Can Lipid Lowering with Atorvastatin Reduce Plaque Disruption and Thrombosis?

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Abstract: Lipid lowering with diet alone in an atherosclerotic rabbit model has been shown to reduce the tissue factor, factor VII, matrix metaloproteinases and other elements responsible for arterial inflammation that can lead to thrombosis. This project design will reveal whether lipid lowering with atorvastatin reduces plaque disruption and thrombosis. [Nature and Science. 2008;6(1):57-62]. ISSN: 1545-0740.

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Introduction

Atherosclerotic plaque rupture and/or disruption with subsequent arterial thrombosis are critical causes for acute coronary ischemic syndromes. We have previously reported on an atherosclerotic rabbit model of pharmacological triggering of plaque disruption and thrombosis (Abela, 1995). This is a modified model based on the earlier work by Constantinides who demonstrated that a vasoconstrictor agent, histamine and a procoagulant, Russell's viper venom (RVV) can trigger a platelet rich thrombus in an atherosclerotic rabbit model (Constantinides, 1981). This modified model has since been used to evaluate a viral agent causing atherosclerosis as well as imaging of thrombosis by magnetic resonance angiography. Although serum cholesterol has been well established as a major risk factor for cardiovascular events, the exact role of tissue cholesterol in the development of acute cardiovascular syndromes has not been defined. The purpose of this study is to determine if tissue cholesterol level influences cardiovascular events. This is performed by measuring tissue cholesterol content in an atherosclerotic model of plaque disruption and thrombosis.

The vulnerable plaque that is responsible for acute has been characterized by a thin fibrous cap and a rich lipid pool. Often, an inflammatory cellular component with few smooth muscle cells and low collagen content has been described (Kinlay, 1998). However, the role of the lipid pool in terms of its contribution to the unstable event has not been well defined. Speculation about the gruel content being highly thrombogenic has been suggested but it remains unclear what role the cholesterol content in the pool contributes to the severity of the acute cardiovascular event. It has also been suggested that reduction in the size of the lipid pool and/or its replacement by fibrous tissue reduces the vulnerability of the plaque (Monroe et al, 2003). Hence, a primary role for HMG-CoA reductase inhibitors is the alteration in the composition of the plaque content (Riessen et al, 1999; White, 1999). This study is conducted to evaluate the effect of the amount of cholesterol in the plaque and the severity of the event as defined by the amount of thrombus formation using an atherosclerotic model of plaque disruption and thrombosis. Furthermore, atorvastatin is used to evaluate the effect of antioxidation as a potential mechanism to reduce the risk of disruption and thrombosis.

Lipid lowering with diet alone in an atherosclerotic rabbit model has been shown to reduce the tissue factor, factor VII, matrix metaloproteinases and other elements responsible for arterial inflammation that can lead to thrombosis (Aikawa, 1999). However, the definitive test demonstrating that reduction of these elements will actually decrease thrombosis has not been demonstrated. It is no longer ethically possible to conduct controlled clinical trials between statin and placebo therapy in patients at high risk for cardiovascular events due to high cholesterol levels. However, in an atherosclerotic rabbit model of plaque disruption and thrombosis that we have developed, it would be possible to test the hypothesis that the reduction in tissue factor and other thrombogenic and inflammatory molecules will reduce the event outcome as measured by extent of thrombosis. Atherosclerosis is a disease characterized by inflammation, beginning with the earliest identifiable lesion (fatty streak) to the advanced vulnerable plaque. Clinical markers of inflammation, including C-reactive protein, modified low-density lipoprotein, homocysteine, tumor necrosis factor, and thermogenicity, have been identified as emerging risk factors that may add prognostic information in patient management. The model of plaque disruption and thrombosis can be triggered pharmacologically to evaluate the effect of various interventions on the outcome of thrombus formation in a prospective fashion (Abela, 1995). In a recent study of plaque disruption using this model

we have demonstrated that the extent of thrombosis is highly correlated with the content of tissue cholesterol (r=0.98) (Ma, 2007). Also, human data suggest that the reduction of inflammatory response by lipid lowering with statins has been associated with lower acute events (Ridker, 1999). Also, in an atherosclerotic rabbit model, atorvastatin is shown to reduce plaque inflammatory acitivity (Bustos, 1998).

