Antisense oligodeoxynucleotide inhibits vascular endothelial growth factor expression in U937 foam cells[1]

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ABSTRACT

AIM: To study the expression of vascular endothelial growth factor (VEGF) induced by oxidized low density liprotein (ox-LDL) and the inhibitory effects of antisense oligodeoxynucleotide (asODN) on the levels of VEGF protein and mRNA in the U937 foam cells. **METHODS:** U937 cells were incubated with ox-LDL 80 mg/L for 48 h, then, the foam cells were treated with asODN (0, 5, 10, and 20 μmol/L). The VEGF concentration in the media was determined by ELISA. The VEGF protein expression level in cells was measured by immuohistochemistry; the positive ratio detected by a morphometrical analysis system was used as the amount of the VEGF expression level. The VEGF mRNA level was examined by Northern blotting. **RESULTS:** After U937 cells were incubated with ox-LDL, VEGF expression level increased greatly both in the cells and in the media. asODN markedly inhibited the increase of VEGF. After treatment with asODN 20 μmol/L, the VEGF protein concentration in the media decreased by 45.0 %, the VEGF positive ratio detected by immuohistochemistry in cells decreased by 64.9 %, and the VEGF mRNA level decreased by 47.1 %. **CONCLUSION:** The expression of VEGF in U937 foam cells was strong. asODN inhibited VEGF expression significantly in U937 foam cells *in vitro*.

INTRODUCTION

Foam cells are characteristic pathological cells in the lesions of atherosclerosis. During the process of atherosclerosis, monocytes and oxidized low density lipoprotein (ox-LDL) appear to play central roles, not only through foam cell formation but also via the introduction of numerous cytokines and growth factors. Once monocytes adhere to the subendothelial space and enter the intima of the artery, ox-LDL and other substances associated with atherogenesis may participate in activation of the monocytes into macrophages. Uptake of ox-LDL by the macrophages through scavenger receptors will lead to foam cells formation[1].

VEGF, also known as vascular permeability factor, has been demonstrated to be involved in normal and pathological processes, including tumor progression, collateral vessel formation in ischemic tissues, and inflammation[2-6]. We have demonstrated that foam cells could secrete VEGF in the lesions of atherosclerosis[7]. In addition, VEGF plays an important role in the formation and development of atherosclerosis through the

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effect on VEGF-induced hyperpermeability in endothelial cells[8].

Antisense oligodeoxynucleotides (asODN) are short stretches of synthetic, chemically modified nucleic acids designed to hybridize to complementary mRNA sequences and block production of proteins encoded by the targeted mRNA transcripts[9]. The asODN of VEGF synthesized in our lab could inhibit the expression of VEGF in several tumor cells[10-12]. However, the effect of this asODN in the cardiovascular system has not been reported.

Since the VEGF expression in foam cells plays an important role during the process of atherosclerosis, it sounds reasonable that asODN of VEGF has elicited interests as potential therapeutic agents for atherosclerosis. In the present study, we established a macrophage-derived foam cell model through incubating the U937 cells with ox-LDL, aiming to determine the effect of asODN on the levels of VEGF protein and mRNA in the U937 foam cells.

MATERIALS AND METHODS

Reagents $CuSO_4$ and ethylenediamine tetraacetic acid disodium salt were purchased from Sigma Chemical Co; VEGF ELISA kit was purchased from Cytimmune Co (USA); VEGF antibody was obtained from San Fransco Co (USA); VEGF cDNA probe was provided by Beijing University.

ODN The sequence of the VEGF asODN was 5'-GCA GTA GCT GCG CTG ATA GTG C-3', complementary to the third extron of human VEGF mRNA. For control, sense and missense ODN were used, which had the sequence of 5'-CTA TCA GCG CAG CTA CTG C-3' and 5'-CCT CGT CAT GAG ACA CGT C-3', respectively. All ODN were synthesized in Shanghai Institute of Biochemistry.

ox-LDL preparation LDL (d=1.019 to 1.063 kg/L, Sigma Co, USA) was sterilized by filtration through 0.45-μm millipore membranes, and stored at 4 °C as described previously[13]. After ethylenediamine tetraacetic acid disodium salt was removed by dialysis, LDL was oxidized by incubation in $CuSO_4$ 10 μmol/L at 37 °C for 16 h, and then dialyzed in phosphate buffered saline (PBS) containing ethylenediamine tetraacetic acid disodium salt 0.1 mmol/L at 4 °C for 24 h.

