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Effect of hypercholesterolemia on transendothelial EBD–albumin permeability and lipid accumulation in porcine iliac arteries

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Abstract

Hypercholesterolemia is associated with increased cardiovascular mortality and is known to promote the advancement of atherosclerotic lesions in experimental animal models. Juvenile swine were fed a normal or high-cholesterol diet, and the transendothelial macromolecular permeability of the external iliac arteries of these animals was assessed by measuring the uptake rate of circulating Evans blue dye (EBD). The extent and patterns of lipid-containing lesions were also determined using en face staining with Oil Red O (ORO). Sites of ORO staining often excluded EBD, possibly via the fragmentation of the internal elastic lamina, to which EBD binds. By spatially averaging the EBD uptake in arterial segments relatively free of ORO-positive lesions, it was found that endothelial permeability to albumin was greater in hypercholesterolemic pigs than in those on a normal diet ($p = 0.056$).

Keywords: Evans blue dye; Permeability; Endothelium; Albumin; Swine; Hypercholesterolemia; Arteries

1. Introduction

Hypercholesterolemia, which is associated with several manifestations of endothelial dysfunction [1], has been epidemiologically linked to increased risk of coronary heart disease [2] and its associated mortality [3,4]. Among the effects of experimental hypercholesterolemia on arterial endothelial function are increased intimal levels of the proliferative transcriptional factor NF-κB, decreased levels of eNOS [5], increased activation of NF-κB in areas prone to lesion development [6], and increased intimal expression of VCAM-1 resulting in augmented leukocyte adherence [7]. In humans, either lipid-lowering therapy [8] or infusion of reconstituted HDL [9] improves endothelial cell function, as assessed by resistance to acetylcholine-induced vasoconstriction, while treatment with L-arginine reverses the attenuation of flow-mediated vasodilation seen in hypercholesterolemia [10].

One of the trademarks of atherosclerosis is the accumulation of intimal lipids. Since hyperlipemia appears to promote endothelial dysfunction, it may also lead, at some point during the progression of the disease, to an alteration in macromolecular permeability. The relation between hypercholesterolemia and macromolecular permeability is yet to be resolved. Early studies concluded that a high-cholesterol diet leads to increased permeability in rabbits [11,12]. Later, in the same species, a similar conclusion was made based on transport rates of radioactively labeled albumin through the carotid artery wall [13]. However, the latter authors later noted a paradoxical decrease in the rate of albumin accumulation in the arterial wall following 1 week of cholesterol feeding, which was hypothesized to reflect larger rates of clearance from the wall, since venous levels of albumin were elevated in these animals [14]. More recent efforts have focused on the localized effects of hypercholesterolemia on albumin permeability around branch points in rabbit aortae, leading to the conclusion that the permeability patterns depend on age and NO bioavailability [15].

Here, we explore the effects of experimental hypercholesterolemia on permeability in pigs. Swine were chosen over rabbits, the other species commonly used in studies of diet-induced atherosclerosis. While both species develop lesions

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during administration of a high-cholesterol diet, only the pig spontaneously develops such lesions as man does [16]. Natural porcine lesion development mimics that of the human both anatomically and temporally, when the latter is adjusted for the expected lifespan of each species [17]. Furthermore, the natural and diet-induced serum lipid profiles of swine are more similar to those of man than are those of rabbit [16,18]. The serum cholesterol level of the juvenile pigs used in this study is more responsive to a high fat diet than that of adult pigs [17].

Albumin transport was determined indirectly by measuring the cumulative uptake of Evans blue dye (EBD). EBD binds to albumin in the blood, is transported across the endothelium, and then dissociates from the protein to bind to components of the subendothelial matrix, primarily free amino groups of the internal elastic lamina (IEL) [19]. The area density of matrix-bound EBD has been shown to be quantitatively related to en face tissue optical density (OD) [20]. In the present work, OD measurements were made on the distal portions of the external iliac arteries of normolipemic and hyperlipemic swine, revealing an elevated permeability in the hyperlipemic pigs relative to controls. During the course of this work, it was discovered that sites of lipid-containing lesions were often devoid of accumulated EBD. As this could potentially introduce error in permeability measurements using EBD, much of this report is devoted to characterizing this phenomenon by histological observation.

