研究报告

Induced Expression of Adipophilin With High Cholesterol Diet in Rabbit Atherosclerotic Lesions and Reduced Lipid Accumulation with Adipophilin Antisense in Mouse Macrophages*

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Abstract Adipophilin is a specific marker for lipid accumulation in a variety of cells and for diseases associated with fat-accumulating cells. Lipid-laden foam cells derived from macrophages play a critical role in the development of atherosclerosis. By immunohistochemistry with specific monoclonal antibody, it was shown that expression of adipophilin is induced by high-cholesterol-diet feeding in rabbit atherosclerotic lesions. New Zealand white rabbits were fed with high cholesterol chow for 12 weeks. The level of serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, and cholesterol content of aortic wall was investigated. The areas of fatty streak of the aortas was measured after staining with Sudan Ⅳ. The aortic, and liver specimens with HE and immunohistochemistry staining were observed with light microscopes. The level of serum total cholesterol, low density lipoprotein cholesterol, and cholesterol of aortic wall was significantly increased and the areas of fatty streak of the aortas was (40.06±7.29)% at the end of 12-week-cholesterol feeding. The fatty streak of the aorta with immunohistochemistry staining was strongly positive for adipophilin in animals fed with high cholesterol chow, and the liver was negative with or without high cholesterol chow. Antisense oligodeoxynucleotides of mouse adipophilin was also constructed, mouse peritoneum macrophages was cultured with oxLDL or oxLDL plus the antisense fragment. The results showed that adipophilin antisense decreased cellular cholesterol and lipid droplet content of the cell. The data suggested that the expression of adipophilin in vessel walls is related to the hypercholesterolemia, and has a potential role in lipid accumulation in macrophages and pathogenesis of atherosclerosis.

Key words adipophilin, atherosclerosis, macrophage, rabbit, oxidized low-density lipoprotein

Adipophilin (also known as adipose differentiation related protein, ADRP) is a protein encoded by a gene initially isolated from differentiating adipocytes[1]. Its expression has now been found in diverse cell types in culture and is associated with the lipid fractions in the cell[2]. Shiffman *et al*.[3] conducted a large scale gene expression analysis of the response of macrophages to exposure to oxidized low density lipoprotein (oxLDL). RNA from oxLDL treated and time-matched control untreated cells was hybridized to microarrays containing 9 808 human genes. They found that among these thousands of genes, adipophilin was the only gene upregulated as early as 2.5 h after the cells were exposed to oxLDL. The expression of adipophilin has also been detected in human atherosclerotic lesions by *in situ* hybridization[4]. The early induction of adipophilin in macrophages by oxLDL potentially makes it a good marker of foam cell formation. However, whether it merely serves to stabilize intracellular lipid droplets, or plays a regulatory role in lipid accumulation of the cell needs to be further investigated.

To further elucidate the potential role of adipophilin in the molecular mechanism of atherogenesis, we fed New Zealand White rabbits with high cholesterol chow for 12 weeks, and used an adipophilin antisense oligodeoxynucleotides in culture of mouse peritoneum macrophages to block the expression of adipophilin, and then observed the effect of cholesterol feeding on expression of adipophilin in rabbit atherosclerotic lesions and the effect of adipophilin antisense oligodeoxynucleotides on lipid accumulation in cultured macrophages.

1 Materials and methods

1.1 Animal experiments

New Zealand White rabbits (male : female = 1 : 1) weighing approximately 2 kg were randomly divided into two groups, a normal control group (n = 12) and a cholesterol-fed group (n = 12). Normal control rabbits were fed regular laboratory

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chow (75 g · kg^{-1} · d^{-1}) and cholesterol-fed animals received regular laboratory chow supplemented with 1% cholesterol (75 g · kg^{-1} · d^{-1}), for 12 weeks. The animals were fed at 9 AM and given free access to tap water. Food consumption was measured daily and body weight was recorded once a month. Blood samples for lipid measurements were withdrawn at week 0, 4, 8, and 12 from auricular veins after an overnight fasting. At the end of the experimental period, the animals were killed by phlebotomy under light anesthesia with 30 mg/kg sodium pentobarbital. The aorta was dissected from the heart to the bifurcation and gently rinsed with normal saline, and the peri-aortic tissue was carefully removed. The liver was also taken out. The samples of the aorta and liver from different groups were fixed with 10% formaldehyde and embedded in paraffin. Paraffin slides of the samples were made and stained with hematoxylin.

