of VEGF exon 2 and exon 3.

**Cell proliferation studies**

Walker-256 carcinosarcoma cells were planted into a 24-well plate at 1×10⁴/well (1 mL/well) and cultured in non-serum medium. Ten µL of 0.25, 0.5, 1.0, 2.0 µmol/L antisense ODNs and sense ODNs was added to the media every 24 h. Ten µL of non-serum medium was added to the blank control wells. Forty-eight hours later, the supernatant fluids were collected. The concentration of VEGF was measured by ELISA. Two hundred µL of supernatant fluids were added triple to a 96-well plate to culture ECV-304 cells (endothelial cell line, purchased from China Center for Type Culture Collection, 1×10⁴/well), equilibrated with 950 mL/L air and 50 mL/L CO₂. Seventy-two hours later, ECV-304 cells were collected. MTT method was used to detect the growth of ECV-304 cells.

**Tumor model and treatment schedule**

Walker-256 carcinosarcoma cells were inoculated subcutaneously in the right flank of rats with 10⁴ tumor cells in approximately 0.1 mL of cell suspension. Tumors were palpable 7 d after transplantation. Fresh tumor tissues were isolated and cut into 1 mm³ pieces. Seventy tumor-bearing rats were randomly divided into 3 groups, 10 rats each. LP group: the hepatic arteries were embolized with 0.2 mm, external diameter 0.61 mm, Neolab, Germany) under a binocular operative microscope (Suzhou Medical Instruments Factory, Jiangsu, China) and infused embolic materials to the left hepatic lobe of rats. Seven days later, after anesthesia and laparotomy, the gastroduodenal artery was ligated and the abdominal cavity was closed. LP+ODNs group: the hepatic arteries were temporarily ligated during infusion. Thirty tumor-bearing rats were randomly divided into 3 groups, 10 rats each. LP group: the hepatic arteries were embolized with 30D antisense ODNs mixed with 0.2 mL lipiodol, control group: 0.2 mL normal saline was infused into hepatic arteries. Then the gastroduodenal artery was ligated and the abdominal cavity was closed.

MR scans were performed on 1.5-Tesla system (Magnetom Vision, Siemens, Germany) supplemented by a cervical coil before and 7 d after TAE. T1-weighted (TR/TE, 450/12 ms) and T2-weighted (TR/TE, 2800/96 ms) transverse SE images (slice thickness 2 mm) were acquired using acquisition times of 7:25 and 6:16 min, respectively. Tumor volume was measured from MR measurements of the largest and smallest diameters and calculated according to the following formula: Tumor volume (mm³, V) = largest diameter (mm) × [smallest diameter (mm)]²/2. The tumor growth rate = Vₜ/V₀×100%.

The rats were sacrificed 7 d after TAE. Liver cancerous and peri-cancerous tissues were dissected. Some of them were frozen at -70 °C for RNA isolation. The reminders were fixed in 40g/L formaldehyde, dehydrated and embedded in paraffin. Five-μm sections were stained with hematoxylin-eosin for light microscopy and measurement of the degree of tumor necrosis.

**RNA isolation and RT-PCR analysis**

Total RNA was extracted from liver cancerous and peri-cancerous tissues using the TRIzol reagent (Gibco). Reverse transcription of 5 µg total RNA was performed in a volume of 20 µL for 60 min at 37 °C, containing AMV 5U, Oligo d'T 0.050 µg, RNasin 20 U, 10 mmol/L dNTP 2 µL. The samples were heated to 95 °C for 5 min to terminate the reverse transcription reaction. By using a Perkin-Elmer DNA thermocycler 2 400 (Perkin-Elmer, Norwalk, CT), 2 µL cDNA mixture obtained from the reverse transcription reaction was then amplified for VEGF and G.PDH. G.PDH was used as a housekeeping gene and amplified with VEGF as control. The amplification reaction mixture consisted of 10×buffer 2.5 µL, 5 mmol/L dNTP 0.5 µL, 25 mmol/L MgCl₂ 1.5 µL, 10 pmol/L each of sense and antisense primers, Taqase 5U. The reaction mixture was first heated at 95 °C for 5 min and amplification was carried out for 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and at 72 °C for 30 s, followed by incubation for 10 min at 72 °C. The PCR primers used were: VEGF, sense 5'-GAA GTG GTG AAG TTC ATG GAT GTC-3' and antisense 5'-GCA TCG TTC TGT ATC AGT CTT TTC-3'; gPDH, sense 5'-TCC TCC AAG ATT GTC AGCA AA-3' and antisense 5'-AGA TCC ACA ACG GAT ACA TT-3'. The length of PCR products for VEGF and G.PDH was 394 bp and 309 bp. PCR products were checked with 15 g/L agarose gel electrophoresis stained with ethidium bromide.