Cell culture The human monocyte line U937 was obtained from the cell bank in Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. U937 cells were cultured in RPMI-1640 containing 10 % fetal bovine serum. For experiments, the U937 cells were incubated with ox-LDL 80 mg/L for 48 h and a macrophage-derived foam cell model was established. Then, the medium was treated with asODN (0, 5, 10, and 20 μmol/L), sense ODN 20 μmol/L, and missense ODN 20 μmol/L for 24 h. The control group was the U937 cells.

Oil Red O dyeing The foam cells were collected and fixed with 4 % paraformalde for 12 h. The cells were then treated with fresh 0.3 % Oil red O for 20 min.

ELISA The supernants were collected and used for the measurement of VEGF protein quantification after the cells were centrifuged at 1000×*g* for 10 min. ELISA procedures and the standard curve were the same as those described[14].

Immunohistochemistry The cells were collected and fixed with 4 % paraformalde for 20 min. Streptavidin-biotin-peroxidase complex (SABC) method was used for VEGF staining[12]. VEGF is shown as yellow gains at the cytoplasm of the cells. The positive ratio was analyzed by CA6300-image-analysis system (Kejijia Co, Beijing).

Northern blotting Total RNA was isolated from the U937 cells using Trizol reagent (Gibco/BRL). RNA (15 μg) was fractionated by denaturing electrophoresis and capilliary blotted to nylon membranes (Boehringer Mannheim). The filters were cross-linked with UV light, followed by prehybridization at 50 °C in prehybridization solution for 4 h . Hybridization was then performed with 5 mg/L of [α-^{32}P]CTP-labeled VEGF cDNA probe at 50 °C for 18 h. After hybridization, the filters were washed and exposed to Fuji imaging plate. The blot films were analyzed with smark view software (Furi Co). The relative mRNA level of VEGF was normalized against the signal of total RNA and the corrected density with smark view software was plotted as the ratio to the signal of control value.

Statistics All results were shown as mean±SD. Statistical analysis was performed with ANOVA.

RESULTS

Morphologic form of the U937 foam cells After treatment with fresh 0.3 % Oil red O, many red pellets were found in the plasma of the U937 foam cells, which showed that U937 cells internalized ox-LDL through scavenger receptor and became foam formation[15].

VEGF protein levels in supernants and the effects of asODN The increase in VEGF protein levels

in the medium after incubation with ox-LDL 80 mg/L was estimated to be about 2-fold, indicating that foam cells could give a strong VEGF protein expression. The VEGF concentration in asODN-treated cells decreased greatly. Especially after treatment with asODN 20 μmol/L, the VEGF protein level decreased by 45 % compared to that of foam cells, while the sense and missense ODN- treated cells remained unchanged (Fig 1).

VEGF protein levels in foam cells and the effects of asODN Before the samples were measured by immunohistochemistry method, the cells were adjusted to the same mount of 1×10^4. Strong expression of

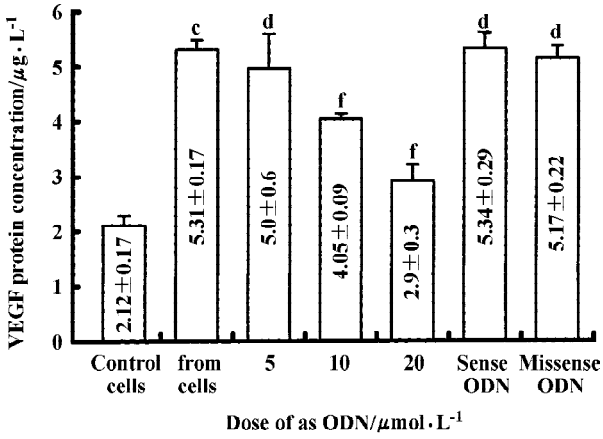

Fig 1. Vascular endothelial growth factor (VEGF) concentration in supernant of foam cells and the inhibitory effect of antisense oligodeoxynucleotide (asODN). *n*=3. Mean±SD. ^{c}*P*<0.01 *vs* control group; ^{d}*P*>0.05, ^{f}*P*<0.01 *vs* foam cell group.