1.2 Assay of plasma lipids

Plasma total cholesterol (Tc), high density lipoprotein cholesterol (HDLc), and triglyceride (TG) were determined by enzymatic methods using the commercially available kits from Shanghai Rongsheng Biotech Inc., Shanghai, China. Low density lipoprotein cholesterol (LDLc) was calculated as LDLc=Tc−(HDLc+TG/5).

1.3 Measurements of atheromatous area

The cleaned aortae were opened longitudinally, stained with Sudan Ⅳ, and the extent of gross atheromatous area was quantified by a dot-counting method. Templates of the vessels were drawn on clear acrylic sheets and superimposed over a dot grid with a 2 mm×2 mm grid size. The number of dots in the lesions areas and in the whole area were counted.

1.4 Measurement of aortic cholesterol content

Immediately after the measurement of atheromatous areas, the aortae were fixed in 10% buffered formalin and refrigerated until used. They were rinsed with 70% isopropanol and then with normal saline. The intima and media were carefully separated from the adventitia, weighed, and minced. The minced tissue was homogenized in 5 ml of $CHCl_3$: MeOH (2 : 1) for each 0.1 g with a Physcotron homogenizer (Nichion Irikakikai, Chiba, Japan). The lipid residue was dissolved in a small amount of isopropanol and sonicated. This solution was mixed with 2% Triton X-100 (approximately seven volumes) and incubated for 20 min at 70℃ before cholesterol analysis. Aortic cholesterol content was determined by enzymatic methods using the above commercially available kit.

1.5 Immunohistochemistry

Immunohistochemistry was performed using the UltraSensitiveTM S-P Kit (Maxim Biotech Inc., USA), according to the procedures as the producer suggested. Briefly, paraffin slides of aorta or liver sections were heated and rehydrated, incubated with 10% normal serum in phosphate-buffered saline (PBS), and treated with primary antibodies (mAB against adipophilin AP125 from mouse IgG, Research Diagnostic Inc., USA) at a dilution of 1 : 20 for 60 min at 37℃. As secondary antibody, a biotinylated anti-mouse IgG antibody (Vector, Burlingame, Calif.) was used at a dilution of 1 : 100, for 30 min at room temperature. Bound antibodies were visualized by the avidin-biotin-peroxidase technique, according to the manufacturer's instructions; the color reaction was performed by using a DBA kit. Sections were then mildly counterstained with hematoxylin solution. Microphotographs were taken on Kodak 200 color film.

1.6 Construction of sense and antisense oligodeoxynucleotides

An antisense fragment (5′-TGCTGCTGCC-ATTTT-3′) (AS-ODNs), a sense fragment (5′-AAAATGGCAGCAGCA-3′) and A missense fragment(5′-GAGGTTGTATCCAGC-3′) of mouse adipophilin mRNA[1] from nucleotide 76～90 and the initial code AUG were designed and synthesized in Shanghai Shengong Bioengineering Inc.

1.7 Cell culture and treatment

Macrophages isolated from C57BL/6J mice according to the method previously described by Brown *et al*.[5] were grown in RPMI1640 medium containing 10% fetal serum (*V*/*V*), penicillin (100 U/ml) and streptomycin (100 mg/L). For oxLDL loading experiments, cells were seeded at a density of 1×10^6/ml in medium for 24 h. The medium was replaced by culture medium with or without 80 mg/L of $CuSO_4$ "fully" oxidized LDL according to the method previously described by Steinbrechert *et al*.[6]. For antisense treatment, the medium was replaced by culture medium with 80 mg/L oxLDL or with 80 mg/L oxLDL plus the above antisense fragment (1 mmol/L), 80 mg/L oxLDL plus the sense fragment (1 mmol/L) and 80 mg/L oxLDL plus the missense fragment (1 mmol/L). Medium was replaced every day during experiment.