**Immunohistochemical analysis**

Five micron paraffin-embedded tissue sections were deparaffinized and rehydrated. Rabbit anti-mouse vWF, VEGF monoclonal antibody and SABC kit were provided by Beijing Zhongshan Biological Technology Co., Ltd. Immunohistochemical studies were performed by SABC methods according to the manufacturer’s instructions. VEGF staining was evaluated semiquantitatively on the basis of the percentage of positive cells, and classified as follows: diffusely positive (+++) when positive cells accounted for more than 50% of the total cells, moderately positive (+) when positive cells were 16-50%, weakly positive (+) when positive cells accounted for 5-15%, and negative (-) when positive cells accounted for less than 5%. For MVD determination, 5 areas were randomly selected and counted at a magnification of 200. Briefly, the stained sections were screened at a magnification of 200 under a light microscope to identify 3 regions of the section with the highest microvessel density. Microvessels were counted in these areas at a magnification of 200, and the average number of microvessels was recorded.

**Statistical analysis**

Experimental results were analyzed with analysis of variance (ANOVA) and Kruskal-Wallis rank test. The difference between 2 groups was analyzed by SNK-q and Dunn test respectively. Statistical significance was determined at P<0.05.

**RESULTS**

**VEGF concentration in supernatant of walker-256 cells**

After incubated with walker-256 cells for 48 h, antisense ODNs decreased the concentration of VEGF in the supernatant of walker-256 cells in a dose-dependent manner, whereas no significant changes were seen in sense ODNs (Table 1).
and composed of collagen fibers, which were caused by the surrounding normal hepatic parenchyma, its capsules were thin detected. The mass had a sharp demarcation from the hyperchromatosis, polymorphism and numerous mitoses were was spherical or ovoid in shape. Tumor cells arranged in specimens showed a poorly differentiated carcinoma, which was not statistically significant.

Table 1 Concentration of VEGF in supernatant of Walker-256 cells (pg/ml)(mean±SD)

| Group           | 0.25 μmol/L | 0.5 μmol/L | 1.0 μmol/L | 2.0 μmol/L |
|-----------------|-------------|------------|------------|------------|
| Antisense ODNs  | 84.2±2.2    | 79.2±2.6   | 74.4±2.1   | 65.2±2.0   |
| Sense ODNs      | 89.2±1.9    | 90.4±2.6   | 88.2±1.9   | 88.6±2.5   |
| Control         | 90.2±1.9    | 90.8±1.8   | 89.4±2.1   | 90.4±1.7   |
| F values        | 7.877       | 23.132     | 49.906     | 133.636    |
| P values        | 0.021       | 0.002      | 0.000      | 0.000      |

1SNK-q test: The difference between sense ODNs and control groups was not statistically significant.

Cell proliferation study
The supernatants of Walker-256 cells that were incubated with antisense ODNs could inhibit the growth of ECV-304 cells in a dose-dependent manner. No significant effects were seen in those incubated with sense ODNs (Table 2).

Table 2 Inhibitory effect of supernatants of Walker-256 cells cultured with ODNs on ECV-304 proliferation (OD) (mean±SD)

| Group           | 0.25 μmol/L | 0.5 μmol/L | 1.0 μmol/L | 2.0 μmol/L |
|-----------------|-------------|------------|------------|------------|
| Antisense ODNs  | 0.339±0.012 | 0.332±0.006| 0.327±0.008| 0.321±0.019|
| Sense ODNs      | 0.345±0.021 | 0.348±0.014| 0.336±0.022| 0.359±0.014|
| Control         | 0.357±0.010 | 0.359±0.008| 0.356±0.005| 0.360±0.009|
| F values        | 2.477       | 5.573      | 12.931     | 7.296      |
| P values        | 0.164       | 0.043      | 0.007      | 0.025      |

1SNK-q test: The difference between sense ODNs and control groups was not statistically significant.

Tumor growth and histopathological findings
Seven days after implantation, the tumor was located in the left lobe of liver as a solitary mass. There was no statistical difference between the volumes of tumors in control group, LP group and LP+ODNs group (212.3±117.5 mm³, 174.6±106.5 mm³, 173.9±91.8 mm³ respectively) before TAE treatment. Seven days after the treatment, all tumors grew. The volumes and growth rates in LP group (286.0±186.4 mm³, 177.9±64.9%, respectively) and LP+ODNs group (337.6±98.1 mm³, 140.1±33.8%, respectively) were significantly less than those in control group (823.3±426.1 mm³, 403.9±69.4%, respectively, P<0.01) (Table 3).  

