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Thrombosis in Association with Atherosclerosis Induced by Dietary Perturbations in Dogs

Abstract. *The distribution, severity, and complications of diet-induced atherosclerosis in dogs can be altered by changing the source of fat in the diet. Thrombosis and thromboembolic disease associated with atherosclerosis occurred with diets containing beef tallow and lard or coconut oil but were absent in dogs fed cottonseed oil as a source of fat. Experimental animals with and without thrombosis are of value as models in elucidating the role of platelets and thrombostatic mechanisms in atherosclerosis.*

Atherosclerosis is the major cause of mortality and morbidity in the United States. It is characterized by local fibromuscular proliferation and lipid deposition in the tissue layer that lines the arteries. These overgrowths of tissue are called atherosclerotic plaques. The disease becomes symptomatic when the size of the plaques causes significant obstruction to blood flow or when thrombi form on the plaques. Despite the identification of various dietary, metabolic, and environmental factors as contributors to the genesis of this disease, little is known on a molecular or cellular level about its causative mechanisms. The roles of the platelet and hemostatic mechanisms in the etiology of the disease have been debated for decades. Most investigators agree that thromboembolic events are frequent and often fatal sequels of atherosclerotic lesions, but there is major controversy concerning the contribution of platelets to the primary or secondary events of the disease process (*1*). Diets high in saturated fat have been implicated in causing thrombosis, and it has been suggested that increased sensitivity of platelets to aggregation results from altered platelet membrane cholesterol or fatty acid content, or both (*2*). However, investigations of the roles of the platelet and coagulation in atherosclerosis have been hampered by lack of a suitable animal model. We now describe an animal model that develops significant thrombosis in association with atherosclerosis and suggest that such a model may be useful in the elucidation of the role of hemostatic processes in atherosclerosis.

The dog is a suitable model for studies of atherosclerosis. There is much to recommend the species, including ease of handling, availability, and a size large enough to provide sufficient blood and arterial tissue for analyses. In addition, dogs do not develop spontaneous atherosclerosis; thus, any disease observed is the direct result of the imposed experimental conditions. However, dogs are resistant to the development of hypercholesterolemia and atherosclerosis and require experimental maneuvers beyond simply feeding them a high cholesterol diet. Two different protocols have been demonstrated to be successful in the production of canine atherosclerosis. In one protocol the hypothyroid dog is fed a high fat, high cholesterol diet in association with a bile acid (*3, 4*). We have used various dietary fats in this protocol to determine the effects of changes in triglyceride content on the type and distribution of atherosclerosis and the occurrence of thrombosis. In the second protocol (*5*), euthyroid dogs are fed a semisynthetic diet containing cholesterol and hydrogenated coconut oil as the only sources of fat. Dogs on this diet have not been reported to develop thrombosis.

Atherosclerosis was shown to develop (*4*) in surgically thyroidectomized dogs fed a commercial diet supplemented with cottonseed oil, cholesterol, cholic acid, and propylthiouracil. The most severe disease occurred in dogs with concentrations of cholesterol in the plasma in excess of 750 mg per 100 ml and a characteristic hyperlipoproteinemia. The atherogenic hypercholesterolemia was characterized by the occurrence of beta very low density lipoproteins (β-VLDL), an increase of low density lipoproteins (LDL), and the presence of a unique lipoprotein, the HDL_c (*6*). A similar type of hyperlipoproteinemia has been described in association with accelerated, cholesterol-induced atherosclerosis in swine (*7*). The atherosclerosis in the dog fed the cottonseed oil diet involved the thoracic and abdominal aorta and the coronary arteries with only moderate peripheral artery disease. The lesions were of the intimal proliferative type and were associated with lipid deposition. Evidence of thrombosis was not observed (*4*).