1.8 Cellular cholesterol measurement

The cells were harvested from culture bottles with the aid of a rubber policeman and transferred to a conical graduated centrifuge tube. The cells were washed three times by suspending them in PBS, centrifuging at 800 *g* for 5 min, and aspirating the fluid. An appropriate volume of isopropyl alcohol (0.5 L for every 1 g protein) was then added to the pellet and the sample was sonicated with a microprobe. After centrifugation for 15 min at 800 *g* the clear supernatant was decanted and

an aliquot was taken for cholesterol and cholesteryl ester determination by spectrophotofluorometer with axenon lamp (Shanghai analytic instrument factory, China. $\lambda_{ex}=325$ nm, $\lambda_{em}=415$ nm. Slits=2 mm.), as described previously[7].

The residue was dissolved in 0.1 mol/L sodium hydroxide and an aliquot was taken for protein determination by the method of Lowry *et al*.[8].

1.9 Oil red O staining

A modified oil red O staining was used to stain lipids in the cultured cells. Briefly, cells were fixed (1 min) using 50% isopropanol and then stained with the working solution of oil red O for 10 min, rinsed with deionised water for 3 times (1 min/time), and counterstained using Mayer's haematoxylin for 60 s to visualize nuclei.

1.10 Statistical analysis

All values are expressed as $(\bar{x}+s)$. The significance of the difference in mean values between groups was evaluated using the unpaired Student's test. All statistics were performed with the aid of a software statistical package (SPSS 10.0). The statistical significance level<0.05.

2 Results

2.1 Time curves of serum lipids

Body mass increased gradually in two groups during the experimental period. There was no difference in body weight between the normal group and the high cholesterol-fed group.

As shown in Table 1, Table 2, and Figure. 1, the time curves of levels of serum lipids are shown, total cholesterol level of the cholesterol-fed group elevated significantly in a time-dependent manner. The total cholesterol level of the control group was almost unchanged during the experimental period. LDLc and TG levels in choleserol-fed group markedly increased as compared with those of the control. HDLc in the cholesterol-feeding group slightly increased, but there was no significant difference between normal and high cholesterol group.

Table 1 Serum triglyceride concentrations of the rabbits

	n	ρ(TG)/(mg · L^{-1})			
		0	4 w	8 w	12 w
Cholesterol-fed	10	827+704	1 066+180*	1 151+212*	1 234+516*
Control	10	787+585	650+54	652+105	752+155

* Compare with the control group, $P<0.05$.

Table 2 Serum HDL cholesterol concentrations of the rabbits

	n	ρ(HDLc)/(mg · L^{-1})			
		0	4 w	8 w	12 w
Cholesterol-fed	10	137+28	181+61	203+36	170+25
Control	10	140+15	166+22	136+16	156+16

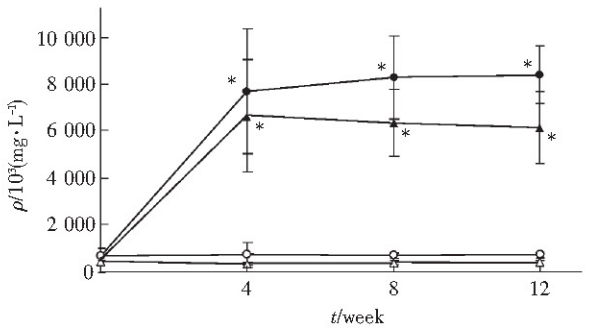

Fig. 1 Time curves of serum total cholesterol (Tc) and LDL cholesterol (LDLc) concentrations

● ●: Tc/cholesterol-fed; ○ ○: Tc/control; ▲ ▲: LDLc/cholesterol-fed; △ △: LDLc/control. * compare with the control group, $P<0.05$. $n=10$.

