Role of Gap Junction in Atherosclerosis and Thrombosis

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Abstract: The scientific objective of this study is to test the hypothesis that the antioxidant β-carotene will prevent or reduce plaque disruption and thrombosis in atherosclerotic rabbits. This protective effect on plaque disruption will be mediated by enhanced gap junctional intercellular communication (GJIC). This article is giving a brief description of the tactics to study the role of gap junction in atherosclerosis and thrombosis. [Nature and Science. 2006;4(4):79-89].

Keywords: artery; blood; cardiovascular diseases; heart; vein

1. Introduction

The scientific objective of this study is to test the hypothesis that the antioxidant β-carotene will prevent or reduce plaque disruption and thrombosis in atherosclerotic rabbits. This protective effect on plaque disruption will be mediated by enhanced gap junctional intercellular communication (GJIC). To test this hypothesis, plaque disruption will be triggered in atherosclerotic rabbits as we described (Abela, et al. 1995; Hage-Korban, et al. 1999; Ma, et al. 2002, 2003) and GJIC will be measured with this rabbit model.

1) Plaques will be induced by the combination of a high cholesterol diet and balloon injury. Plaque disruption and thrombosis will be less in atherosclerotic arteries pretreated with β-carotene compared to non-treated control arteries. Despite a normal appearing endothelium after eight months following balloon injury, balloon de-endothelialized arteries are still vulnerable to disruption. However, our preliminary data suggest that β-carotene may protect the atherosclerotic arteries from plaque disruption and thrombosis.
2) Gap junction function in endothelial cells will be enhanced in arteries pretreated with the antioxidant β-carotene. Since gap junction function can be altered by oxidized LDL cholesterol and antioxidant treatment can modify oxidized LDL in atherosclerotic plaque, it is expected that the GJIC will be greater in β-carotene treated rabbit arteries.
3) Antioxidants have been shown to preserve endothelium-derived relaxing factor (Mugge, et al. 1991; Drexler, et al. 1999). Atherosclerotic rabbit arteries pretreated with β-carotene will preserve vasodilatation to acetylcholine challenge in a physiologic chamber when compared to control (non β-carotene treated rabbit arteries).
4) GJIC will be inhibited in β-carotene treated rabbits to evaluate if the GJIC is the key way for the β-carotene protection on atherosclerotic arteries from plaque disruption and arterial vasodilatation.

The proposed studies address an area of the critical importance to unstable cardiovascular syndromes. The applicant is a potential independent research scientist who has successfully accomplished many related projects and trained many students and medical research-fellows under the supervision of Dr. George S. Abela.

2. Background and Significance

Plaque disruption and thrombosis are the major events leading to unstable cardiovascular syndromes. Studies of pathologic specimens have shown convincingly that plaque fissuring is an important cause of acute myocardial infarction, crescendo angina and sudden ischemic death (Davies and Thomas, 1984). Early studies have demonstrated that plaque disruption frequently occurred in patients with fatal coronary thrombosis. Studies using coronary angiography and angioscopy have shown that plaque fissuring and disruption are common findings in patients with unstable angina and unstable events and that ulcerated plaque is much more commonly associated with occlusive thrombus that are smooth and non ulcerated plaque (Mizuno, et al. 1991).

Clinical trial have demonstrated that individuals receiving β-carotene had 49% fewer total vascular events and 44% fewer major coronary events including myocardial infarction and sudden death than did control subjects receiving placebo (Gaziano, et al. 1990). Also, vitamin E has been shown to reduce coronary events in both men and women on long-term supplementation (Rim, et al. 1993).

Evidence suggests that oxidation of LDL is important in the development of atherosclerosis (Steinberg, et al. 1989). Oxidized LDL is more readily taken up by macrophages than the native LDL. Also, oxidized LDL is chemotactic for circulating monocytes and may be cytotoxic to endothelial cells. The presence in plasma of an electronegative LDL subfraction cytotoxic for endothelial cells has been reported and the LDL shows proinflammatory activity on endothelial cells and may contribute to early atherosclerotic events (De-Castellarnau, et al. 2000). Antioxidants such as vitamin E have been shown to increase resistance to LDL oxidation

(Esterbauer, et al. 1991) and reduce platelet adhesion (Jandak, et al. 1988).

LDL cholesterol alters endothelial function resulting in abnormal vasoreactivity of human coronary arteries (Vita, et al. 1990). This is related to reduced endothelial mediated endothelium-derived relaxing factor (EDRF) production result in regional vasospasm at sites of dysfunction appears to persist up to 4 weeks following balloon injury. Duration of the endothelial dysfunction is related to severity of the initial vascular injury (Weidinger, et al. 1990). The aorta of the rabbits with balloon injury is highly vulnerable to disruption and thrombosis for up to 8 months following injury (Abela, et al. 1995). This is despite the presence of a normally appearing endothelium by scanning electron microscopy.

The presence of a thin collagenous cap overlying a lipid rich pool is associated with the morphology often seen in disrupted plaque with overlying platelet thrombus (Falk, 1989). In the proposed study, we hypothesize that antioxidants will prevent or reduce plaque disruption and thrombosis by preserving EDRF mediated vasodilator response and enhanced gap junction protein activity. This is expected to provide stabilization of vulnerable atherosclerotic plaques.

Both behavior and growth of individual cells depend on the cells with which they are in contact. Cell-to-cell communication is important for the maintenance of tissue homeostasis and control of growth and differentiated function. Since the gap junction is the only known structure by which the interior of adjacent cells are connected, it has been postulated that factors important for control of intercell communication is exchanged through these protein channels (Lowenstein, et al. 1979; Trosko and Chang, 2000).

