The Effect of Hypercholesterolemia on Early Atherosclerotic Lesions Initiated by Fibrinopeptide B1,2

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Hypercholesterolemia and thrombosis have been implicated as factors in the development of atherosclerosis. Fibrinopeptide B (FPB) is a short chain peptide cleaved from fibrinogen during the production of fibrin. FPB is a known chemoattractant and has been shown to produce experimental atherosclerotic lesions in association with hypercholesterolemia. The present study was designed to examine the role of hypercholesterolemia in this process and to study the time course of the development of these lesions. Twelve New Zealand White rabbits were placed on an atherogenic diet and had suture carrying either FPB, fibrinopeptide A (FPA), or saline (controls) implanted in the adventitia of the femoral arteries and were sacrificed at 14 days. An equal number of animals were left on a standard diet and underwent similar treatment. Eleven animals were treated as the hypercholesterolemic group but were sacrificed at 2, 4, and 7 days. The thickness of the intima was measured adjacent to the suture in the animals sacrificed at 14 days, and the hypercholesterolemic FPB sites were thicker (12.23 μ ± 6.60) than either hypercholesterolemic FPA (6.06 μ ± 3.72), saline (4.94 μ ± 1.42), or the normocholesterolemic FPB (6.99 μ ± 4.61), FPA (3.89 μ ± 2.20), or saline (3.97 μ ± 1.83) (P < 0.05 for all groups). Transmission electron microscopy of the hypercholesterolemic FPB group showed evidence of macrophages, actively secreting smooth muscle cells with newly deposited elastin, and foam cells by 7 days. We conclude that FPB attracts or stimulates cellular and extracellular proliferation favors early atherosclerotic lesion formation in the presence of hypercholesterolemia.

INTRODUCTION

Recent clinical studies have indicated that serum fibrinogen levels are associated with an increased incidence of symptomatic atherosclerosis-related disease. Data from the Framingham study have revealed that individuals with elevated serum fibrinogen levels are at an increased risk of developing stroke and ischemic heart disease [1]. Fibrin and fibrinogen-related antigens have been found to be elevated in patients with acute myocardial infarction and unstable angina compared to patients with stable angina or noncardiac pain [2]. There is reason to believe that fibrinogen and its products are involved in the initiation of the atherogenic process at the tissue level, for fibrin and fibrinogen have found to be quantitatively present in the intima of both normal vessels and in vessels with early plaque formation [3].

The conversion of fibrinogen to fibrin results in the hydrolysis of peptide residues from fibrinogen with the release of short chain peptides. These peptides include fibrinopeptide A (FPA) and fibrinopeptide B (FPB). Under physiologic conditions, the release of FPA is almost complete before the release of FPB occurs and the rate of release of FPA from intact fibrinogen is almost 30 times as great as the release of FPB [4]. Thus, the release of FPB is not necessary for the initial stages of blood coagulation. In previous studies, it was shown that trehalose dimycolate of the mycobacterial cell wall, a known inflammatory agent, adsorbs fibrinogen, and the action of thrombin results in preferential release of FPA, associated with an inflammatory response [5, 6]. Other investigators have shown that FPB is characteristic for macrophages [7]. We have previously demonstrated the ability of FPB to produce early atheroscle-
rotic lesions in the intima of hypercholesterolemic rabbits [8]. The present study was designed to test whether FPB promotes the development of atherosclerosis in hypercholesterolemic animals and to study the mechanism of this effect at the ultrastructural level.

METHODS AND MATERIALS

Experimental Design

The experimental design has been previously reported in detail [4]. Briefly, normal and hypercholesterolemic New Zealand White rabbits were given, in a operative procedure, a perifemoral artery suture impregnated either with FPB or FPA; control groups of rabbits received a sham operation and a suture treated only with physiologic saline, i.e., no peptide.

The materials to be studied included FPB and two controls. The controls included normal saline solution (saline) and FPA. Three different groups were studied. Group 1 animals were hypercholesterolemic. These animals underwent the operative procedure and were then begun on an atherogenic diet containing rabbit chow with 4% corn oil and 1% cholesterol (n = 12). Group 2 was designed to study the effect of FPB independently of hypercholesterolemia and was maintained on standard rabbit chow only (n = 12). Both of these groups were sacrificed at 2 weeks. Group 3 was identical to group 1, except that it was designed to study the time course of early changes; hence, animals were sacrificed at 2 days (n = 3), 4 days (n = 4), and 7 days (n = 4).

The test substances were delivered to the vessel wall by soaking 4-O silk suture material in solutions of each substance. FPB and FPA were made in 0.1 mg/ml concentrations in saline. The test sutures were soaked in the solution for 24 hr in a vacuum and then dessicated in a vertical hood. The compound-embedded sutures were routinely prepared for surgical implantation and sterilized.