2.2 Atheromatous areas and aortic lipid contents

Relative atheromatous area (% of whole area) percent rate which was measured by a dot-counting method was (40.06 + 7.29)% in the high cholesterol group, and the area was 0 in the control group ($P<0.05$). The values of the atheromatous area between groups were significantly different. The atherosclerotic lesions were widely distributed in the thoracic and abdominal aorta of the high cholesterol-fed rabbits. The surface of the aorta isolated from the cholesterol-fed rabbits had fatty lesions protruding to the lumen.

Aortic cholesterol content was (13.6+2.4)mg/g tissue in cholesterol-fed rabbits and (1.3+0.5)mg/g in the control rabbits ($P<0.05$)(Figure. 2).

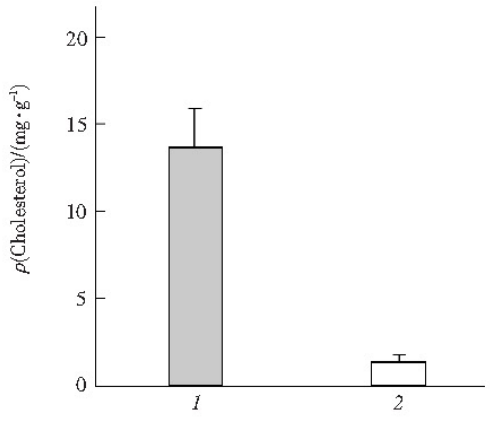

Fig. 2 Aortic contents of total cholesterol
1: cholesterol fed; *2*: control. * compare with the control group, $P<0.05$. $n=12$.

2.3 Detection of adipophilin in aorta by immunostaining

Paraffin slides of the aorta and liver samples were stained with IIE. With light microscopy, we observed that there were thickness of the intima of aorta and a lot of foam cells in the intima of the aorta in cholesterol-fed animals (Figure. 3a). Monoantibody against adipophilin produced consistent and strong immunostaining in the aortic atherosclerotic lesions of the cholesterol-fed rabbits, the intima was full of chocolate-like brown granules which represented immunostained adipophilin. Under highly magnification, it could be seen that these chocolate brown granules were distributed within the cytoplasm of the foam cells (Figure. 3b). The media and adventitia were immunostaining negative. Vascular vessel walls from control rabbits were also negative (Figure. 3c). This meant that cholesterol-feeding induced adipophilin expression and it was localized within the foam cells in the intima of the aortic wall. In the liver specimens, the liver structure of both groups were morphologically normal and immunostaining negative (data were not shown).

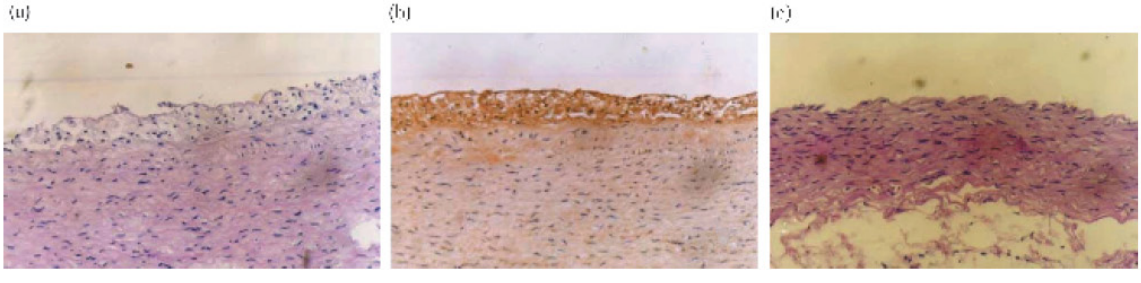

Fig. 3 Microphotos of aortic specimen from the two groups
(a) From cholesterol fed group, stained with haematoxylin, the intima was hyperplastic and full of foam cells. (b) From cholesterol fed group, immunohistochemical staining positive (chocolatebrown), indicating high expression of adipophilin. (c) From control group, stained with haematoxylin, appeared morphologically normal. (×100).