Structural and functional studies have identified communicating gap junctions in endothelial cells and smooth muscle cells in vivo and in vitro (Little, et al. 1995). The gap junctional channel has been shown to admit low molecular weight molecules including anions, cations, camp, IP3 and calcium, but to exclude proteins and nucleic acids. For example, elevation of intracellular calcium concentrations has been associated with decreased GJIC and increased levels of camp with increased GJIC (Hossain, et al. 1989). These channels have been implicated in the tumor-promotion phase of carcinogenesis and more recently in the atherosclerotic process. Inhibition of GJIC has been demonstrated in cultured smooth muscle cells by the cytokine TNF-α and upregulation of GJIC by β-carotene in cultured human fibroblasts (Mensink, et al. 1995; Zhang, et al. 1995). Currently, little is known of the effects of there agents on vascular endothelium. The present proposal will investigate the effect of oxidized LDL on GJIC in atherosclerotic arteries and the effect on physiologic function of the vessels.

Unsaturated fatty acids inhibit GJIC in a reversible fashion and at doses above particular chemical thresholds (Trosko and Chang, 2001). In cultures of normal aortic cells the number of coupled cells is significantly higher than in cultures of atherosclerotic cells and gap junctional communication between cells loaded with lipid inclusions is lower than that between cells free of excess of intracellular lipids. In cultures of human skin fibroblasts the rate of intercellular communication is comparable to that in cultures of atherosclerotic cells. It is hypothesized that the reduced gap junctional communication in atherosclerotic human aorta is associated with alterations in the degree of smooth muscle cell differentiation (Andreeva, et al. 1996).

But, other reports show that in atherosclerosis and hypertension, vascular smooth muscle cells are stimulated to proliferate and exhibit enhanced gap junction protein expression (Kurjiaka, et al. 1998). Immunohistochemical staining followed by *in situ* hybridization on sections of human atherosclerotic carotid arteries revealed strong expression of gap junction connexin43 messenger RNA by macrophage foam cells. These results suggest that tissue-specific conditions present in atherosclerotic arteries induce expression of connexin43 messenger RNA in monocyte/macrophages (Polacek, et al. 1993).

It is reported that β-carotene (1-10 μM, 1-5 days treatment durations) did not affect GJIC, gap junction protein (connexin43, Cx43) expression, or growth in vitro of non-transformed (C10) or neoplastic (E9 and 82-132) murine lung epithelial cells, but β-carotene enhanced GJIC and Cx43 expression and reduced the growth of C3H10T1/2 murine fibroblasts (Banoub, et al. 1996). These indicate that the effects of β-carotene on GJIC and growth are cell-specific. Although the antioxidant properties of lycopene are thought to be primarily responsible for its beneficial properties, evidence is accumulating to suggest other mechanisms such as modulation of intercellular gap junction communication, hormonal and immune system and metabolic pathways may also be involved (Rao and Agarwal, 2000). Studies on vascular reactivity in atherosclerotic rabbits have demonstrated that vitamin E at low doses improves the EDRF mediated vasodilatory response (Keaney, et al. 1994). The organ chamber system that we have used is designed to study vasoreactivity of whole artery preparation under systemic perfusion pressures. We will use the same system to evaluate vasoreactivity of rabbit arteries in this proposal.

Atherosclerosis is associated with inflammation and acute coronary events (Li, 2004). CRP, IL-6, PAI-1, tissue factors may provide links between systemic inflammation and the outcomes at a localized cardiovascular event. Studies have demonstrated that several arteries may be involved in the acute event other than the one that has occluded (Buffon, 2002; Mukherjee, 2002). In this study we will also determine if there is an association among the expression of inflammation factors, acute coronary events and gap junction.

This proposed project would reveal the GJIC in the atherosclerosis, plaque disruption and thrombosis procedures and the link of the β-carotene and GJIC in these procedures.

3. Preliminary Studies

Working with George S. Abela, the applicant has done many experiments related this proposed project. All the techniques required by atherosclerotic rabbit model and thrombosis triggering have been done by the applicant (Abela, et al. 1995; Hage-Korban, et al. 1999; Ma, et al. 2002, 2003, 2004) and GJIC measurements by the project consultants (Trosko, et al. 2000).

1) Rabbit Atherosclerosis, Thrombosis and Vasoactivity

Twenty-four New Zealand white (NZW) rabbits were divided into 4 groups: normal rabbits as control-control (Group I, n=4)**;** atherosclerosis (induced by diet of 1% cholesterol plus arterial wall balloon injury), non-thrombus triggered and non-β-carotene treatment as control (Group II, n=4); atherosclerosis, thrombus triggered by Russell's viper venom (RVV, 0.15 mg/kg, IP) and histamine (0.02 mg/kg, IV) but non-β-carotene treatment (Group III, n=8); atherosclerosis, thrombus triggering and β-carotene (30 mg/kg, i.v.) treatment (Group IV, n=8). After rabbits were killed isolated carotid arteries were placed in a dual perfusion chamber and tissues were kept in liquid nitrogen for biochemical measurements. Both carotid arteries from each rabbit were perfused with oxygenated physiologic buffered solution at 37^{o}C and 60 mmHg. Baseline vasodilation was determined using norepinephrine (NE, 1×10^{-6} M) preconstriction, and pharmacological challenge was performed with acetylcholine (Ach, 1×10^{-5} M) and sodium nitroprusside (SN, 1×10^{-5} M). Vessel diameter was measured by a computer planimetry system.