Thus, it would be important to link the reduction of acute events is to a lowered inflammatory process. This could be demonstrated if atrovastatin by lowering the inflammatory activity and oxidative stress can reduce the thrombosis rate in our unique model of pharmacologically triggered plaque disruption and thrombosis (Ma, 2006).

Studies

For this project, atherosclerotic NZW rabbits are evaluated. Rabbits are made atherosclerotic by using a high cholesterol diet and balloon endothelial debridement. After feeding a cholesterol enriched diet for 6 months, the rabbits are thrombus triggered with Russell's viper venom (RVV) and histamine as previously reported (Abela, 1995). RVV activates clotting Factors V and X and histamine induces vasoconstriction in rabbits. Both these interventions are critical for the development of plaque disruption and thrombosis.

Atherosclerotic rabbits are thrombus triggered at 6 months. Group I rabbits are kept on a cholesterol enriched diet for 6 months prior to triggering. Group II rabbits are on a cholesterol enriched diet and atorvastatin for 6 months prior to triggering (Bustos, 1998).

After thrombus triggering, the rabbits are given heparin and then killed with euthanasia solution. The heparin prevents post-mortem thrombi from forming. The aortas will be exposed to evaluate the extent of thrombosis by counting the number of thrombi and planimetery of thrombus surface area. Tissues from the thoracic and abdominal aorta will be sampled for 1) measurement of tissue cholesterol levels by enzymatic techniques (Carr, 1993; Folch, 1975); 2) quantitative RT-PCR or Northern blot analyses of genes encoding the inflammatory molecules and thrombus; 3) Western blot and immuno-histochemistry study of inflammatory molecules and thrombus; and 4) lucigenin chemiluminescence determination of superoxide generation and plasma nitrite/nitrate levels using the griess reaction. Inflammation markers C-reactive protein, low-density lipoprotein and homocysteine are detected by immunohistochemistry methods in this project. These studies help elucidate the mechanism of reduction of plaque disruption and thrombosis.

The study details are as the following:

Study Groups: Eighty, male, New Zealand White rabbits weighing between 2.5 to 3.2 kg are divided into 4 groups. The method of establishing the atherosclerotic rabbit model and thrombus triggering are described previously (Abela, 1985; 1995).

Atherosclerosis Inducing: Briefly, the control-control group (Group I, 8=20) consisted of four normal rabbits that are fed a regular diet for 6 months. Rabbits in Groups II, III and IV (n=20, 20 and 20, respectively) underwent balloon deendothelialization and are then maintained on a 1% cholesterol enriched diet (Harlan-Sprague Dawley, Inc., Indianapolis, IN, USA) alternating with regular diet every month for a total of 6 months. Under general anesthesia (ketamine 50 mg/kg and xylazine 20 mg/kg, i.m.) balloon-induced deendothelialization of the aorta is performed using a 4F Fogarty arterial embolectomy catheter (Baxter Healthcare Corporation, Irvine, CA, USA) introduced through the right femoral artery cutdown. The catheter is advanced in a retrograde fashion to the ascending aorta and pulled back three times.

Pharmacological Triggering: Only the atherosclerotic rabbits (Groups II, III and IV) had pharmacological triggering since previous studies have not shown thrombosis to occur in normal arteries. Thrombus triggering is induced by RVV (0.15 mg/kg, i.p., Sigma Chemical Co., St. Louis, MO, USA) and histamine (0.02 mg/kg, i.v., Sigma Chemical Co., St. Louis, MO, USA) given at 48 and 24 hours prior sacrifice. In Group IV, atorvastatin (30 mg/kg) given 8 days prior to sacrifice. Heparin sulfate (1000 IU/rabbit, i.v., Sigma Chemical Co., St. Louis, MO, USA) is given 30 minutes prior to sacrifice to prevent postmortem clotting. Rabbits are sacrificed with an overdose of pentobarbital (50 mg/ml, i.v., Abbot Laboratories, North Chicago, IL, USA). Tissue samples from the heart, liver and kidney are stored immediately in liquid nitrogen until biochemical measurements. Procedures are performed according to Michigan State University's Animal Care and Use Committee approved protocol.