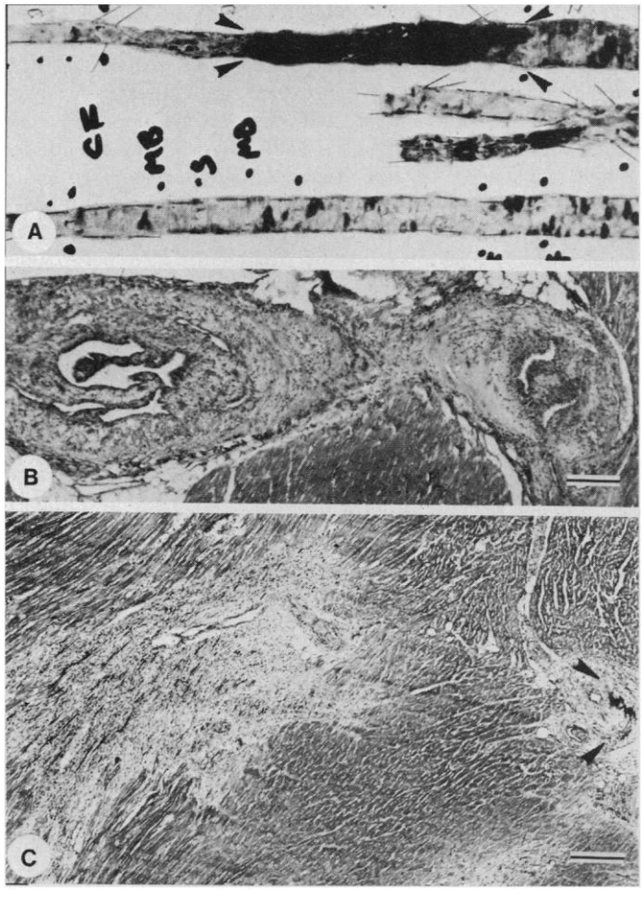
In sharp contrast to the above, when beef tallow or a mixture of beef tallow and lard was substituted for the cottonseed oil in the diet, there were significant changes in both the type and distribution of the disease. Moreover, thrombosis became a prominent component of the atherosclerotic disease process. To investigate the role of saturated fats in the production of atherosclerosis, 48 purebred foxhounds (NIH colony) were divided into two groups, which were maintained for 12 months on diet. One group of 24 euthyroid dogs was fed (*8*) a standard dog chow supplemented with fat (an equal mixture of lard and tallow). The other group of 24 dogs was surgically thyroidectomized and placed on the same diet except that cholesterol, taurocholic acid, and propylthiouracil were added (*8*). The euthyroid dogs did not develop significant hypercholesterolemia or atherosclerosis. The hypothyroid dogs developed atherosclerosis which was associated with a hyperlipoproteinemia that was similar to that of the dogs fed cottonseed oil. However, the distribution of this disease, unlike that in dogs fed the cottonseed oil, was restricted primarily to the terminal abdominal aorta, while the thoracic aorta was spared. An additional, not previously seen, difference in the pattern of disease was an extensive, often occlusive, involvement of the peripheral arteries, including the iliofemorals, mesenterics, internal carotids, and coronaries. Histologic study of the lesions of the terminal aorta revealed severe intimal proliferative disease with lipid deposition, occasional necrosis, calcification, and adventitial inflammatory response. The peripheral arteries showed an even more fulminant disease process associated with extensive necrosis, lipid deposition, and rapid progression to medial involvement. Of particular interest was the occurrence of thrombosis in association with many of these atherosclerotic lesions.

At autopsy, grossly visible thrombosis was found in association with arterial lesions in six of the 24 dogs. This occurred in the iliofemorals, terminal aorta, and internal carotids. A large, virtually occlusive thrombus in the iliofemoral artery is shown in Fig. 1A. Histologic examination of the arteries of many of the ani-

mals showed some evidence of prior occurrence of thrombosis in association with lesions in the terminal aorta and the peripheral and coronary arteries. For example, many recanalized peripheral and coronary arteries were found (Fig. 1B). Myocardial infarction was seen on routine histologic examination of one heart (Fig. 1C). Associated with this infarct was a small intramyocardial arteriole, which was occluded and appeared to have three recanalized channels. Another example of thromboembolic disease was the occurrence, in one dog, of gangrene of the foot, which required amputation of a toe.