2.4 Effects of adipophilin antisense on cellular cholesterol and lipid droplet

After 72 h incubation with 80 mg/L oxLDL, cellular cholesterol of the cultured preritoneum macrophages increased significantly (Table 3), from (2.5 ± 2.2) mg/L protein to (46.6 ± 3.4) mg/L. Whereas, the cells incubated with 80 mg/L oxLDL plus 1 mmol/L adipophilin AS-ODNs for 72 h contained significantly lower cholesterol, (19.9 ± 1.9) mg/g. The cellular cholesterol of the cells cultured with oxLDL + sense or oxLDL + missense were almost unchanged.

Neutral lipids in cells appeared red with oil red O staining, and the nucleolus appeared blue when counterstained with hematoxylin. The cells incubated with oxLDL alone contained the most red lipid droplets (Figure. 4a). The cells incubated with oxLDL + antisense had fewer lipid droplets (Figure. 4b). The cells cultured without oxLDL contained no lipid droplets (Figure. 4c).

Table 3 Cellular cholesterol content

Group	*n*	Cellular cholesterol/(mg · g $^{-1}$)		
		Free	Total	Ester
Control	10	40.1±3.1	42.6±2.0	2.5±2.2
OxLDL	10	51.2±1.7	97.8±3.9	46.6±3.4
OxLDL+antisense	10	45.5±2.1	65.4±1.7*	19.9±1.9*
OxLDL+sense	10	43.6±1.1	89.2±2.8	45.6±1.8
OxLDL+missense	10	47.1±3.3	90.5±1.6	43.4±2.9

* Compare with the oxLDL group, $P<0.05$.

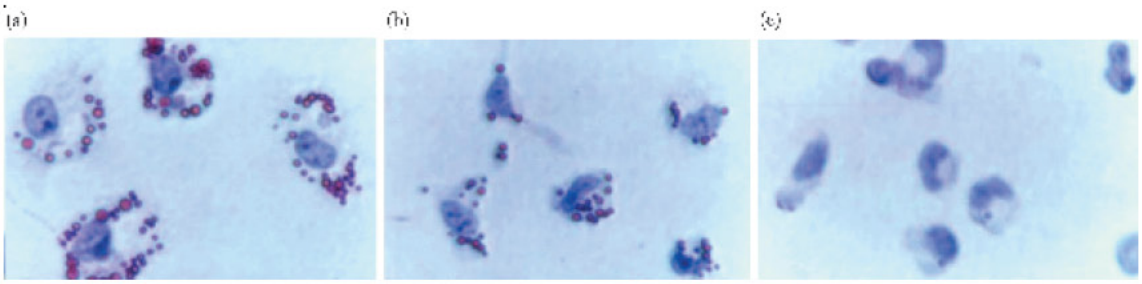

Fig. 4 Microphotos of mouse peritoneum macrophages stained with oil red O

Neutral lipids in cells appeared red with oil red O staining, and the nucleolus appeared blue counterstained with hematoxylin. (a) The cells incubated with oxLDL alone showed the most red lipid droplets. (b) The cells incubated with oxLDL+antisense against adipophilin had less lipid droplets. (c) The cells cultured without oxLDL contained no lipid droplet. (×400).

3 Discussion

Adipophilin (ADRP) was first identified by Serrero, *et al*[1]. who showed that ADRP mRNA is expressed most strongly in adipose tissue and is induced very early during adipocyte differentiation. It has been shown that adipophilin is a marker of lipid loading in macrophages and forms a major component of the coating for the intracellular lipid vesicles[2]. Lipid storage is facilitated by the production of large amounts of lipid vesicle coating proteins such as adipophilin, perilipin, *et al*[9]. Brasaemle *et al*.[10] reported that adipophilin is a ubiquitously expressed lipid storage droplet associated protein. Steiner *et al*.[11] demonstrated that drug induction of liver lipid deposits also induces the appearance of adipophilin. Wang *et al*.[4] identified a cDNA encoding adipophilin in cultured human macrophages stimulated with oxLDL. They also localized adipophilin mRNA expression in a subset of lipid rich macrophages of human atherosclerotic lesions. However, an important question unanswered is whether induced adipophilin promotes lipid deposition or if it merely reflects the increased storage of lipid. In the present study, we first linked induced expression of adipophilin in rabbit vessel wall to hypercholesreolemia, and demonstrated that adipophilin antisense oligodeoxynucleotides decreased lipid accumulation of mouse peritoneum macrophages cultured with oxLDL. Our results suggest that expression of adipophilin has a promotive role in atherogenesis. Since the high cholesterol diet did not induce significant change in hepatic lipid content, adipophilin expression was not detectable in the liver of the rabbits in our study (data were not shown).