The experimental results showed that all the normal rabbits had no atherosclerosis but all the rabbits in other three groups had atherosclerosis. The average weight of aorta for Group I was 1.62±0.55 g. The average weight of aorta for Groups II, III and IV was 3.65±0.37 g and there was no significant difference within the 3 groups. The average weight of aorta arteries for Groups II, III and IV was about 2.25 times heavier than that of Group I ($p<0.01$). Half of the triggered rabbits developed thrombosis. The ratio of the thrombus surface area on the aorta in Group III rabbits to that on the aorta in Group IV rabbits was 1.56. Group II rabbits got atherosclerosis, but no thrombus because rabbits of this group had no thrombus triggering by RVV and histamine. Group I rabbits got neither atherosclerosis nor thrombus. Thrombus surface area on aorta of Group III rabbits was higher than that of Group II rabbits and Group IV rabbits. The number of thrombi in Group III rabbits was two times as that in β-carotene treatment rabbits. The average of total cholesterol in aorta was 2729±334 μg/g for rabbit Groups II, III and IV, and 458±83 μg/g for Group I. There was no significant difference for aorta cholesterol among Group II, III and IV but was significant difference between the average of the above 3 groups and Group I ($p<0.01$). The vasodilation activity of artery in response to norepinephrine, acetylcholine and sodium nitroprusside was Group I>Group II>Group IV>Group III (inter-group ratio of the arterial vasodilation after pharmacological challenge was 1.2-3.5, $p<0.01$-0.05). There was no significant difference of glucose and protein content in aorta arteries among 4 groups. β-Carotene has potential pharmacological effects on atherosclerotic rabbits. β-Carotene reduced thrombosis triggering but not cholesterol content in the artery. This gives the possibly that β-carotene was involved other ways such as enhancing GJIC between the atherosclerostic artery cells.

This results in a platelet rich thrombus on a disrupted plaque in over 70% of treated rabbits (Figure 1) (Ma, et al. 2003).

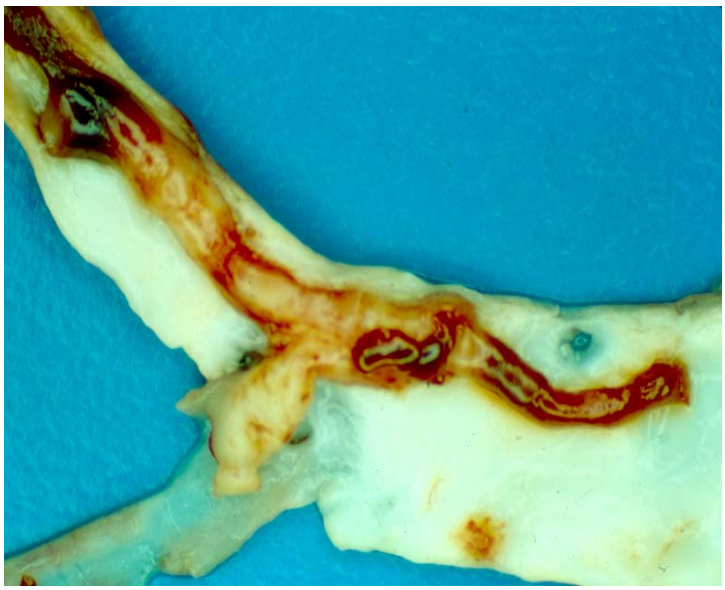

Figure 1. Gross examination: White thrombi with attached fibrin rich thrombi can be seen on the intimal surface of the aorta in more than half the triggered rabbits.

Consultants have measured the effect of LDL and GJIC in endothelial cells. These data have demonstrated that LDL inhibits GJIC in cultured porcine endothelial cells (Scaffidi, 1992).

2) C-reactive Protein Measurement

Atherosclerosis was induced in 17 NZW rabbits using balloon deendothelialization and feeding a high cholesterol alternating with normal chow for 9 months. Triggering was induced by Russell viper venom (0.15 mg/kg; i.p.) and histamine (0.02 mg/kg; i.v.) given at 48 and 24 hr prior to sacrifice.

Materials and Methods

Serum CRP levels were evaluated under three conditions:

1. **Normal rabbits**
2. **After induction of atherosclerosis**
3. **Following pharmacological triggering**

Model of Plaque Disruption and Thrombosis:

Atherosclerosis was induced in 17 NZW rabbits using balloon deendothelialization and feeding a high cholesterol alternating with normal chow for 9 months.

Triggering was induced by Russell viper venom (0.15 mg/kg; i.p.) and histamine (0.02 mg/kg; i.v.) given at 48 and 24 hr prior to sacrifice. This results in a platelet rich thrombus on a disrupted plaque in over 70% of treated rabbits (Figure 1) (Abela, 1995).

CRP Levels:

Rabbit specific high sensitivity ELISA was developed to detect the levels of serum CRP concentrations.

Serum samples were obtained by venous puncture of the ear veins in control rabbits (n=3), and atherosclerotic rabbits, before (n=6) and after triggering (n=8).

4. Results

Serum CRP levels increased significantly following each intervention. Levels on normal chow were very low and more than quadrupled after cholesterol feeding and were more than ten fold after thrombus triggering (Figure 2). Furthermore, CRP was significantly higher in rabbits that had thrombi than those that did not develop thrombi after triggering (Figure 3).