The second dietary protocol with which thrombosis and atherosclerosis were associated in dogs was a semisynthetic diet containing 16 percent coconut oil and 5 percent cholesterol [Teklad Mills, Madison, Wisconsin (5)]. Seven purebred, euthyroid foxhounds were fed this diet for up to 12 months. The hypercholesterolemia and hyperlipoproteinemia were similar to those in the hypothyroid dogs on cottonseed oil and tallow-lard diets. In three of the seven dogs the cholesterol in the plasma was, on the average, less than 700 mg/100 ml, and the disease, as judged by gross examination, was only minimal. In the other four dogs, the cholesterol in the plasma was between 700 and 1200 mg/100 ml. These four dogs had significant atherosclerosis, which involved the terminal abdominal aorta, the iliofemorals, mesenterics, internal carotids, cerebral arteries, and coronaries. The topographic distribution and morphologic characteristics of this disease closely resembled those found in the group of dogs fed the tallow and lard diet. Gross thrombosis was observed in the internal carotid arteries of two dogs and in the basilar artery in one dog. The basilar artery (Fig. 2A) was virtually occluded at several locations. Gross hemorrhage had occurred into several of these plaques, with thrombotic occlusion of the vessel. An example of a thrombus that totally occluded the internal carotid artery is shown in Fig. 2, B and C.

The above result—that an apparently identical disease was produced in euthyroid and hypothyroid animals with and without added dietary bile salts—suggests that the bile salts and the hypothyroidism were not essential to the pathogenesis of the observed events and points instead to factors associated with the manipulation of the diet. Two factors common to both diets are the deficiency in essential fatty acids and the high con-



