Oxidized LDL at low concentrations inhibits *in vitro* angiogenesis of human coronary artery endothelial cells.

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Abstract

Aims: To investigate the effects of low concentrations of oxidized LDL (oxLDL) on in vitro cell angiogenesis, represented by tube formation from human coronary artery endothelial cells (HCAEC). **Methods and Results:** Cultures of HCAEC were treated with low concentrations of native LDL (nLDL) isolated from human plasma, electronegative LDL [LDL(-)], a sub-fraction of LDL with minimal oxidative characteristics, and chemical minimally oxidized LDL, through methods of low degree and times of oxidation; the effects were compared. We established the ability of nLDL, LDL(-), oxLDL(ENO-HCO3-/1') and oxLDL(ENO-HCO3-/10') in low concentrations, of 1 and 4 µg/mL, to investigate their effects on tube formation from HCAECs in Matrigel™. No inhibiting effect nLDL was observed on tube formation from HCAECs. LDL(-), oxLDL(ENO-HCO3-/1') or oxLDL(ENO-HCO3-/10') inhibited the formation of capillary-like structures in a dose-dependent manner. **Conclusion:** The effects of oxLDL on the coronary endothelial cells in culture, inhibiting the process of angiogenesis are proportional to the concentration and degree of oxidation of LDL. Our data, however, should be interpreted in light of the limitations inherent in in vitro experimentation, albeit their value in exploring pathophysiological mechanisms. Yet, these resuts furnish new insight into mechanisms by which OxLDL may impair endothelial functions related to atherogenesis and its clinical complications.

Key words: oxidized LDL, angiogenesis, coronary artery endothelial cells.

Introduction

Angiogenesis, defined as formation of new capillaries, is a physiological process necessary for embryonic development and wound repair as well as in various pathologic events such as tissue ischemia, cancer, diabetic retinopathy, and chronic inflammatory states including atherosclerosis. This highly regulated process involves degradation of extra-cellular matrix, disruption of cell-cell contacts, migration and proliferation, and capillary tube formation from endothelial cells.[1]

High plasma levels of low-density lipoprotein (LDL) contribute causally to the development of cardiovascular atherosclerotic disease. The response to injury hypothesis of atherosclerosis proposes that the first step in atherogenesis is endothelial dysfunction induced by the action of risk factors, especially the exposure of the vascular endothelium to oxidized LDL (oxLDL).[2]

Preliminary studies revealed that preparations of isolated human LDL have toxic effects on endothelial cells in culture.[3,5] We investigated the effects of oxLDL at low concentrations on the proliferation and spontaneous motility of human coronary artery endothelial cells (HCAECs) in culture. In our experiments, oxLDL inhibited in vitro proliferation and cell migration of HCAECs, essential mechanisms in the processes of reestablishing vascular integrity after injury and angiogenesis. This detrimental effect was proportional to the concentration and degree of oxidation of LDL.[6]

Some studies have shown that the lipid components of oxLDL can paradoxically increase the production of VEGF by endothelial cells in culture, which may be a protective mechanism in face of cellular injury.[7,8] OxLDL had a biphasic effect on endothelial replication: stimulation of cell proliferation at low concentrations (5 to 10 µg/mL), but causing apoptosis at concentrations above 50 µg/mL.[9,10] Recent data, have shown that oxLDL at low concentration, under 5 µg/mL, could promote capillary tube formation using HCAECs.[11,12]

The precise mechanisms and clinical relevance of the formation of capillaries in response to oxLDL, require further examination experimentally and in humans.

Objective

Considering the limited interpretation data from studies using several lineages of endothelial cells in the literature, and aiming at contributing to a better understanding of the basic processes involved in the interaction between oxLDL and endothelial cell, we carried out a systematic experimental study using HCAECs, which have a primordial role in coronary atherogenesis.

This study investigated the effects of low concentrations of nLDL, electronegative LDL [LDL(-)], a sub-fraction of LDL with minimal oxidative characteristics, and chemical minimally oxidized LDL, through methods of low degree and times of oxidation, on in vitro cell angiogenesis, represented by tube formation from HCAECs.