VEGF was noted in foam cells with the positive ratio of 9.52 % (Fig 2). After the foam cells were treated with asODN 20 μmol/L, VEGF protein expression decreased by 64.9 % compared with the positive ratio of U937 foam cells. The treatment of the sense and missense ODN had no effect on VEGF expression.

VEGF mRNA levels in foam cells and the effects of asODN The relative mRNA level of VEGF was normalized against the signal of total RNA (18 S, 28 S) and the result was expressed as a ratio relative to the control expression level. After incubation with ox-LDL 80 mg/L, the increase of VEGF mRNA expression was estimated to be about 22-fold (Fig 3). After treatment with VEGF asODN 20 μmol/L and incubation with ox-LDL, the VEGF mRNA level decreased by 47.1 % compared to that of foam cells. No change was found in VEGF level of the cells treated with sense ODN and missense ODN.

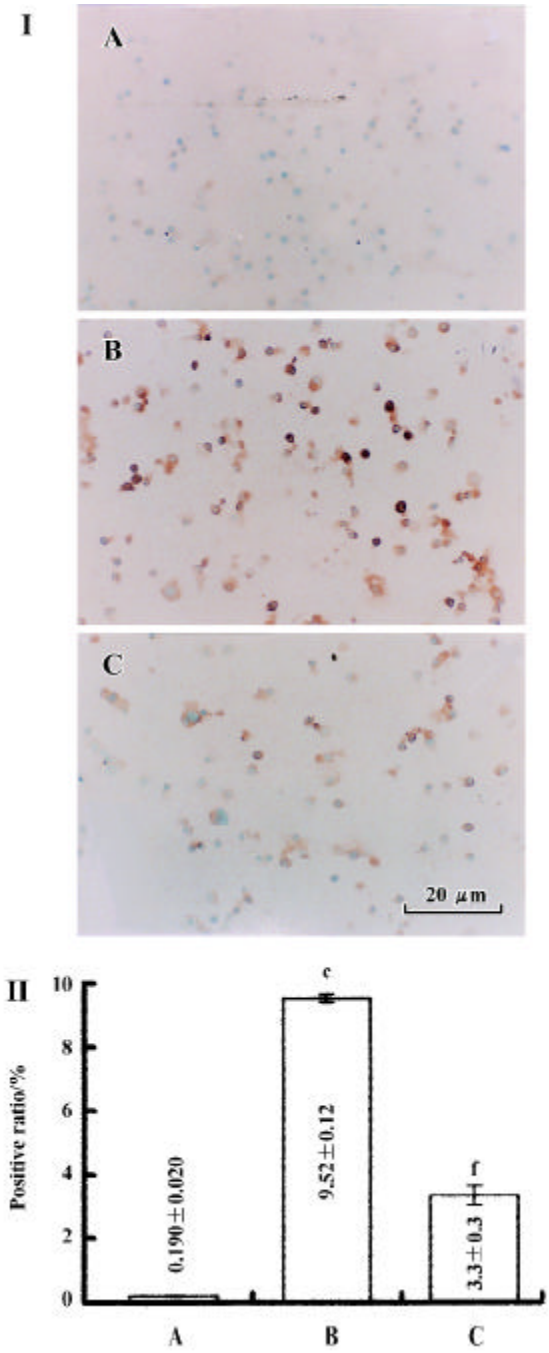

Fig 2. Expression of VEGF protein in U937 foam cells and the inhibitory effect of asODN. (I) VEGF was shown as yellow gains at the cytoplasm of the cells. (Immunohistochemistry, ×200). (II) The positive ratio detected by CA6300-image-analysis system was used as the total protein expression. A: U937 cells (control); B: U937 foam cells; C: the foam cells treated with asODN 20 μmol/L. *n*=3. Mean±SD. ^{c}*P*<0.01 *vs* control group. ^{f}*P*<0.01 *vs* foam cell group.