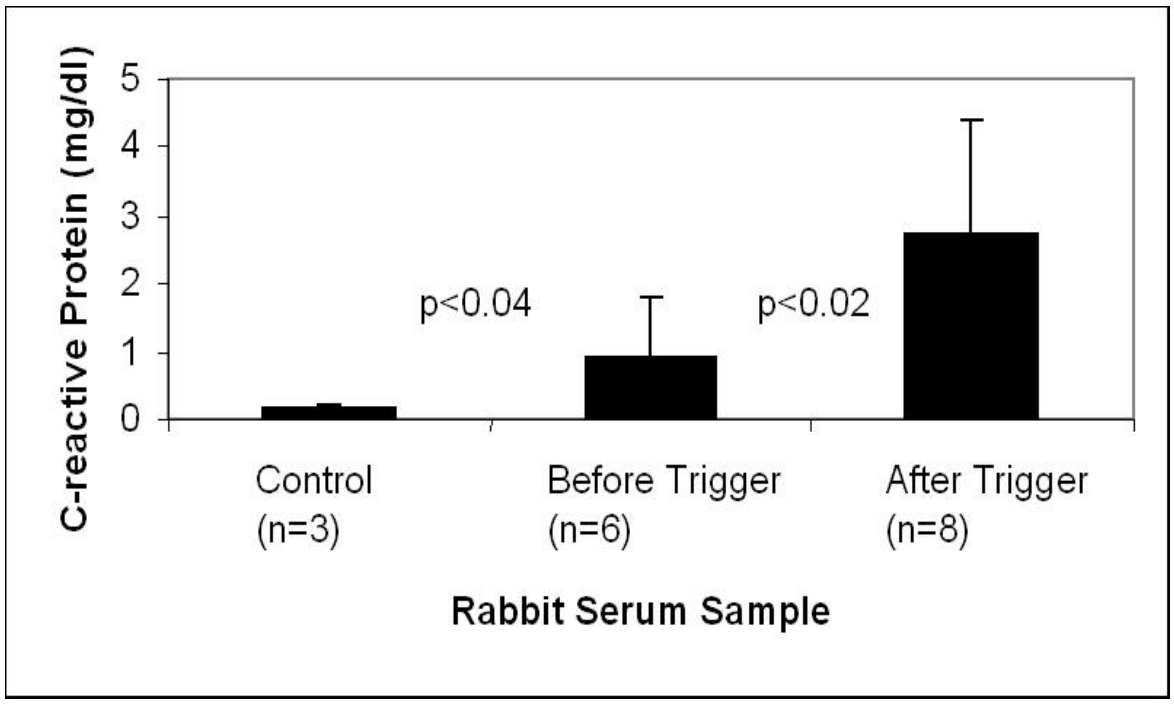

Figure 2. CRP levels were lowest at baseline (Control) and rose following feeding a high cholesterol diet (Before Trigger) and jumped further after triggering (After Trigger).

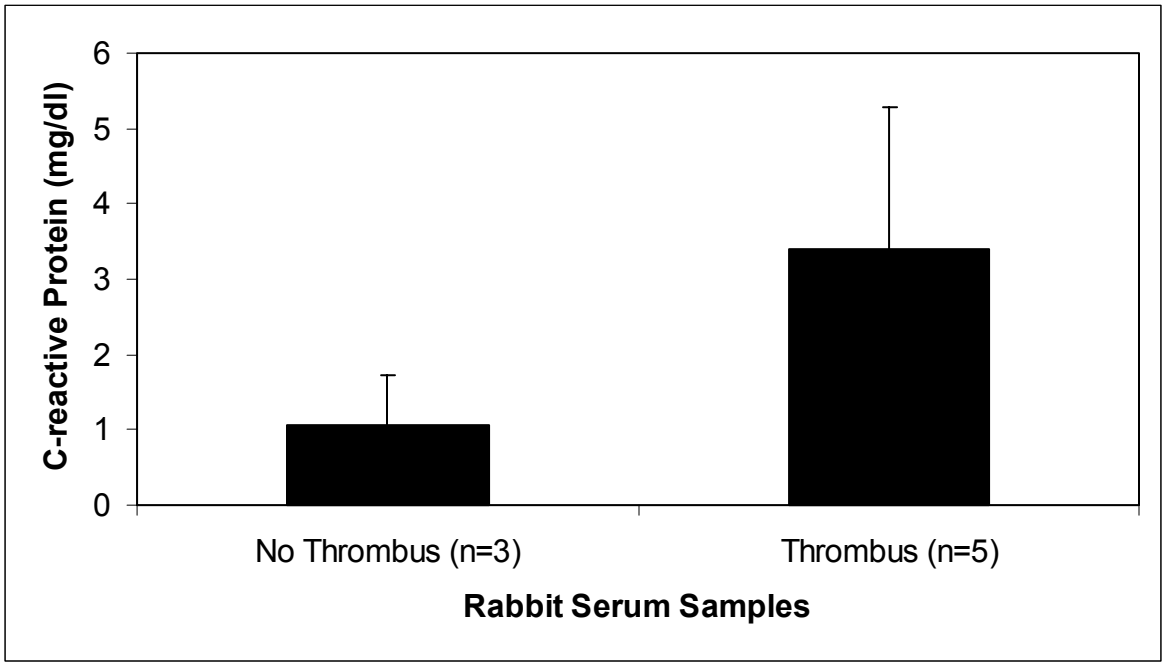

Figure 3. CRP levels in rabbits that developed thrombus were significantly greater than those without a thrombus ($p<0.01$).