Methods

The nLDL and LDL(-) samples were extracted from human plasma and purified as described [6] and were stored at a temperature below -70ºC until use. The LDL samples (0.5 µg/mL of protein) underwent oxidation with 1.0 µM of spermine nonoate (SNO) plus HCO_3^- (SNO-HCO_3^-) (SIGMA). The samples were incubated in a water bath (37°C) under constant agitation for 1 or 10 minutes. The reaction was stopped by addition of 100 mM of diethylene tetramine pentacetic acid, 100 mM of butylhydroxytoluene, 125 units/mL of superoxide dismutase, and 125 units/mL of catalase.[13] All samples were maintained at -20°C until the time of the experiments.

HCAECs were obtained from CLONETICS (BioWhittaker Inc., Walkersville, MD, USA). The culture medium used was MCDB- 131 (GIBCO) with the addition of the following: EGF (INTERGEN), 10 µg/mL; hydrocortisone (SIGMA), 1.0 µg/mL; amphotericin B (GIBCO), 50 µg/mL; penicillin, 100 U/mL; streptomycin (GIBCO), 100 U/mL; and 10% fetal bovine serum (GIBCO) with 1 µM L- glutamine (GIBCO). The experiments were performed in culture media with 5% fetal bovine serum.

The assay of capillary tube formation in Matrigel™ was performed according the in vitro angiogenesis protocol established by GRANT et al. (1989) [14], and modified by GAGLIARDI et al. (1997) [15] adapted to culture plates with 96 wells. A cold solution (4ºC) of Matrigel™ (200 µl) in a cold (4ºC) culture medium 1:1

without fetal bovine serum was placed in culture plates with 96 wells. Pipettes and plates used were previously cooled. After incubating the Matrigel™ gel for 30 min, at 37ºC, about 30 thousand cells in 200 µl culture medium, were placed on the Matrigel™. After 18 hours, culture was fixed and stained. Capillary tube formation was quantified in three microscopic fields for each well with four samples for each experimental treatment.

The substances investigated, nLDL, LDL(-), oxLDL(ENO-HCO_3^-/1') and oxLDL(ENO-HCO_3^-/10') were added to HCAECs cultures depending on the protocol of each experiment, in concentrations of 1 and 4 µg/mL, and added to Matrigel™ as described, by 18 hours. The number of capillary tubes was quantified under microscopy (NIKON-TMS) with an increase of 100 times in three representative fields for each well culture plate with four samples for each experimental treatment.

Statistical analysis

The Kruskal-Wallis nonparametric test compared the concentrations, because it is considered the most appropriate test for independent samples, and the Dunn test was used for multiple comparisons. Nonparametric tests were used because the supposition of data normality was rejected. The significance level adopted for the tests was 5%.[16]

Results

We established the ability of nLDL, LDL(-), oxLDL(ENO-HCO_3^-/1') and oxLDL(ENO-HCO_3^-/10') in low concentrations, of 1 and 4 µg/mL, to investigate their effects on tube formation from HCAECs in Matrigel™. Figure 1 shows no inhibiting effect nLDL on tube formation from HCAECs. LDL(-), oxLDL(ENO-HCO_3^-/1') or oxLDL(ENO-HCO_3^-/10') inhibited the formation of capillary-like structures in a dose-dependent manner (Figures 2, 3 and 4). Microphotography (40X magnification) showed this inhibitory effect (Figure 5).