New Zealand White male rabbits weighing 3.0 to 3.5 kg were preanesthetized with ketamine (30-40 mg/kg) and xylazine (5 mg/kg). They were then anesthetized by inhalation of 1 to 2.5% halothane. The animals were then sacrificed at 2 days. The animals were sacrificed by cerebral perfusion with 4% paraformaldehyde for 30 min. Histologic sections were prepared with hematoxalin and eosin and Weigert-van Giesson and Gomori-trichrome aldehyde fucsin stains. The histologic sections were studied for evidence of intimal thickening or disruption or damage to the media or intima. Groups 1 and 2 were studied to find the thickest portion of the intima in approximation to the suture site which was measured with the image of the vessel section projected onto a Numonics 2200 digitizing tablet online with an 80286 based personal computer employing digitizing software (Sigmascan, Jandel Scientific). The measurements for intimal thickness were compared by use of an analysis of variance followed by independent t tests.

Groups 1 and 3 specimens underwent further study with ultra-thin sections to guide transmission electron microscopy. Electron microscopy was employed to identify cell morphology and type.

High Pressure Liquid Chromatography

Human synthetic FPA and FPB were obtained from Sigma Chemical Company (St. Louis, MO). The purity and yield of these peptides were verified by amino acid analysis and analytic reverse-phase high performance liquid chromatography (HPLC) [10]. A peptide standard was similarly prepared and analyzed. The quantity of FPA and FPB in the sample was determined by comparing the expected to the actual areas under the curves for each amino acid residue compared to the standard.

Reverse phase HPLC was also used to quantitate the peptides. Standard curves were produced from solutions of FPA and FPB from which the concentration had been determined by amino acid analysis. Fibrinopeptide solutions were then quantitated by chromatographing the solutions on an IBM octadecylsilane column (25 x 0.45 cm) using the following gradient: time 0 min: 10% A, 90% B; time 5 min: 10% A, 90% B; time 35 min: 40% A, 60% B; time 40 min: 40% A, 60% B (A = 0.05 M sodium acetate, pH 6.0, and B = acetonitrile). Peak areas obtained were then compared to the standard curve to obtain the concentration of fibrinopeptide.

Sutures were prepared as follows: Four such sutures, each 24 inches in length were soaked in 0.5 M ammonium acetate for 24 hr. The sutures were removed from the solution; the remaining solution was lyophylized and the powder was redissolved in 100 μl of 0.5 M ammonium acetate of which 50 μl was injected into the ODS column to quantitate peptide in the solution.

RESULTS

HPLC

The quantity of FPB on the surface of the suture material was 1.02 x 10⁻³ nmoles/cm and for FPA was 1.85 x 10⁻³ nmoles/cm.
The cholesterol levels in Group 1 were normal (51.1 mg/dl ± 15.1) at implantation and greatly increased to 1204.6 mg/dl ± 247.1 at time of sacrifice at 2 weeks. The Group 2 animals displayed normal cholesterol levels at both implantation (50.6 mg/dl ± 30.6) and sacrifice (63.0 mg/dl ± 28.3). Group 3 animals began with cholesterol levels of 68.4 mg/dl ± 26.5 and by 4 days these levels were increased to 761.8 mg/dl ± 62.7.

Intimal Thickening (Table 2)

The endothelium and media of all sections were without cell necrosis or interruption. There were, however, differences in intimal thickness. Analysis of variance between the six different treatments of Groups 1 and 2 showed that there was a difference (P = 0.001) between means of the 2-week specimens in regard to intimal thickening. In Group 1 (hypercholesterolemic group), the FPB group (12.23 μ 6.60) was thickened compared to saline (4.94 μ 1.42, P = 0.009) and FPA (6.06 μ 3.72, P = 0.037). In Group 2 (normocholesterolemic group) the FPB (5.99 μ 4.61) was not thicker than either saline (3.97 μ 1.83) or FPA (3.89 μ 2.20). Furthermore, the Group 1 FPB was thicker than the saline (P = 0.007), FPA (P = 0.004), and FPB (P = 0.045) of Group 2. There were no other differences between groups.

Ultrastructure

Groups 1 and 3 underwent electron microscopy with specimens taken at 2, 4, 7, and 14 days. There was no disruption of the intima or media in any of the sections studied from any treatment group or time frame. The endothelium remained directly on the internal elastic lamina in those arteries exposed to FPA and saline at all four time frames studied (Fig. 1). At 4 days the FPB group displayed actively secreting smooth muscle cells with abundant rough endoplasmic reticulum and deposition of newly formed elastin in the extracellular matrix. At 7 days this process was more pronounced and included both smooth muscle cells and macrophages laden with lipid vacuoles (Fig. 2). By 14 days, the smooth muscle cells and extracellular matrix was prominent, but the lipid laden cells were less prominent.