Table 1. Preparation of the four rabbit groups

Groups	Treatments	n	Normal Diet	Cholesterol Diet (1%)	Balloon Trauma	RVV and Histamine	β-carotene
I	Control-control	40	Yes	No	No	No	No
II	Control	40	Yes	Yes	Yes	No	No
III	Non-β-carotene	40	Yes	Yes	Yes	Yes	No
IV	β-carotene	40	Yes	Yes	Yes	Yes	Yes

5. Research Design and Methods

1) Atherosclerosis and Thrombosis

A. Atherosclerotic Rabbit Model:

To study the function of gap junction in the protection of β-carotene on atherosclerosis and plaque disruption, the first step is to induce atherosclerosis in rabbit model.

One hundred and sixty, male, New Zealand White rabbits weighing between 2.5 and 3.2 kg (Harlan-Sprague Dawley, Inc., Indianapolis, Indiana) are divided into 4 groups as shown in the Table. The method of establishing atherosclerotic rabbit model and thrombus triggering is done as described previously (Abela, et al. 1995; Hage-Korban, et al. 1999).

The control-control group (Group I) will be fed a regular diet (Harlan-Sprague Dawley, Inc., Indianapolis, Indiana) for 6 months and without pharmacological thrombus triggering. Rabbits in Groups II, III and IV will have balloon-induced arterial injury, then will be maintained on a 1% diet (Harlan-Sprague Dawley, Inc., Indianapolis, Indiana) for 1 month followed by an alternated regular diet for another 1 month for total of 6 months up to pharmacological thrombus triggering.

The catheter will be advanced in a retrograde fashion to the aortic valve and then withdraw 3 cm. The balloon is inflated with 1.5 cm^3 of air, and the catheter is retracted down to the iliofemoral artery. This will be repeated three times in each rabbit. Rabbits are anesthetized with ketamine (50 mg/kg, IM, Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (20 mg/kg, IM, The Butler Company, Columbus, Ohio) in this surgery process.

Briefly, one week after initiating the atherogenic diet, balloon-induced arterial wall injury of the aorta (endothelial debridement) will be performed with a 4F Fogarty Arterial Embolectomy catheter (0.9×40 cm, Baxter Healthcare Corporation, Irvine, California) introduced through the right femoral artery cutdown. Atherosclerosis will be achieved by feeding a 1% cholesterol diet for 1 month, followed by a one-month period on a normal diet. This cycle will be repeated for a total of 6 months during the three years period of the proposal. Serum cholesterol will be checked prior to killing.

B. Thrombosis of Pharmacological Triggering:

Only the rabbits that are underwent balloon-induced arterial injury and are maintained on an alternative 1% cholesterol diet (Groups II, III and IV) had atherosclerosis then could be triggered thrombus. For thrombus triggering in atherosclerotic rabbits (Groups III and IV), Russell's viper venom (RVV, 0.15 mg/kg, Sigma Chemical Co., St. Louis, Missouri) is given by intraperitoneal injection at 48 and 24 hours before the rabbits are sacrificed. Thirty minutes after each RVV injection, histamine (0.02 mg/kg, Sigma Chemical Co., St. Louis, Missouri) is administered intravenously on an ear. For rabbits of Group IV, β-carotene (30 mg/kg, BASF Corporation, Mount Olive, New Jersey) is injected intravenously 8 days before sacrificed. After intravenous administration of heparin sulfate (1000 U/rabbit, IV, Elkins-Sinn, Inc., Cherry Hill, New Jersey) to prevent postmortem clotting, rabbits are anesthetized by injecting Nembutal sodium solution (pentobarbital 50 mg/ml, 1 ml/kg rabbit, Abbot Laboratories, North Chicago, Illinois) through a marginal ear vein. Procedures are performed according to Michigan State University's Animal Care and Use Committee approved protocol.

C. Aorta artery and Thrombus Evaluation and Quantitation:

Aorta, left femoral arteries and both carotid arteries are removed immediately after the rabbits are sacrificed. The artery diameter vasodilation, thrombus, plaque and total surface area of aorta are measured. The tissues of heart, liver and kidney are stored immediately in liquid nitrogen until biochemical measurements.

The total surface area of aorta, the surface of aorta covered with atherosclerotic plaques, the surface area of aorta covered with *ante mortem* thrombus, the number and weight of thrombi on the aorta from the aortic arch to the distal common iliac branches are evaluated. The surface area is evaluated by a color charge-coupled device camera (TM 54, Pulnix, Sunnyvale, California) 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.

Measurements will be made of the total surface area of the aorta and the iliofemoral branches, the surface area covered with atherosclerotic plaque, and the surface area covered with thrombus. Images of the arterial surface will be collected with a Pulnix TMC-7 color video camera and digitized by an Apple Macintosh Quadra 950 computer equipped with a NuVista 2M color graphics card and color image processing cubsystem. The digitized images will be calibrated using a graticule, and surface areas measured using a quantitative image analysis package. Tissue samples will be taken from the ascending, upper and lower abdominal aorta. Tissue preparation will be performed as indicated for the tests described below. Arterial tissue samples will be obtained after sacrifice and snap frozen for immunoperoxidase studies.

Arterial samples will then be processed for light microscopy using hematoxulin and eosin, Masson's trichrome, and Verhoffe's elastic stain. It is expected that two tissue blocks will be obtained per rabbit. This will result in an estimate of 180 samples. Our laboratory has the expertise to process these tissues as demonstrated by the preliminary data.