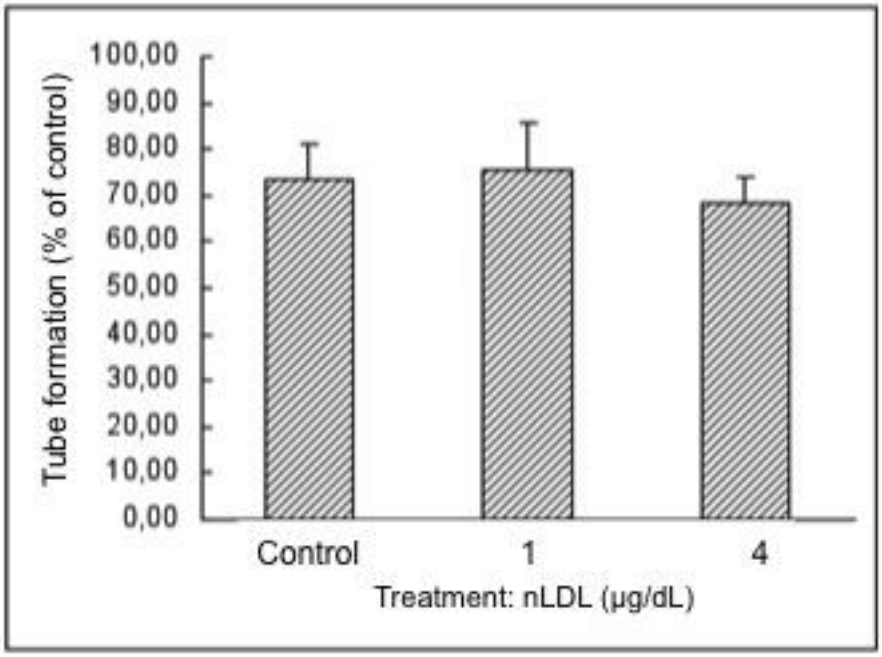


Figure 1. Effect of nLDL on tube formation from HCAECs; n=4 samples for each independent experiment. Control versus treatments (p=0,5264)

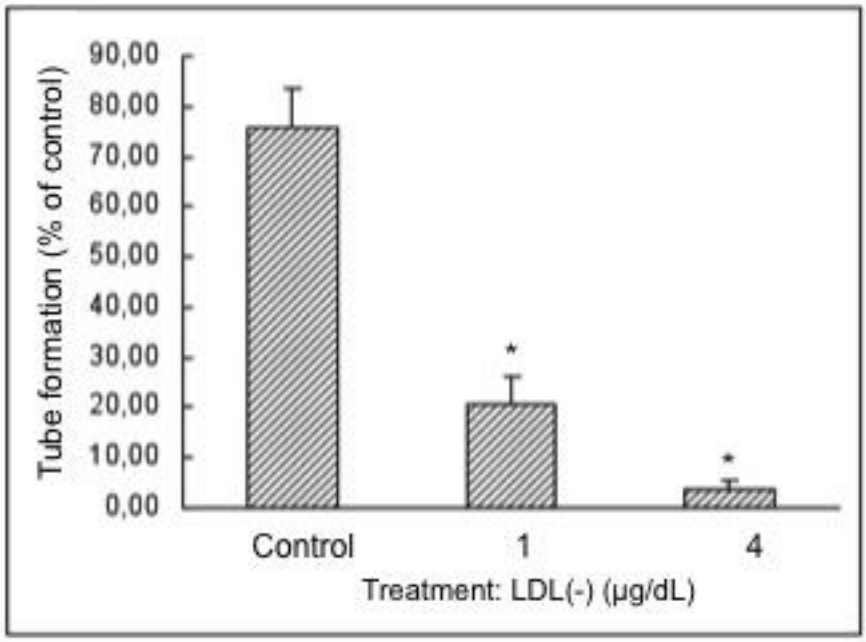


Figure 2. Effect of LDL(-) on tube formation from HCAECs; n=4 samples for each independent experiment. Control versus treatments *($p < 0.05$)

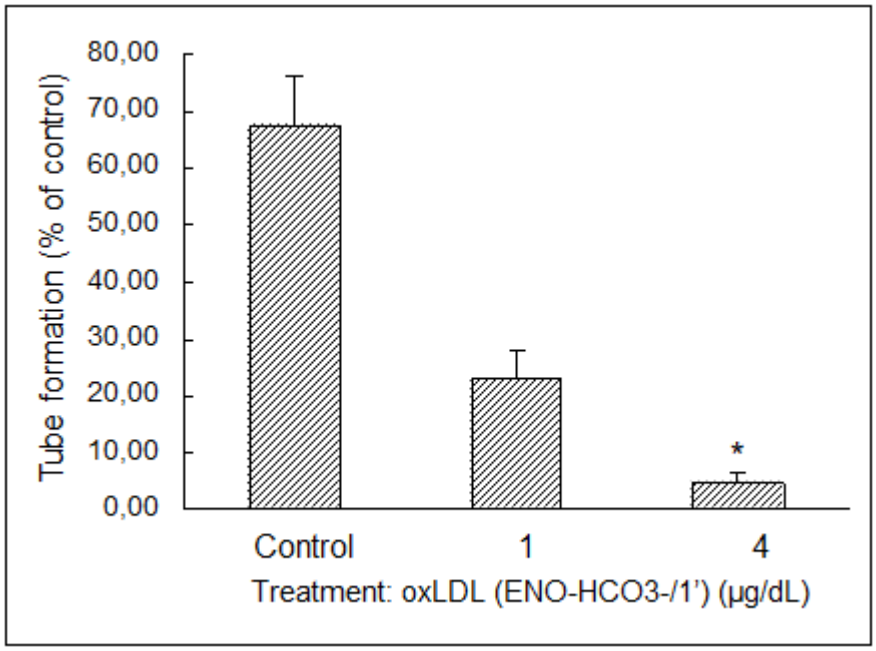


Figure 3. Effect of LDL-ox (ENO-HCO3-/1') on tube formation from HCAECs; n=4 samples for each independent experiment. Control versus treatments *($p < 0.05$)