Table 3 Volumes and growth rates of transplanted liver tumors (mean±SD)

| Group            | Volume of pre-TAE(mm³) | Volume of post-TAE(mm³) | Growth rate (%) |
|------------------|-------------------------|-------------------------|-----------------|
| Control          | 212.3±117.5             | 823.3±426.1             | 403.9±69.4      |
| LP               | 174.6±106.5             | 286.0±186.4             | 177.9±64.9      |
| LP+ODNs          | 173.9±91.8              | 337.6±98.1              | 140.1±33.8      |
| F values         | 0.432                   | 14.319                  | 60.019          |
| P values         | 0.653                   | 0.000                   | 0.000           |

1SNK-q test: The difference between antisense LP and LP+ODNs groups was not statistically significant.

Hematoxylin-eosin (H & E) stained sections of the liver specimens showed a poorly differentiated carcinoma, which was spherical or ovoid in shape. Tumor cells arranged in irregular nests and signs of malignancy including hyperchromatosis, polymorphism and numerous mitoses were detected. The mass had a sharp demarcation from the surrounding normal hepatic parenchyma, its capsules were thin and composed of collagen fibers, which were caused by the compression of the tumor. The tumor showed inhomogeneous signs of hypervascularization consisting mainly of small arteries and capillaries. Seven days after therapy, spotty and scattered necrosis were seen in all cases of control group (Figure 1A). Satellite nodules could be seen in some tumors. In LP group, the necrotic area was increased. Many patched necrotic zones were seen in tumor tissues (Figure 1B). In LP+ODNs group, the tumor necrotic area was much wider. It showed a big area of central necrosis. The residual tumors could be seen only in the margin of tumor (Figure 1C).

Figure 1 Pathological changes of tumor tissues 7d after TAE. A: Control group showing spotty and scattered necrosis. B: LP group showing many patched necrotic zones. C: LP+ODNs group showing big areas of central necrosis. Hematoxylin-eosin ×40.

Figure 2 RT-PCR analysis of VEGF mRNA level in cancerous and peri-cancerous tissues using G3PDH as internal control. Control group: 1 tumor, 2 peri-tumor. LP group: 3 tumor, 4 peri-tumor. LP+ODNs group: 5 tumor, 6 peri-tumor, 7 marker.
RT-PCR analysis of VEGF mRNA expression

VEGF mRNA expression was detected in cancerous and peri-cancerous tissues. The expression level in cancerous tissue was higher than that in peri-cancerous tissues. The VEGF mRNA levels in cancerous and peri-cancerous tissues in LP group were higher than those in control group, and those in LP+ODNs group were lower than those in control and LP groups (Figure 2).

| Table 4 Expression of VEGF and MVD by immunohistochemical staining (n=10) |
|-----------------|---|---|---|---|---|
| Group           | MVD (mean±SD) | +++ | ++ | +  | -  | Positive rate (%) |
| Control         | 73.2±20.4      | 3   | 2  | 2  | 3  | 70                  |
| LP              | 80.3±18.5      | 3   | 4  | 2  | 1  | 90                  |
| LP+ODNs         | 53.1±18.4      | 1   | 2  | 2  | 5  | 50                  |
| Test values     | F=5.440        |     |    |    |    |                     |
| P values        | 0.010          |     |    |    |    |                     |

1SNK-q test: The difference between LP and control groups was not statistically significant.

Immunohistochemical analysis of VEGF protein expression and MVD

VEGF immunoreactivity was observed mainly in the cytoplasm of tumor cells, and also frequently in hepatic cells in peri-cancerous tissues. The distribution of strong VEGF-staining zones and microvessels was mainly in the survival nidui and margins of the tumor. The positive rate of VEGF in LP group, control group and LP+ODNs group was 90%, 70% and 50% respectively, and the difference was not statistically significant (P=0.065) (Table 4, Figure 3). The MVD in LP+ODNs group (53.1±18.4) was significantly less than that in control group (73.2±20.4) and LP group (80.3±18.5). Although the MVD in control group and LP group showed no significant difference, but abundant tumor vessels were seen in residual nidui in LP group (Table 4, Figure 4).