D. Artery diameter responding evaluation:

After rabbits are killed the both isolated carotid arteries from each rabbit are placed in a dual perfusion 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) under 60 mmHg flow pressure and 2.5 ml/min flow rate at 37°C. Baseline vasodilation is determined using norepinephrine (1×10^{-6} M) preconstriction, and pharmacological challenge is performed with acetylcholine (1×10^{-5} M) and sodium nitroprusside (1×10^{-5} M) successively. Vessel diameter is measured by a computer planimetry system. 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.

E. Tissue Culture:

In this project, primary cells will be cultured as the bypass observations. Endothelial cells and smooth muscle cells isolated from aorta and femoral arteries of all four rabbit groups will be cultured under the standard tissue culture technique (Davies, 1990). The GJIC and biochemical examinations will be made for the cultured cells.

2) Gap Junction Intercellular Communication

Gap junctional intercellular communication (GJIC) plays an important role in the regulation of cell growth, migration, and differentiation. How β-carotene influences the gap junction intercellular communication (GJIC) in atherosclerotic rabbit arteries and plaque disruption condition will be the key question in this project. GJIC in all the rabbit groups and cultured cells will be measured. The technique of GJIC measurement will be supported in Drs. Chang and Upham's lab (Trosko, et al. 2000).

GJIC will be assessed in β-carotene treated and non-treated rabbit aorta using the scrape-loading dye transfer method. The test demonstrates the intact function of the gap junction protein by visualizing the crossing of a dye (Licifer Yellow) from cells injured by a cutting blade to adjacent intact cells. Although the test has been used primarily in co-cultures of various cell types, it has also been used in intact specimen (El-Foulty, 1987; Christ, et al. 1995). Gap junction will be measure in cultured cells and isolated tissues (Emdad, et al.2001).

Immediately after sacrificing the rabbits with an overdose of pentobarbital, the aorta will be excised, a longitudinal incision will be made and the specimen will be

pinned flat on a cork board. The sample will be washed with phosphate buffered solution (PBS: 137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 nM KH_2PO_4, 0.68 mM $CaCl_2$, 0.49 mM $MgCl_2$) and incubated in Dulbeccos modified Eagle's Medium (DMEM) containing 10% fetal calf serum at 37°C and 5% CO_2 atmosphere prior to the experiments. Each sample will be inspected under a dissection microscope for morphological uniformity of the endothelium prior to dye studies, and atherosclerotic-appearing, normal-appearing and thrombus-involved areas of each animal will be identified and marked. Cells will be rinsed with PBS and covered with PBS containing 0.05% Lucifer Yellow and tetramethyl rhodamine dextran (Molecular Probes, Inc., Eugen, Ore). The dye will be scraped-loaded by scalpel incision into cells in the previously marked areas and incubated for 3 minutes at room temperature. Because of its low molecular weight, Lucifer Yellow can be transmitted between adjacent cells via gap junctions, but does not diffuse through intact plasma membranes. The high molecular weight rhodamine Dextran (10,000 daltons) cannot cross the gap junction channels or diffuse through intact plasma membranes, and therefore, serves to identify the primary loaded cells.

Following the scrape-loading procedure, samples will be rinsed several times in PBS to remove excess dye, fixed in formalin to trap the dye in place, and examined under a Nikon epifluorescence microscope for evidence of dye transfer from scrape-loaded cells to contiguous cells. Using the ACAS 570 laser cytometer, the data will be digitized and expressed as the average (percent) fluorescence SEM and normalized using an arcsin square root transformation procedure.

3) Biochemical and Histological Examinations

A. G-6-P, G-6-Pase, ATP/ADP/AMP, cAmp, cGMP, Adenylyl Cyclase, Guanylyl Cyclase, GTPase, and G-proteins:

There are often cell-cell communications through gap junction. Besides gap junction-mediated intercellular communication through GJIC, other signal pathways such as cAMP pathway, also play important roles in the intercellular signal transduction mechanism (Romanello, et al. 2001). In order to determine if β-carotene treatment alters the signal pathways in the rabbit with atherosclerosis, thrombosis and plaque disruption condition, and in order to find if there is relationship between GJIC and the other signal pathways in atherosclerotic rabbits under the antioxidant β-carotene treatment, the following measurements will be performed on homogenates of the arteries from all the rabbit groups and cultured cells.

Glucose-6-Phosphate (G-6-P) (Beutler, 1984): 0.5 ml of Tris-HCl (1 M, with 5 mM EDTA, pH 9.0) added to 1 ml of NADP (2 nM), 0.01 ml of β-mercaptoethanol, 1.2 ml of homogenized tissue extract, and 1.3 ml of H_2O. Read baseline absorbance at 340 nm then add 5 l of G-6-PD (150 U/ml). Record fluorescence till stable reading is obtained. Add glucose phosphate isomerase 10 l (1500 U/ml) and repeat record fluorescence. Add 10 l of G-6-P standard (50 M) and record fluorescence, and then add 20 l of G-6-P standard (50 M) and read fluorescence.

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.

AMP and ADP (Beutler, 1984): 50 µl of Tris-HCl (1 M, with 5 mM EDTA, pH8.0) added to 20 µl of $MgCl_2$ (0.1 M), 200 µl of NADP (2 mM), 700 µl of homogenized tissue extract, 5 µl of H_2O, 50 µl of phosphoenolpyruvate (15 mM), 100 µl of NADH (2 mM), 50 µl of lactate dehydrogenase (240 U/ml) and 5 µl of ATP (20 mM). Read baseline absorbance at 340 nm at 37°C. Then add 10 µl of pyruvate kinase (Type II, 140 U/ml) and read absorbance at 340 nm for AMP content. Then add 10 µl of adenylate kinase (myokinase, 725 U/ml) and read 340 nm for ADP content.