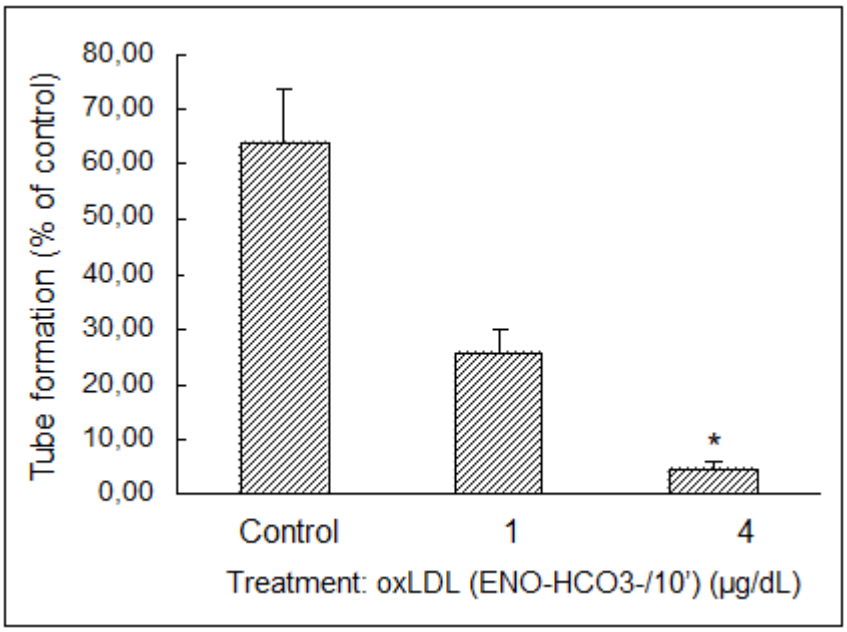


Figure 4. Effect of LDL-ox (ENO-HCO3-/10') on tube formation from HCAECs; n=4 samples for each independent experiment. Control versus treatments *($p < 0.05$)

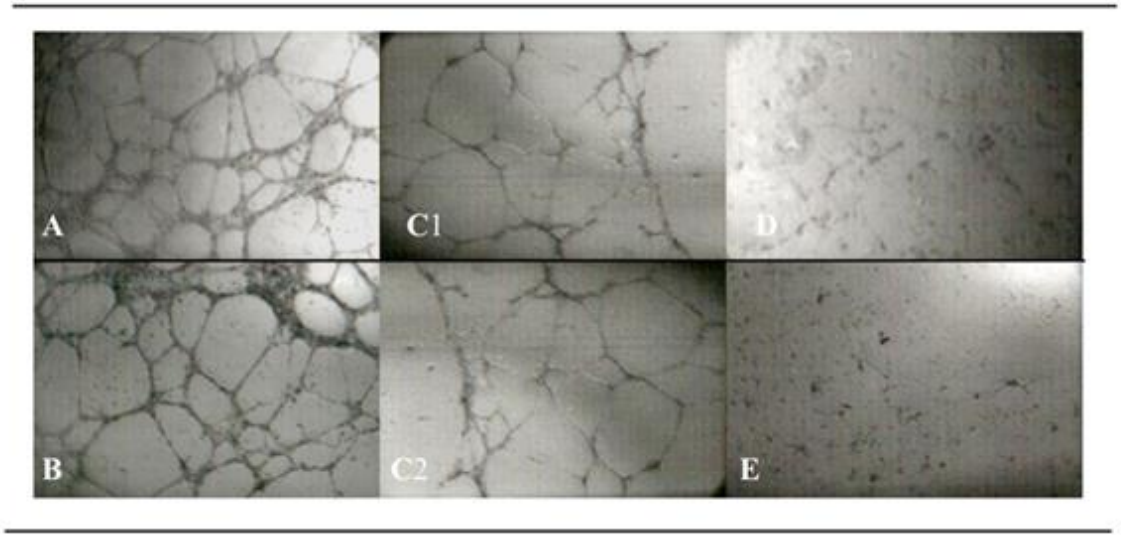


Figure 5. Microphotography of the formation of capillary-like structures representing in vitro angiogenesis on a total of 48 samples**. A**: control; **B**: nLDL; **C1** and **C2**: LDL(-); **D**: treatment oxLDL(ENO-HCO3-/1'); **E**: treatment oxLDL(ENO-HCO3-/10'); (40X magnification)