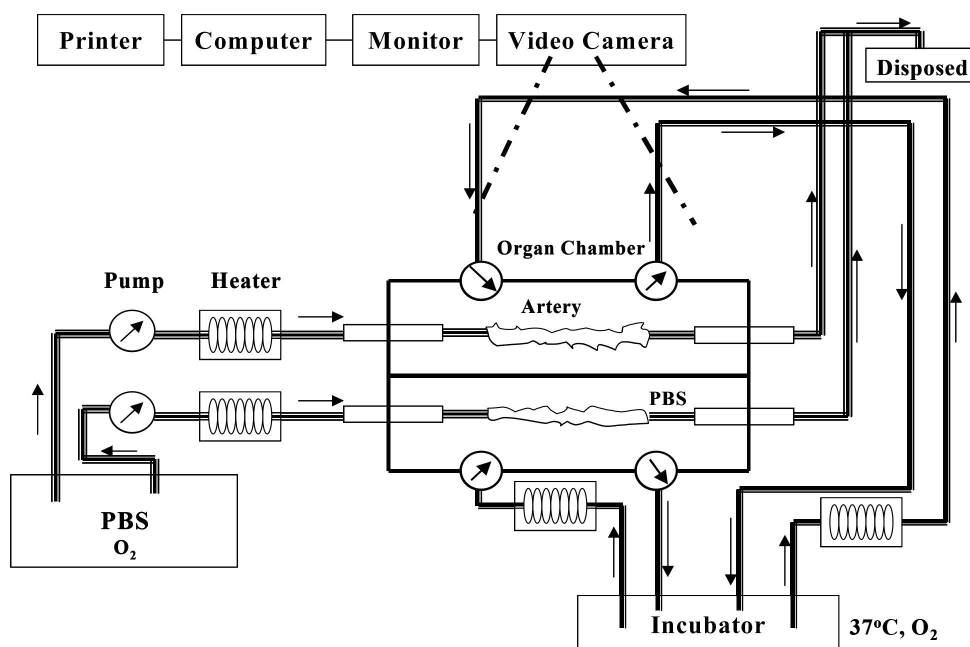

Figure 1. Dual organ chamber with separate perfusion using oxygenated physiological buffered solution at 37°C. Vessel diameter is measured by a computer planimetry system.

Quantitation of Thrombosis: The total surface area of the aorta, the surface area of aorta covered with atherosclerotic plaque, the surface area of aorta covered with *ante mortem* thrombus are measured. The surface area is measured from images obtained by a color charge-coupled device camera (TM 54, Pulnix, Sunnyvale, CA, USA) and digitized by an IBM PC/AT computer with a color image processing subsystem. The digitized images are calibrated by use of a graticule. Surface area is measured by use of a customized quantitative image analysis package. Also, the number of thrombi on the aortic arch to the distal common iliac branches is counted.

Artery Diameter Respond Evaluation: After rabbits are sacrificed both carotid arteries are isolated from each rabbit and placed in a dual organ chamber and perfused with oxygenated physiologic buffered solution (PBS) (NaCl 119 mM, KCl 4.7 mM, $CaCl_2$ 2 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 22.6 mM, glucose 11.1 mM and Na_2EDTA 0.03 mM) at 60 mmHg and 2.5 ml/minute flow rate at 37°C. Baseline vasodilation is determined using norepinephrine (NE, 1×10^{-6} M) preconstriction and pharmacological challenge is then performed with acetylcholine (Ach, 1×10^{-5} M) and sodium nitroprusside (SN, 1×10^{-5} M) successively. Vessel diameter is measured by a computer planimetry system (Figure 1). The data are calculated according to the formulas: Ach-NE (%)=(Ach-NE)/NE×100 and SN-NE (%)=(SN-NE)/NE×100 separately, where Ach, NE and SN represented the diameter (mm) of the arteries that are perfused by the PBS containing a corresponding chemical.