ATP (Beutler, 1984): 100 µl of Tris-HCl (1 M, with 5 mM EDTA, pH8.0) added to 20 µl of $MgCl_2$ (0.1 M), 200 µl of NADP (2 mM), 50 µl of glucose (20 mM), 200 µl of homogenized tissue extract, 400 µl of H_2O, and 5 µl of G-6-PD (60 U/ml diluted in β-mercaptoethanol-EDTA stabilizing solution). Read baseline absorbance at 340 nm at 37°C. Then add 10 µl of hexokinase (400 U/ml) and read absorbance at 340 nm until constant value is obtained.

cAMP and cGMP (Armbruster, 1990; Sambrook, 1989): cAMP and cGMP will be measured by standard ELISA and Western Blotting method. The cAMP and cGMP antibodies will be bought from Sigma (Product No. A0670 and G4899).

Adenylyl cyclase catalytic activity (Johnson and Salomon, 1991): 250 l of the reaction solution is incubated at 30°C for 10 min, which contains 20 mM Tris-HCl, pH7.5, 1 mM [α-^{32}P]ATP (10 cpm/pmol), 2 mM cAMP, 5 mM MgCl2, 1 mM EDTA, 1 mM beta-mercaptoethanol and 0.1% (w/v) BSA. After the incubation 600 µl of 120 mM zinc acetate is added to stop the reaction. cAMP is isolated with Dowex AG-50W-X8 column (BioRad) and the radio labeled product [α-^{32}P]cAMP is measured by a scintillation counter.

Guanylyl cyclase catalytic activity (Domino, et al. 1991): The assay buffer (3 ml) is prepared as the following: 600 µl of 200 mM 2-(N-morpholino)ethanesulfonic acid (MES), 180 µl of 100 mM $MnCl_2$ or $MgCl_2$, 600 µl of 10 mM 1-methyl-3-isobutylxanthine (MIX), 600 l of 10 mM cGMP, 300 l of 10 mg/ml bovine serum albumin and 720

μl of H_2O, 1.5 mg of creatine kinase and 33.1 mg of creatine phosphate (disodium salt). Take 50 μl of the buffer and add 25 μl of 2 mM GTP (50 nmol) containing 500,000 cpm [α-^{32}P]GTP, incubate at 37^{o}C for 5 min. Start the assay with the addition of 25 μl of the enzyme source (tissue extract solution). Controls will not be incubated and the reaction stopped by adding 500 μl of 120 mM zinc acetate prior to the adding the enzyme. The control is used to subtract background radioactivity when calculating the amount of cGMP formed. Non-enzymatic formation of cGMP will be tested by adding 25 μl of the buffer from the enzyme source or 25 μl of enzyme boiled in a 100^{o}C water bath for 5 min in separate reaction mixtures. The assay reaction will be stopped by adding 500 μl of 120 mM zinc acetate. Samples are then placed in an ice bath. Once all of the assay reactions have been stopped, 600 μl of 144 mM sodium carbonate is added to precipitate 5'-nucleotides, including unreacted [α-^{32}P]GTP. Centrifuge at 2000×g for 10 minutes. Samples could be frozen and thawed prior to centrifugation. Pour the sample over a neutral alumina column (0.7×15 cm, Econo-Column from BioRad). Elute [α-^{32}P]GTP with 5 ml of 100 mM Tris-HCl, pH7.5, into scintillation vials. Add 10 ml of scintillation fluid or H_2O and determine the radioactivity of the samples in a scintillation counter. Recoveries will be determined by measuring the absorbance at 252 nm of aliquots from each sample before and after column separation or with a tracer amount of cGMP. Recovery is usually between 60%-70%.

GTPase (Kikuchi, 1988): GTPase is the enzyme for the degradation of GTP. GTPase can be measured by the detection of ^{32}P from [^{32}P]GTP. The GTPase activity is determined by a modification of the method of Kikuchi (1988). Briefly, 20 μl of the sample is incubated for 30 min at 30^{o}C in 80 l of the reaction mixture containing 20 mM Tris-HCl at pH7.5, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.8 M NaCl, 3 mM L-α-dimyristoylphosphatidylcholine, and 1 μM [^{35}P]GTP (2000-3000 cpm/pmol). After the incubation, 50 μl of aliquots is added to 0.75 ml of ice-cold 5% (w/v) charcoal in 50 mM NaH_2PO_4. The mixture are centrifuged at 1000×g for 10 min at room temperature, The amount of ^{32}Pi released from [γ-^{32}P]GTP is then estimated by counting the radioactivity of 0.5 ml of the clear supernatant.

G Proteins (Ohmori, et al. 1989): G proteins are GTP-binding proteins. GTP combines with G proteins specifically. Incubate sample with radioactive-labeled GTP and isolate free GTP then measure radioactivity in the sample to measure G protein amount. Samples are incubated for 2 hours at 30^{o}C in 40 μl of the reaction mixture containing 20 mM HEPES at pH8.0, 1 mM EDTA, 1 mM DTT, 0.8 mM NaCl, 6 mM $MgCl_2$, 3 mM L-α-dimyristoylphosphatidylcholine, and 1 μM [^{35}S]GTPγS (1000-2000 cpm/pmol, Boehringer Mannheim). The reaction will be stopped by the addition of about 2 ml of the ice-cold 20 mM Tris-HCl at pH8.0 containing 100 mM NaCl and 25 mM $MgCl_2$ followed by rapid filtration on nitrocellulose filters. Filters are washed five times with the same ice-cold buffer. After the filter are dissolved in 8 ml of scintillation mixture, the radioactivity will be counted, G-proteins will be measured in endothelial cell suspension in normoglycemic and hyperglycemic culture medium.