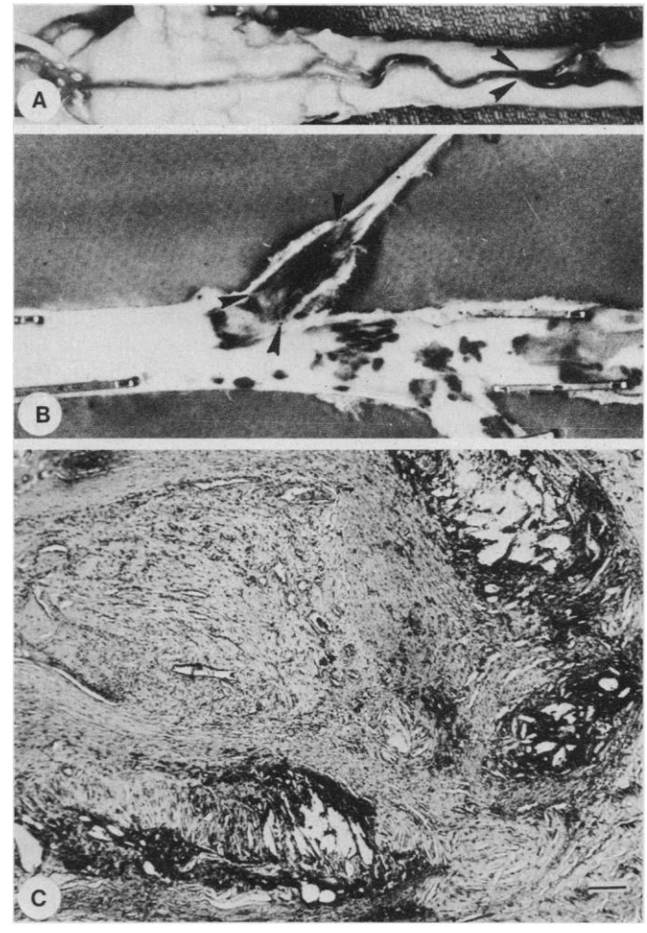


Fig. 1 (left). Tissue from dogs fed the tallow-lard and cholesterol diets. (A) The left iliofemoral artery (top of photograph), opened and pinned out, reveals a large, occlusive thrombus (arrowheads). The trifurcation of the aorta with the internal iliacs (middle) and the right iliofemoral artery (bottom) reveals small thrombi and plaque hemorrhages (dark patches). (B) Recanalized coronary arteries indicative of the resolution of thrombi (scale bar, 250 μm). (C) Myocardial infarction (left center) approximately 2 months old as dated by histologic reaction. Note the recanalized, partially calcified intramyocardial arteriole (arrowheads) (scale bar, 250 μm). Fig. 2 (right). Tissue from dogs fed the coconut oil and cholesterol diet. (A) Extensive thrombosis at the junction of the vertebral and basilar arteries (arrowhead) and at several sites in the basilar artery as it crosses the brainstem. When viewed through a microscope, the vessel showed severe atherosclerosis associated with hemorrhage and occlusion associated with recanalization. (B) Sudan staining of the opened carotid artery and major branches reveals atherosclerotic lesions (dark patches). An occlusive thrombus (arrowheads), which prevented opening of the vessel, is present in the internal carotid. (C) Frozen section, stained with oil red 0 and hematoxylin, of the internal carotid artery pictured in (B). The original lumen (upper left) is extensively recanalized. Severe atherosclerosis with lipid deposition and cleft formation involves the entire wall of this vessel (scale bar, 250 μm).

tent of saturated fat. Thus it is possible that these or other yet unidentified dietary factors present in both regimes are responsible for the thrombosis.

Our model provides an opportunity to determine whether the thrombotic tendency is the result of altered hemostatic mechanisms and therefore is a possible progenitor of the atherosclerotic lesion itself, or whether this tendency is simply secondary to a particularly virulent form of atherosclerosis induced by these dietary factors. Apart from the fulminant nature of the disease that occurred under the two dietary regimes, the observed pathologic events appear to be similar to those that occur in man. The availability of an animal model in which it is possible to study these important questions may represent a key discovery that will allow final clarification of the role of hemostasis in the genesis, progression, morbidity, and mortality of atherosclerosis.

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8. Dogs were given free access to food. The basic dog chow was a mixture of 60 percent growth diet and 40 percent meat-bone meal (Riviana Foods, Topeka). To the diet of the euthyroid dogs were added equal amounts of beef tallow (Litvak Meat Packing Co., Denver) and lard (Loveland Packing Co., Loveland) to a final concentration of 22 percent fat by weight in the diet. The hypothyroid dogs received the same basic chow supplemented with a mixture of tallow and lard (total fat in diet, 22 percent) and 0.75 percent taurocholic acid. The cholesterol in the diet was varied from 1 to 5 percent by weight in an attempt to regulate the concentration of cholesterol in the plasma to between 750 and 1500 mg per 100 ml. The propylthiouracil was fed at a level of 1.5 mg per kilogram of body weight per day but was increased to 15 mg per kilogram per day after 28 weeks. Propylthiouracil, in addition to surgical thyroidectomy, was necessary to suppress the function of aberrant thyroid tissue, which occurred frequently in the dogs. The supplement was prepared by solubilizing the tallow and lard at 70°C and adding the cholesterol, taurocholic acid, and propylthiouracil slowly and with stirring.
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Chloramine Mutagenesis in *Bacillus subtilis*

Abstract. *Chloramine (which occurs widely as a by-product of sanitary chlorination of water supplies) is shown to be a weak mutagen, when reversion of* trpC *to* trp$^+$ *in* Bacillus subtilis *is used as an assay. Some DNA-repair mutants appear to be more sensitive to chloramine, suggesting the involvement of DNA targets in bactericide. The influence of plating media on survival of cells treated with chloramine suggests a bacterial repair system acting upon potentially lethal lesions induced by chloramine.*