Metallothionein (MT): Metallothionein concentration as an index of oxidation is measured with Cd-hemoglobin saturation method (Eaton, 1991). Tissues are removed and rinsed in ice-cold Tris-HCl buffer (0.05 M, pH 8.6) then homogenized in 3 volume of the Tris-HCl buffer. The homogenate is centrifuged at 8,000×g for 10 minutes at 4°C and the supernatant fraction is heated for 90 seconds at 100°C. The heated samples are centrifuged at 8,000×g for 5 minutes at 4°C to remove precipitates. 100 µl of 400 ppm $CdCl_2$ solution is added into 200 µl of the supernatant and allowed to incubate at room temperature for 10 minutes. 150 µl of 2% bovine hemoglobin solution (w/v) is added into the sample, then the sample is mixed and heated in boiling water for 2 minutes. The boiled samples are placed in ice for 3

minutes and centrifuged at 8,000×g for 5 minutes at 4°C. Another 150 μl of 2% hemoglobin is added into the supernatant, then heating, cooling and centrifuging are repeated, and the supernatant is collected. The Cd concentration in the supernatant is measured using a flame atomic absorption equipment (Philips Electronics UK Ltd., Croydon, Surrey, England) and MT concentration is calculated from the Cd concentration measured in the supernatant (1 mg Cd represented 8.93 mg MT).

Glucose-6-phosphatase (G-6-Pase): Glucose-6-phosphatase measurement is followed Harper method (Harper, 1965). 0.1 ml of tissue homogenate (100 mg tissue/ml) in citrate buffer (0.1 M, pH 6.5) is added into a test tube and incubated at 37°C for 5 minutes. 0.1 ml of glucose-6-phosphate (0.08 M) is added and the sample is incubated at 37°C for 5 minutes, then 5 ml of trichloroacetic acid (10%, w/v) is added and centrifuged at 9,000×g at 4°C for 5 minutes. 1 ml of the supernatant is taken into a test tube and 5 ml of ammonium molybdate solution (2 mM) then 1 ml of reducing solution (42 mM 1-amino-2-naphthol-4-sulphonic acid, 560 mM SO_3) is added. The sample is incubated at room temperature for 30 minutes then absorption is measured at 660 nm.

Tissue Cholesterol: One cm^2 mid-thoracic and mid-abdominal aortic tissue samples are obtained. Total cholesterol (free and individual ester) in the tissue is measured by high-performance liquid chromatography (HPLC) (Kim, 1984). Each sample of aorta is ground to a fine powder with anhydrous sodium sulfate and extracted twice with 5 ml of chloroform and methanol mixture (2:1). The extract is dried under nitrogen and re-dissolved in 5 ml of isopropanol. A portion of isopropanol extract is filtered, dried and re-dissolved in the mobile phase. The sample (0.1 ml) is injected into the HPLC column and separated by using a Waters Radial-Pack C18 column eluted isocratically with acetonitrile:isopropanol (45:55 by volume) at 2 ml/min. The absorbance of the eluate is measured at 210 nm with a UV detector. Total cholesterol concentration is calculated by comparing the peak areas of samples with those obtained from the standard (Sigma Chemical Co., St. Louis, MO) (Witztum, 1985).

Serum cholesterol: Total serum cholesterol is obtained by enzymatic assays of blood samples collected from the rabbits prior to sacrifice. This is performed using a Sigma Diagnostics Kit for cholesterol (Sigma Chemical Co., St. Louis, MO).

Glucose Concentration: Sigma Glucose Diagnostic Kit (Sigma Chemical Co., St. Louis, MO, USA) is used for the serum and tissue glucose concentration measurements. The method of the instruction by Sigma is followed for this evaluation.

Light Microscopy: Arterial tissue specimen are embedded in paraffin, cut and mounted on glass slides. The sections are then stained with hematoxylin and eosin and Masson's trichrome stains. These are then examined using a light microscope.

Electron Microscopy: The tissue samples are fixed overnight in 4% glutaraldehyde (Fisher Scientific, Pittsburgh, PA, USA) with 0.1 M phosphate buffer (pH 7.4). Arterial segments (5 mm long) are subjected to critical point drying in liquid CO_2, mounted on stubs and gold-coated in a sputter coater. The intimal surface is examined using a JEOL scanning electron microscope (JEOL Ltd, Model JSM-6400V, Tokyo, Japan). Tissue sections are obtained and processed routinely for ultrastructural examination. Thin sections are stained with uranyl acetate and lead citrate and then examined with a transmission electron microscope (BEI preamplifier, Au Evirotech Company, Germany).