2. Methods

2.1. Animal surgery

In vivo experiments were performed using eight 59–68 kg commercial juvenile female swine. Of these, four were fed a diet of standard pig chow, and four were fed a special diet consisting, by weight, of 2% cholesterol and 15% lard (Harlan Teklad, Madison, WI). Animals were maintained on the high-fat diet for 5–8 weeks prior to experimentation. Experiments were performed in random order.

The surgical methodology was adapted from a previously described protocol [21] and was in accordance with guidelines for humane treatment set by the Institutional Animal Care and Use Committee of Duke University. Animals were maintained under anesthesia using inhaled isoflurane. A carotid artery was cannulated to measure blood pressure during the experiment and to provide an inlet for a post-mortem arterial flush. In both legs, the iliac–femoral arteries were exposed, and blood flows were measured for 15 min using Transonic S-series perivascular flow probes (Transonic Systems, Ithaca, NY). EBD injectate was prepared by filtering a solution consisting of 25.3 mg dye/kg swine in 60 ml of phosphate-buffered saline (PBS, Fisher) through 0.45 μm filters. To the filtrate, 75.8 mg/kg of bovine serum albumin (BSA, fraction V, heat shock treated, FisherBiotech) was added. The final molar ratio of EBD-to-albumin in the injectate was approximately 1:20, and the ratio of EBD-to-serum albumin in the animal was approximately 1:1. The injectate was administered via a port through a jugular vein.

Specimens of arterial blood were collected immediately prior to EBD injection, 5 min after EBD injection, and every 30 min thereafter during a 180-min EBD exposure. Serum was isolated in Vacutainer tubes containing clot activator. A lipid profile was conducted on the initial serum sample by Laboratory Corporation of America (Research Triangle Park, NC). To assay for EBD in the blood, the absorbance of the serum samples was measured spectrophotometrically at 595 nm. The absorbance of the pre-injection blood sample was subtracted from the subsequent measurements. To determine the corresponding EBD concentrations, the absorbance of several EBD solutions of known concentration was determined, and a calibration curve was constructed, in accordance with Beer's law. To confirm that no unbound EBD was present in the serum, spectrophotometric analysis was also performed on EBD-exposed serum samples following protein precipitation using trichloroacetic acid.

Following the 180-min exposure, the animals were euthanized and the femoral arteries cannulated to facilitate flushing of the arterial tree. Five liters of phenol-free Dulbecco's Modified Eagle Medium (Sigma) was perfused into the carotid artery inlet and the effluent was collected at the femoral artery outlets. A hydrostatic pressure of 100 mmHg was maintained at the carotid artery inlet. This was sufficient to maintain near physiological pressure in the iliac arteries since the largest pressure drop occurred at the narrow femoral artery outlets. Following the rinse, the posterior arterial tree, from the abdominal aorta to the distal femoral arteries, was dissected out.

2.2. EBD uptake quantification

The amount of EBD retained in the intima of the iliac arteries was quantified using previously described methods [21] to obtain the average optical density of the distal portion of each vessel segment; OD can be related to EBD concentration in the wall as discussed below. Briefly, the procedure was as follows. Vessels were cleaned of their connective tissue, adventitia, and deep medial layers, to remove any EBD that might have entered the tissue from the adventitial side. The vessels were cut longitudinally along their dorsal aspect, and unrolled and pinned out on a smooth white silicone block, intimal side up, adjacent to a standardized gray scale (Kodak Publication Q-13). The vessels were submerged in PBS and placed in a photographic stand with uniform lighting. Eight-bit gray scale digital photographs were taken of the vessels using a Nikon Coolpix 990 digital camera (Nikon Corp.) at a resolution of 1200 × 1500 through a Tiffen Red #29 filter (Tiffen, Hauppauge, NY).

The digital images were rescaled to OD images using the pixel intensities of the images of the standardized gray scale. The arterial regions of interest were segmented manually,