DISCUSSION

Atherosclerosis is not merely a disease in its own

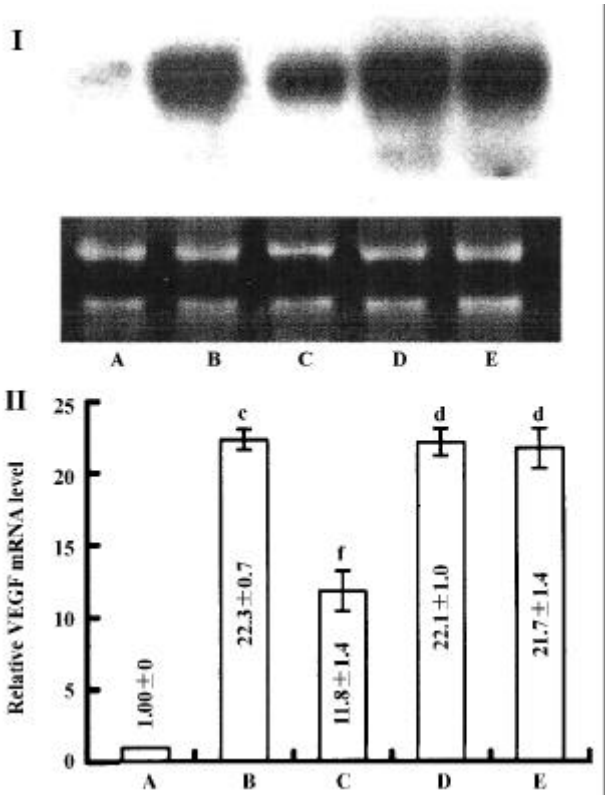

Fig 3. Northern blotting analysis of the expression of VEGF mRNA level in U937 foam cells and the effect of the VEGF asODN. (I) VEGF mRNA level was detected by Northern blotting. The total RNA was separated by 1 % agarose and the 18 S, 28 S were shown at the bottom. (II) The relative mRNA level of VEGF was normalized against the signal of 18 S, 28 S. Corrected density with smark view software was plotted as the ratio to the signal of control value. A: U937 cells (control); B: U937 foam cells; C: the foam cells treated with asODN 20 μmol/L; D: the foam cells treated with sense ODN 20 μmol/L; E: the foam cells treated with missense ODN 20 μmol/L. *n*=3. Mean±SD. $^{c}P<0.01$ *vs* control group. $^{d}P>0.05$, $^{f}P<0.01$ *vs* foam cell group.

right, but a process that contributes the principally contributor to the pathogenesis of myocardial and cerebral infarction, gangrene, and loss of function in the extremities. The macrophage-derived foam cells not only result in formation of fatty streaks, which are believed to represent the earliest type of atherosclerotic plaque, but also play roles in the fibroproliferative process by their capacity to form numerous growth factors especially PDGF[16,17], VEGF[7], IL-1[18], as well as TNFα[19]. Among these factors, VEGF arouses more and more attention because it is not only a growth factor but also as a vascular permeability factor, which can aggravate the development of atherosclerosis through enhancing LDL permeability of endothelial cells.

The present study demonstrated strong expression of VEGF in U937 foam cells and high level of VEGF protein in supernatants of U937 foam cells. In the early atherosclerotic lesion (fatty streak), VEGF staining was frequently seen in subendothelial macrophage-rich regions, foam cells may be the main source of VEGF[20].

The asODN is relatively simple to design and easy to control, showing a broad potential applicability for therapeutic proposal[21]. The asODN synthesized in our lab has shown great promise as a sequence-specific inhibitor of VEGF expression in tumor cells both *in vitro* and *in vivo*. In this study, we proved that this 19-mer asODN of VEGF could inhibit the expression of VEGF in foam cells. As VEGF plays an important role in the formation and development of atherosclerosis through mediating neovascularization and hyperpermeability in the atherosclerotic plaque, the decrease of VEGF expression in the foam cells may prevent progress of the atherosclerotic process. Therefore, our study provides primary evidence of antisense ODN in atherosclerosis gene therapy.

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