DISCUSSION

We have shown that FPB favors early atherosclerotic lesion formation in the presence of hypercholesterolemia. A suture impregnated with FPB caused endothelial proliferation and macrophage and smooth muscle foam cell formation. A similar suture impregnated with FPA, or one containing no peptide, failed to produce these effects, even in hypercholesterolemic animals. These changes are compatible with promotion of early atherosclerotic changes by FPB, but not by FPA or by the surgical procedure itself. Furthermore, normocholesterolemic animals showed none of these early atherosclerotic lesions.

Eckhard et al. [11] found that the average level of FPB in human patients with a variety of disease processes was between 1 and 23 × 10^-4 nmoles/ml. Thus, we have delivered a focal increase of FPB that is within the biologic range. Serum fibrinogen levels have been related to atherosclerosis-related diseases including stroke and ischemic heart disease. Hypercholesterolemia and especially high plasma levels of LDL and of apoprotein B are
FIG. 2. Transmission electron microscopy of a vessel treated with hypercholesterolemia and Fibrinopeptide B at 7 days. The intima contains many cells, including those with lipid vacuoles (foam cells) and large amounts of rough endoplasmic reticulum. The extracellular matrix contains newly formed elastin. (LU, lumen; EN, endothelium; IEL, internal elastic lamina; original magnification 15,000×)

a major epidemiologic risk factor for atherosclerosis [12, 13]. Smith et al. have shown that significant amounts of fibrinogen and fibrin are present in atherosclerotic lesions [3]. Shainoff and Page have also shown that unpolymerized fibrinogen, capable of yielding fibrinopeptides are present in atherosclerotic lesions in human aortas [14]. Kao and Wissler [15] have shown that fibrinogen is closely related to lipoproteins within atherosclerotic lesions. Thus, there is abundant clinical and circumstantial evidence suggesting that fibrinogen and its derivative play a major role in atherogenesis and atherosclerotic cardiovascular disease.

Previous studies of trehalose dimycolate-coated surface have suggested a role for FPB in the development of inflammation in response to bioincompatible surfaces [5, 6]. Fibrinogen rapidly adsorbs onto many lipid-coated surfaces. The orientation of the fibrinogen, however, differs from biocompatible to bioincompatible surfaces. Thus, fibrinogen adsorbs into tightly packed egg lecithin monolayers—a noninflammatory surface—in such a way that thrombosis causes preferential release of FPA. On the other hand, when fibrinogen adsorbs onto trehalose dimycolate—an inflammatory surface—thrombin causes preferential release of FPB. The role of FPB in the subsequent inflammation is not known, but may relate in part to the fact that FPB is chemotactic for macrophages. Prescott, et al. has shown that the migration of leukocytes into the vascular wall can stimulate smooth muscle cell migration into the intima and produce intimal lesions [16]. They have also shown that blocking this inflammation with dexamethasone prevents lesion formation. In our results, FPB did not cause massive migration of macrophages into the lesion area. Furthermore, there was obvious proliferation of intimal cells, ultrastructurally compatible with smooth muscle cells. Although these data do not prove that FPB was the cause of smooth muscle cell proliferation, it is notable that such changes did not occur in animals treated with FPA. Fibrinogen-derived products have been shown to stimulate the release of endothelial-derived growth factors that were mitogenic for both endothelial cells and fibroblasts [17]. Thus, fibrinopeptide B may have a direct effect on smooth muscle cells independent of its chemotactic properties.

The role of the coagulation system in the development of atherosclerosis has recently been reviewed by Thompson and Smith [18] who point out that the role of fibrinogen in atherosclerotic lesion formation may be through the association of LDL or Lp(a) with fibrin directly. However, they indicate that the relationship between macrophages and intra- or extracellular lipid accumulation remain obscure. They conclude that “arterial fibrin deposition (is) the essential feature of the etiology and pathogenesis of atherosclerosis.”

With this background we hypothesize that in the face of hyperlipidemia, lipids, lipoproteins, and fibrinogen are taken in the intima. The interactions between these substances lead to the conversion of fibrinogen to fibrin and release fibrinopeptide B. The fibrin and/or the altered lipids may not be able to pass out of the vessel. FPB either attracts or stimulates the macrophages and smooth muscle cells to cope with the excess lipid influx. Other leukocytes may play an intermediary role here as well. The cellular and extracellular proliferation in the intima leads to intimal thickening, as was found in the present study. Whether and why progression or regression of the lesion from this point forward occurs remains to be determined. Studies involving earlier and later intervals with hyperlipemic animals are necessary to elucidate further the relationship between fibrinopeptide B and atherosclerotic lesion formation.

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