Oxidized LDL is a stimulus for lipid accumulation in macrophages, and it was recently demonstrated to activate macrophage gene expression through members of the nuclear hormone receptor family, peroxisome proliferator activator receptor δ and γ (PPARδ and PPARγ)[12]. Vosper *et al*.[13] used highly selective agonists to demonstrate that both PPARδ and PPARγ differentially regulate the lipid accumulation in macrophages, with PPARδ activation promoting lipid accumulation and PPARγ promoting lipid clearance. They showed that activation of PPARδ promotes lipid accumulation in primary human macrophages and in the monocytic cell line THP 1; and that adipophilin is very selectively and highly induced by a specific PPARδ agonist, compound F, throughout the experiment. Herein, taken our data together, we speculate that high cholesterol diet could have increased oxLDL level *in vivo*, then oxLDL induces its own uptake mechanism, this may concomitantly induce adipophilin (through activation of PPARδ) to handle lipid storage. Buechler *et al*.[14] also showed that PPARγ may mediate the induction of adipophilin expression in human blood monocytes. In addition, as shown in our present study, adipophilin antisense oligodeoxynucleotides decreased lipid accumulation of mouse peritoneum macrophages cultured with oxLDL. Therefore, our results suggest that expression of adipophilin has a promotive role in atherogenesis. Further study of the regulation of adipophilin function in macrophages and other cells of vessel wall may provide a therapeutic opportunity for the treatment of atherosclerosis.

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Adipophilin 在动脉粥样硬化病变和脂质负荷细胞中的作用研究*

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摘要 Adipophilin 是细胞内脂质聚集和与脂质聚集有关疾病的标志物，巨噬细胞源性泡沫细胞的形成是动脉粥样硬化性疾病发生的重要环节. 为了探讨 adipophilin 在动脉粥样硬化性疾病的作用，通过高胆固醇饲料喂养新西兰白兔 12 周，复制动脉粥样硬化疾病模型，同时测定血脂的变化和动脉壁胆固醇，使用 HE 染色、苏丹Ⅳ染色观察动脉粥样硬化病变的形成，使用免疫组织化学的方法观察动脉粥样硬化病变处和动物肝脏中 adipophilin 的表达. 结果发现，高胆固醇饲料喂养组血清总胆固醇、低密度脂蛋白胆固醇和动脉壁胆固醇明显增高，动脉粥样硬化病变面积增加到 (40.06±7.29)%，动脉粥样硬化病变处 adipophilin 表达呈阳性；而 adipophilin 在肝脏中的表达无论是高胆固醇饲料喂养组或对照组均为阴性. 使用 80 mg/L OxLDL 与小鼠腹膜巨噬细胞共孵育，复制脂质负荷细胞，然后把构建的 1 mmol/L adipophilin 反义寡核苷酸与该细胞共孵育. 结果发现，使用油红 O 染色观察的细胞内脂滴明显减少，生化测定细胞内胆固醇酯显著降低，与对照组相比，差别有显著性. 说明 adipophilin 与动脉粥样硬化病变有密切的关系，控制 adipophilin 的表达能够减少巨噬细胞细胞内胆固醇酯的聚集.

关键词 Adipophilin，动脉粥样硬化，巨噬细胞，兔，氧化低密度脂蛋白
学科分类号 R361.3

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