DISCUSSION

Many authors have reported the effects of TAE on VEGF expression of HCC. An et al[27] found that preoperative TAE enhanced VEGF expression in both HCC cells and non-carcinoma liver cells. Kobayashi et al[28] reported that the frequency of Bcl-2 positive cells was higher in HCCs undergone TAE than that in HCCs not undergone TAE, and the immunohistochemical staining intensity for VEGF was higher in Bcl-2 positive than in Bcl-2 negative area. Suzuki et al[29] reported that the serum levels of VEGF in HCC patients increased significantly 7 d after TAE. Guo et al[30] reported that blockage of hepatic arterial blood supply resulted in decreased blood perfusion and increased expression of metastasis-associated genes VEGF and MMP-1 of transplanted liver cancer in rats. The results in this study showed that VEGF mRNA and protein had an increasing tendency after TAE. These findings suggested that TAE could enhance the expression of VEGF in HCC cells. The rationale is based on the following points: TAE was hard to lead to total tumor necrosis and to make the tumor tissue anoxia further. Hypoxia induced transcription of VEGF mRNA was mediated by the binding of hypoxia-inducible factor 1 (HIF-1) to an HIF-1 binding site located in the VEGF promoter[31-33]. VEGF plays an important role in each stage of tumor angiogenesis. The overexpression of VEGF in cancerous and peri-cancerous tissues of HCC could certainly promote tumor
angiogenesis and collateral vessels formation, and enhance the possibility of recurrence and metastasis[34-37]. In this study, although we found that the MVD in LP group was higher than that in control group, but the difference was not significant and abundant tumor vessels were seen in residual nodule in LP group. It must be due to tumor angiogenesis enhancement caused by VEGF overexpression after TAE. In clinical setting, we also found that the collateral vessels were increased with the time of increased TAE. These vessels are very small and hard to catheterize, resulting in the emboziled HCC tissue receiving blood and escaping from anoxia stress. Some HCC vessels not emboziled completely would grow acceleratedly and were prone to recurrence and metastasis. So it is very important to reduce VEGF expression and collateral vessel formation after TAE.

Antisense ODNs have shown great efficacy in selective inhibition of gene expression. They are designed and synthesized artificially, and can enter cells directly to hybridize with complementary mRNA and decrease protein expression. They have been used as an important tool to inhibit the expression of oncogenes and/or growth factors and some of them have been used as drugs in tumor gene therapies[38-41]. In this study, antisense ODNs were designed to complement the region between exon 2 and exon 3 of VEGF gene. They could inhibit 4 kinds of VEGF molecules’ expression[42,43]. To enforce their stability, ODNs were modified by phosphorothioate. Our previous study showed fluorescence labeled ODNs could transfecnt Walker-256 cells and keep in them for about 48 h (in press). The in vivo experimental results in this study showed that VEGF antisense ODNs could decrease VEGF expression of cultured Walker-256 cells in a dose-dependent manner. Similar effect has been reported on other tumor cell lines[44-46]. In in vivo study, we also found that VEGF antisense ODNs could inhibit VEGF expression of liver tumor tissues after TAE, reduce tumor MVD and growth rate in walker-256 cell transplanted rat liver cancer models. These imply that VEGF antisense ODNs could be used as an antiangiogenesis agent to inhibit HCC’s overexpression and collateral vessel formation after TAE.

Intravenous injection is the routine means for phosphorothioate ODNs in clinical administration. However, intravenous infusion is not an ideal route for VEGF antisense ODNs in HCC treatment. First, it has a very short lifetime after injection into animal bodies. To enhance the target cell transfection rate and therapeutic effect, it is needed to increase the dose of antisense ODNs. A higher dose of ODNs may result in more side effects such as dose-dependent hypotension, complement activation, and transient prolongation of thromboplastin time[47-50]. Second, systemic administration of VEGF antisense ODNs might inhibit physiological angiogenesis, such as wound healing, menstruation. In this study, we mixed antisense ODNs with lipiodol and used them as an embolic agent in liver tumor TAE therapy. To infuse the agents into the left hepatic artery (the tumor was planted in the left lobe of the rat liver) and to make each tumor receive the same amount of agents, we catheterized the gastroduodenal artery retrograde and ligated the common hepatic artery and right hepatic artery temporarily during infusion. The results showed that antisense ODNs mixed with lipiodol was better in inhibiting tumor growth rate, VEGF expression and MVD than lipiodol alone. These indicated that antisense ODNs used in combination with lipiodol and transcatheter artery embolization were the ideal route in HCC treatment. The rationale is based on the following points. The blood supply of HCC is mainly from hepatic artery. ODNs hepatic artery infusion could increase the concentration in tumor tissue and reduce the dosage and systemic side-effects. When injected to the hepatic artery, lipiodol could stay in tumor tissue for a long time (several months), and could even be absorbed by tumor cells[51,52]. When mixed with lipiodol, the latter could act as a carrier, ODNs would give off slowly from it. It would prolong the contact time of ODNs and tumor cells, which is very important to increase the transfact rate. Our previous in vivo experimental study showed that fluorescence labeled ODNs could stay in tumor tissue for about 6 d when used in combination with lipiodol hepatic artery embolization on Walker-256 cell transplanted rat liver cancer models (in press). This is why the long-term inhibitory effect on VEGF expression and MVD was achieved in this study.

In conclusion, TAE can increase the formation of microvessels in residual tumor tissues. VEGF plays an important role in liver cancer angiogenesis and collateral vascular formation after TAE treatment. VEGF antisense ODNs are able to inhibit tumor cells’ VEGF expression. VEGF antisense ODNs can inhibit the residual tumor angiogenesis and growth and reduce the possibility of metastasis and recurrence. The combination of TAE and VEGF antisense ODNs will be a hopeful strategy for HCC treatment.

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