Atherosclerosis is associated with inflammation and acute coronary events (Li, 2004). C-reactive protein (CRP), a non-specific inflammatory marker may provide a link between systemic inflammation and the outcomes at a localized cardiovascular event. Studies have demonstrated that several arteries may be involved in the acute event other than the one that has occluded (Buffon, 2002; Mukherjee, 2002). In this study we try to determine if there is an association between the systemic inflammatory response and an acute event. This was performed using an atherosclerotic model of plaque disruption and thrombosis that has been previously reported (Abela, 1995).

Elevated levels of C-reactive protein (CRP) have been associated with increased risk for development of cardiovascular events. In order to follow the trend of CRP over the course leading to an acute event, we evaluated CRP levels under three conditions: normal rabbits, atherosclerotic rabbits before and after pharmacological triggering of plaque rupture and thrombosis. As previously reported, plaque rupture and thrombosis is induced using Russell viper venom (RVV) and histamine in an atherosclerotic rabbit model. Methods: Atherosclerosis was induced with balloon deendothelialization and feeding a high cholesterol diet for 9 months. Serum samples were obtained from control rabbits (n=3), and atherosclerotic rabbits, before (n=6) and 48 hours after RVV and histamine-induced thrombosis (n=8). Rabbit specific high sensitivity ELISA was developed to detect the levels of serum CRP concentrations. Results: CRP levels were significantly lower in control normal rabbits compared to rabbits with atherosclerotic plaques. Our results further demonstrate that rabbits with RVV and histamine-triggered thrombosis had significantly higher levels of serum CRP than non-triggered atherosclerotic rabbits. Conclusion: The rise of serum CRP levels both after cholesterol feeding and the sudden rise after pharmacological triggering of thrombus may help using of CRP to evaluate not only the long-term risk but also a more short-term risk of events if CRP levels increase acutely.

B. Total Tissue Cholesterol and Serum Cholesterol:

Cholesterol plays a key role in the atheroclerosis. To distinguish the rabbit atherosclerotic situation, total tissue cholesterol and serum cholesterol will be measured.

Midthoractic and midabdomical aorta tissues are sampled. Total cholesterol (free and individual ester) in the tissue is measured by high-performance liquid chromatography (HPLC) (Kim and Chung, 1984). Each sample of aorta is ground to a fine powder with anhydrous sodium sulfate and extracted twice with 5 ml of chloroform:methanol (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. 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 elute 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, Missouri) (Witztum, et al. 1985).

Total serum cholesterol is obtained by enzymatic assays of blood samples collected from the rabbits before they are killed. This is done with a Sigma Diagnostics Kit for cholesterol (Sigma Chemical Co., St. Louis, Missouri).

C. Glucose Concentration:

Sigma Glucose Diagnostic Kit (Sigma Chemical Co., St. Louis, Missouri) is used. The method of the instruction by Sigma is followed for this evaluation.

D. Total Protein Concentration:

Bio-Rad Protein Assay Dye Reagent Kit (Bio-Rad Laboratories, Hercules, California) is used and the kit instruction is followed.

E. Inflammation Factors Detection:

Inflammation factors CRP, PAI-1, tissue factor, and IL are measured by ELISA.

F. Electron Microscopy:

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

4) Statistical Analysis

With Jandel Scientific program, SigmaStat (Sigma Chemical Co., St. Louis, Missouri) will be used for data statistical analysis. Data will be presented as mean ± Std.Dev. $P<0.05$ is considered statistically significant difference. Intergroup comparisons will be conducted using multivaritate analysis ANOVA. It is expected that atherosclerosis and plaque will reduce the GJIC function and β-carotene treated arteries will relatively enhance GJIC function.

6. Potential Difficulties and Limitations and the Alternative Approaches to Achieve the Aims

A. Platelet adhesion maybe altered by antioxidants. Studies have demonstrated that platelet adhesion is lowered in patients on vitamin E (Jandak, et al. 1988). It maybe that this mechanism is an important contributor to the early observation of a reduced thrombus size in rabbits with disrupted plaques in β-carotene. In order to address this potential confounding issue, we will perform a bleeding time on the rabbits as described by Blajachman et al. (1979). In brief, a standard incision through the ear of the rabbit is done after warming for 5 minutes to 37°C by immersion into a bath that contains 0.9% NaCl. A 6 mm full thickness incision is made through the ear with a scalpel blade. The selected site avoided areas with visible vessels. The incised ear is then immediately reimmersed in the saline bath and the time for the bleeding to cease is recorded.

B. Attenuation of fluorescence has been shown to occur with β-carotene (Ye, et al. 1993). However, at the concentrations of Lucifer Yellow present in the cells, this has not been a limiting factor during scrape loading/dye transfer experiments (Hossain, et al. 1989). Also, the quenching effect by carotinoid has been shown to be negligible in experiments conducted in Drs. Chang and Upham's lab. Nevertheless, if we find this to be a problem, then an alternative would be to substitute α-tocopherol for β-carotene as the antioxidant agent.

7. **Conclusion and Discussions**

The elevation of CRP levels following cholesterol feeding and pharmacological triggering may provide a means to help monitor progression of acute events. The fact that presence of thrombosis is associated with higher levels of CRP may provide a means to help predict severity of events or even possibly recurrence of events as well.

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