THE EFFECT OF EARLY INTIMAL GROWTH ON PATTERNS OF EVANS BLUE DYE IN MACROMOLECULAR TRANSPORT STUDIES

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INTRODUCTION

Evans blue dye (EBD) has been used for many years as an indicator of vascular macromolecular transport due to its unique binding properties. In the blood, EBD has a high affinity for the large protein albumin. However, once inside the arterial wall, EBD has a higher affinity for the internal elastic lamina (IEL), the structural layer subjacent to the endothelium in non-diseased arteries. Therefore, if an EBD-albumin complex crosses the endothelium, the EBD molecule will remain in the arterial wall as a marker of a single transport event. Thus, the total amount of EBD retained in an artery over a given amount of time is a measure of macromolecular uptake. For these reasons, EBD is a seemingly valuable tool in the assessment of endothelial barrier function. However, there are data to indicate that the IEL may be compromised in experimental hypercholesterolemia [1], which could potentially produce erroneous results when performing transport studies with EBD. Observations in our lab show that EBD is abundant in regions with some degree of intimal thickening, but is absent from more advanced lesions where the IEL is disrupted and lipid has accumulated.

METHODS

Domestic juvenile swine (60-100 kg) were fed a diet of either normal pig chow or a high cholesterol/high fat diet for durations of 10-16 weeks. The surgical methodology used was adapted from a previously described protocol [2]. EBD was intravenously administered in anesthetized animals at a 1:1 molar concentration ratio with blood serum albumin and allowed to circulate for various time periods. Immediately following sacrifice, the arterial tree was flushed with PBS and the posterior arterial tree, from the abdominal aorta to the distal femoral arteries, was dissected and placed in 10% neutral-buffered formalin overnight.

The external iliac arteries were cleaned of their adventitia, cut along their dorsal aspect, and pinned out intimal side up. The vessels were photographed in PBS using a Nikon Coolpix 990 digital camera (Nikon Corp.) to assess the distribution of EBD retained in the intima. Following photography for EBD assessment, the iliac arteries were treated with the lipophilic dye oil red O (ORO). The specimens were rinsed twice in PBS and then immersed for 5 min in a solution of 3% w/v ORO (Sigma, St. Louis) in 60% isopropanol. ORO was removed and the tissue was immersed in the isopropanol for an additional 30 sec, followed by rehydration in PBS for 15 min. The arteries were then photographed again for comparison with the EBD photographs. Regions of interest were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

RESULTS

The relationship between the distributions of EBD and ORO staining is demonstrated in Fig. 1. In general, regions in which ORO staining is positive appear to exclude EBD accumulation, but are often characterized by EBD staining in the nearby vicinity. Near major branches of the iliac artery, EBD staining was confined to the neighborhood of the flow divider lip of each ostium, while ORO staining extended much further distally, as well as lateral to (Fig. 1B), and in rare instances proximal to (Fig. 1D), the ostium.

Histological analysis of a site of EBD accumulation in a normolipemic pig (92 mg/dl total cholesterol) is shown in Fig. 2. Although a slight bluing occurs deeper into the tissue, the majority of EBD is confined to a thin region at the surface of the artery thought to be the IEL (Fig. 2A-B). A cross-section of the tissue at this location was stained with H&E and viewed under normal light (Fig. 2C) and fluorescence (Fig. 2D). As demonstrated near the margin and observed throughout, the EBD-positive site was characterized by modest thickening of the intima (Fig. 2C) and an intact IEL (Fig. 2D).

The distribution of EBD and ORO in the iliac artery of a severely hyperlipemic pig (328 mg/dl total cholesterol, Fig. 3A-B) follows the same patterns as those viewed microscopically. Namely, EBD accumulation does not occur at exact locations of ORO staining, but are common in the vicinity. One lesion with positive ORO staining is characterized histologically by significant intimal thickening (Fig. 3C, E) and an IEL that is intact at the margins of the lesion (Fig. 3D) but thin and highly fragmented within the lesion (Fig. 3E).