Discussion

The products of cholesterol oxidation generated by the oxidative modification of LDL, the cholesterol hydroperoxides, confer cytotoxicity on the particle, are implicated in the pathogenesis and progression of atherosclerosis, and localize in atheroma plaques and human plasma.[17,18]

The concentrations of oxLDL commonly used in the experiments (25 to 100 µg/mL) exceed considerably the levels of oxLDL found in the human plasma (0.1 µg/mL).[19,20] Yet in atherosclerotic lesions, oxLDL localizes in the subendothelial space, because it is retained in the extracellular matrix during atherogenesis. Therefore, large quantities of oxLDL, much higher than plasma concentration, accumulate in that region. The LDL retained in that microenvironment, ideal for oxidation, is more intensely oxidized and has characteristics similar to those of LDL oxidized by various experimental procedures.[21] This study used low concentrations (1 to 4 µg/ mL) of LDL, aiming to obtain values closer to those in the physiological situation, and, therefore, at investigating the biological effects of LDL on *in vitro* angiogenesis of HCAECs.

Our results suggest the occurrence of similar effects of the

oxLDL present in vascular walls, atherosclerotic plaques, and even circulating in the plasma, reinforcing the data in the literature that implicate oxLDL in cardiovascular events. [22,23] Recently, elevated oxLDL plasma levels were shown for the first time to directly relate to plaque instability in atherosclerotic lesions of human coronary arteries. OxLDL levels were measured in patients with acute myocardial infarction, unstable angina, stable angina, and controls, revealing a positive correlation with the severity of acute coronary syndrome. The serum levels of oxLDL were 4 times more elevated in patients with acute myocardial infarction when compared with those of controls, suggesting that circulating oxLDL may be a marker of severity in cardiovascular events.[24]

Our results indicate that oxLDL concentrations similar to those found in the acute phase of coronary syndromes inhibit *in vitro* angiogenesis as assessed by tubule formation by HCAECs. Thus elevated levels of oxLDL, due to its cytotoxicity, may negatively interfere not only with instability of the atherosclerotic plaque, but also with the reestablishment of the post injury vascular integrity, worsening the patient outcomes. The effects of oxLDL on the coronary endothelial cells in culture, inhibiting cell proliferation and motility, key mechanisms in reendothelization of injured areas of the wall[6] and in the process of angiogenesis are proportional to the concentration and degree of oxidation of LDL. One could infer therefore that clinical interventions to control and preserve the integrity of these variables should be pursued to minimize the deleterious effects and change the natural history of coronary artery disease.

Our data, however, should be interpreted in light of the limitations inherent in *in vitro* experimentation, albeit their value in exploring pathophysiological mechanisms. Yet, these results furnish new insight into mechanisms by which OxLDL may impair endothelial functions related to atherogenesis and its clinical complications.

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Conflict of Interest

None declared.

References

1. Ushio-Fukai M. Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res* 2006; **71**:226 –235.

2. Ross R, Glonset JA. The pathogenesis of atherosclerosis. *N Engl J Med* 1976; **295**:369-377.

3. Henriksen T, Evensen SA, Carlander B. Injury to human endothelial cells in culture induced by low density lipoproteins. *Scand J Clin Lab Invest* 1979; **39**:361-8.

4. Henriksen T, Evensen SA, Carlander B. Injury to cultured endothelial cells in culture induced by LDL: protection by HDL. *Scand J Clin Lab Invest* 1979; **39**:369-75.

5. Hessler JR, Robertson AL, Chisolm GM. LDL-induced cytotoxicity and its inhibition in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis* 1979; **32**:213-29.

6. Xavier HT, Abdalla DSP, Martinez TLR, Gagliardi ART, Ramires JAF. Effects of oxidized LDL on in-vitro proliferation and spontaneous motility of human coronary artery endothelial cells. *Arq Bras Cardiol* 2004; **83**(6):493-497.