Statistics

Data analysis is performed using multivariate analysis comparing the two groups relative to the extent of thrombosis as related to cholesterol tissue content and presence and absence of tissue inflammatory markers. Using a 70% event rate we determined that 20 rabbits in each group could yield enough difference to detect a significant change between the two groups. With Jandel Scientific program, SigmaStat (Sigma Chemical Co., St. Louis, MO, USA) is used for data statistical analysis. $P<0.05$ is considered statistically significant difference. Measured data are reported as mean±SD. The student t-test is used for comparison.

Discussions

Atherosclerosis, or "hardening of the arteries", is the process that causes heart attacks and most strokes. It is characterized by the progressive build-up of fatty plaques in blood vessels. One major component of the atherosclerotic plaque is cells loaded with cholesterol called foam cells. It is currently believed that cholesterol, especially the low-density lipoproteins (LDL), must be modified or oxidized before they can be taken up to cause foam cells. Antioxidants such as vitamin C, vitamin E, and carotenoids can prevent the oxidative modification of LDL in the laboratory. This has given rise to the concept that these vitamins could decrease the risk of heart disease by preventing oxidation of LDL in the body. Myocardial infarction in human cases a triggering activity such as physical exertion precipitates the acute onset of the disorder (Mittleman 1993; Muller 1989; Tofle1990), but it is difficult to be studied in human. This study demonstrated that vulnerable plaques could be produced, the plaque disruption and platelet-rich arterial thrombus formation could be triggered pharmacologically in the atherosclerotic rabbits. Rabbits in the three groups which are balloon induced arterial injury and then maintained in an alternative 1% of cholesterol diet for a total of six months clearly caught atherosclerosis.

When cells use oxygen for energy purpose, they produce by-products called free radicals. Free radicals damage cells and tissues during a process called oxidation - a factor in many chronic illnesses, including some forms of cancer, cataracts, arthritis and cardiovascular disease. LDL, known as the "bad cholesterol", is actually a protein that carries cholesterol throughout the body. The cholesterol carried by LDL deserves its bad reputation, however. It often ends up in our arteries, causing clots that can lead to heart attacks. Oxidation of LDL-cholesterol contributes to the plaque build-up in arteries, a process called atherosclerosis that can cause blockages and reduced blood flow. The process also plays a role in the loss of elasticity in arteries. Antioxidants help to neutralize free radicals and prevent them from causing cellular damage. Once oxidized, the cholesterol is less apt to be expelled by the body's cleaning mechanisms and more likely to be stored in arteries.

In animal liver, glycogen is broken down into glucose-1-phosphate by liver phosphorylase and then converted into glucose-6-phosphate. Glucose-6-phosphate is dephosphorylated by G-6-Pase to yield free D-glucose, which passes into the systemic blood to be transported to other tissues.

MT is a ubiquitous class of low molecular weight and cysteine-rich proteins (about 1/3 of amino acid in MT is cysteine) binding unusually high amounts of metal ions, such as Ag, Cd, Cu, Hg and Zn. The most conspicuous biological feature of MT is its inducibility by a variety of agents and conditions. The biosynthesis of MT is induced by various factors such as heavy metals, certain hormones, cytokines, growth factors, tumor promoters, coldness, heat, hanger, radiation and diseases (Brady, 1982). Most of the inducing factors of MT biosynthesis are adverse factors. MT is thought to play an important role the homeostasis of metal ions and to be involved in the detoxification of heavy metals, scavenge free radical, etc. (Kagi, 1991).

The activity of G-6-Pase in liver of these four rabbit groups had the consistent magnitude result with glucose content in blood. This hinted that the alteration of glucose level in blood is adjusted by the activity of G-6-Pase in liver.

Carotenoid pigments are widely distributed in nature, where they play an important role in protecting cells and organisms against oxidation and free radical (Palozza, 1992).

This project design will reveal whether lipid lowering with atorvastatin reduces plaque disruption and thrombosis. Through the studies it will get valuable references for the atorvastatin researches and clinical application.

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