Chloramine (NH_2Cl), the reaction product of chlorine and ammonia in aqueous solution, is widely used directly for, or is generated as a by-product of, the disinfection of public water supplies and swimming pools. Nevertheless, little is known of the biological mechanism of action of chlorine or of possible genetic effects. Our own study (*1*) has shown that chloramine reacted with *Bacillus subtilis* DNA in vivo and in vitro; however, Boyle (*2*) did not obtain auxotrophs by treating *Escherichia coli* cells with chloramine; and a preliminary experiment with bacteriophage lambda by Hayatsu (*3*) did not show mutagenicity of hypochlorite. On the other hand, while this manuscript was in preparation, Wlodkowski and Rosenkranz (*4*) reported that sodium hypochlorite was a weak base-substitution mutagen in *Salmonella typhimurium*. We now report the mutagenicity of chloramine, using reversion of *trp*C to *trp*$^+$ in *B. subtilis* as an assay.

The strains used in this study were derivatives of indole-requiring *B. subtilis* strain 168 (*5*). As an index of the involvement of DNA targets in bactericide by chloramine, the sensitivity of different *B. subtilis* strains carrying various DNA-repair mutations was examined. Cell concentrations of each mutant and of its control for chloramine treatment were adjusted to about the same. In addition, the mixed cultures of strains 168 + SB879 and strains BD170 + BD194 were treated with chloramine and their survivors were sorted out by their respective nutritional markers. The surviving fractions of a representative test for each mutant as a function of chloramine doses are shown in Fig. 1. While *uvr* (SB879) and *rec*B (BD191) showed no evident increases in sensitivity, *rec*3 (BD193), *rec*A (BD194), and *pol*A5 (SB1060) were consistently

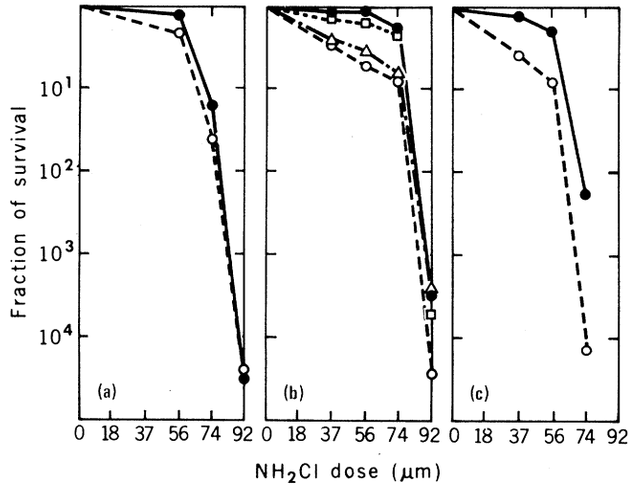

Fig. 1. Chloramine sensitivity of different *B. subtilis* strains. (a) ●, 168 (*uvr*$^+$, *trp*C); ○, SB879 (*uvr*, *trp*C, *his*B); (b) ●, BD170 (*rec*$^+$, *trp*C, *threo5*); □, BD191 (*rec*B, *trp*C, *threo5*); △, BD193 (*rec*3, *trp*C, *threo5*); ○, BD194 (*rec*A, *trp*C); (c) ●, SB1058 (*pol*$^+$, *phe*A, *trp*C, *his*B); ○, SB1060 (*pol*A5, *phe*A, *trp*C, *his*B). For the chloramine treatment, NaOCl was diluted in 0.05*M* phosphate buffer, *p*H 7, to different concentrations. Chloramine (NH_2Cl) was formed by incubating eight parts of NaOCl and one part of 0.1*M* NH_4Cl at 37°C for 1 hour. The prepared early stationary phase cells (*1*) were diluted ten times into chloramine solution and treated for 30 minutes at 37°C. The reaction was stopped by the addition of one volume of 0.02*M* sodium thiosulfate. Viable cell counts were scored by averaging the colonies of two plates (amino acid media, see Table 1) after incubation at 37°C for 2 days. The minimum number of colonies per plate was 161 ± 8 percent.