7. Dulak J, Jozkowicz A, Dichtl W, Alber H, Schwarzacher SP, Pachinger O, Weidinger F. VEGF synthesis in vascular smooth muscle cells is enhanced by 7-ketocholesterol and lysophospha-tridylcholine
indepemdently of their effect on nitric oxide generation. *Atherosclerosis* 2001; **159**(2):325-32.

8. Dulak J, Jozkowicz A. Regulation of vascular endothelial growth factors synthesis by nitric oxide: facts and controversies. *Antioxd Redox Signal* 2003; **5**(1):123-32.

9. Yano Y, Seishima M. Stimulatory effects of lipoprotein (a) and low-density lipoprotein on human umbilical vein endothelial cell migration and proliferation are partially mediated by fibroblast growth factor-2. *Biochim Biophys Acta* 1998; **1391**(1):26-34.

10. Galle J, Heinloth A, Wanner C, Heermeier K. Dual effect of oxidized LDL on cell cycle in human endothelial cells trough oxidative stress. *Kidney Int Suppl* 2001; **78**:s120-3.

11. Danadapat A, Hu C, Sun L, Mehta JL. Small concentrations of oxLDL induce capillary tube formation from endothelial cells via LOX-1-dependent

redox-sensitive pathway. *Arterioscler Thromb Vas Biol* 2007; **27**:2435-2442.

12. Yu S, Wong SL, Law CW, Huang Y, Yu CM. Oxidized LDL at low concentration promotes in-vitro angiogenesis and activates nitric oxide synthase through PI3K/Akt/eNOS pathway in human coronary artery endothelial cells. *Biochem Biophys Res Commun* 2011; **407**(1):44-48.

13. Oliveira JMA. *Efeito da suplementação com vitamina E sobre os níveis de LDL eletronegativa (LDL-), de auto-anticorpos anti-LDL oxidada e marcadores da modificação oxidativa de lipídios e proteínas em pacientes ateroscleróticos*. In: São Paulo 2002; Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

14. Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR, Kleinman HK. Two different laminin domains mediate the differentiation of human endothelial cells into capillary like structures in vitro. *Cell* 1989; **58**:933-43.

15. Gagliardi A, Ludewig G, Munn RK, Collins DC. Tamoxifen inhibits endothelial cell migration and *in vitro* angiogenesis. American Association for Cancer Research, San Diego, California, 12-16 Abril 1997 (Abstract).

16. Rosner B. *Fundamentals of Biostatistics*. PWS Publishers, 2nd ed.,1986.

17. Crisby M, Kallin B, Thyberg J, Zhivotovsky B, Orrenius S, Kostulas V, Nilsson J. Cell death in human atherosclerotic plaques involves both oncosis and apoptosis. *Atherosclerosis* 1997; **130**(1-2):17-27.

18. Yasunobu Y, Hayashi K, Shingu T, Yamagata T, Kajiyama G, Kambe M. Coronary atherosclerosis and oxidative stress as reflected by autoantibodies against oxidized low-density lipoprotein and oxysterols. *Atherosclerosis* 2001; **155**(2):445-53.

19. Itabe H, Yamamoto H, Imanaka T, Shimamura K, Uchiyama H, Kimura J, Sanaka T, Hata Y, Takano T. Sensitive detection of oxidatively modified low density lipoprotein using a monoclonal antibody. *J Lipid Res* 1996; **37**:45-53.

20. Holvoet P, Donck J, Landeloos M, Brouwers E, Luijtens K, Arnout J, Lesaffre E, Vanrenterghem Y, Collen D. Correlation between oxidized low density lipoproteins and von

Willebrand factor in chronic renal failure. *Thromb Haemost* 1996; **76**:663-9.

21. Yla-Herttuala S, Palinski W, Rosenfeld ME, Parthasarathy S, Carew TE, Butler S, Witztum JL, Steinberg D. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 1989; **84**:1086-95.

22. Chen CH, Henry PD. Atherosclerosis as a microvascular disease: impaired angiogenesis mediated by suppressed basic fibroblast growth factor expression. *Proc Assoc Am Physicians* 1997; **109**:351-61.

.

23. Bucal M, Nguy J, Barrios R, Chen CH, Henry PD. Impaired adaptive vascular growth in hypercholesterolemic rabbit. *Atherosclerosis* 1998; **139**:243-51.

24. Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M, Komatsu R, Matsuo T, Itabe H, Takano T, Tsukamoto Y, Yoshiyama M, Takeuchi K, Yoshikawa J, Becker AE. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity acute coronary syndromes. Circulation 2001; **103**:1955-60.