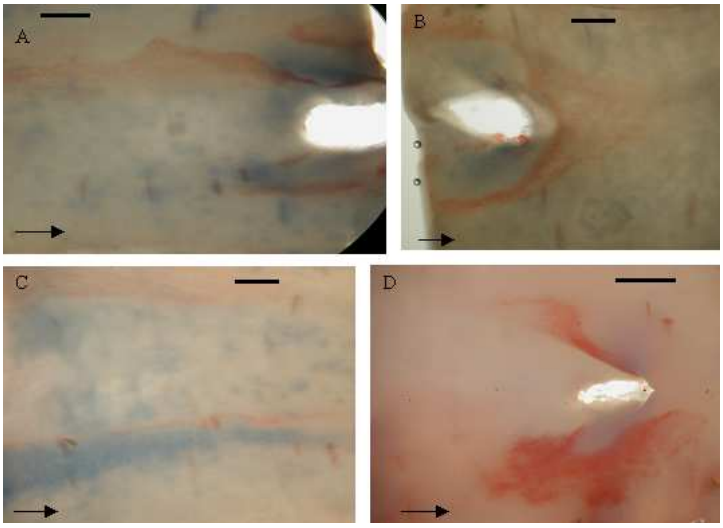

Figure 1: En face photomicrographs of iliac artery following ORO treatment. Specimens are from hyperlipemic pigs (A, B, C) or from a normolipemic pig (D). A. left proximal iliac near circumflex ostium. B. right proximal iliac near circumflex ostium. C. left proximal iliac near inlet region. D. left proximal iliac near circumflex ostium. Arrow indicates direction of bulk flow. Bar: 1 mm.

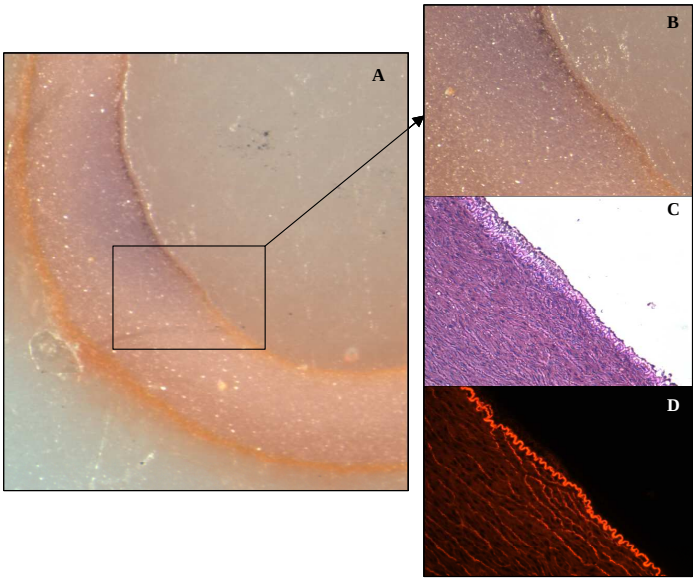

Figure 2: Photomicrographs of the iliac artery of a normolipemic pig in a region of high EBD uptake as viewed end-on in a paraffin-embedded block (A, B) or as a cross-sectional slice following staining with H&E (C, D). D shows the same field as C, but with fluorescence optics (excitation 510-560 nm, emission >590 nm) to accentuate the IEL.

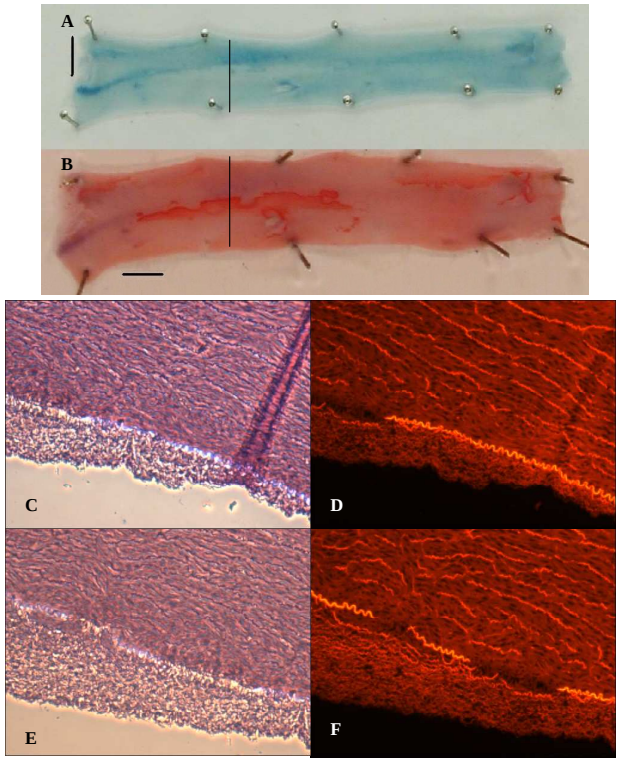

Figure 3: Iliac artery of a hyperlipemic pig. A-B. Photographs of tissue before (A) and after (B) staining with ORO. Bar: 5 mm; flow is from left to right. C-F. Photomicrographs of a section corresponding to the line through the tissue shown in A-B following staining with H&E and viewed with normal (C, E) or fluorescence (D, F, as in Fig. 2) optics. C-D show a field at the margin of the lesion, while E-F show a field at the interior of the lesion.

DISCUSSION

The data presented here are consistent with the hypothesis that early endothelial dysfunction results in modest intimal thickening; however, the IEL remains intact, allowing EBD accumulation. As lesions progress, a process that is accelerated in animals on a high-cholesterol diet, the intima becomes thicker and laden with lipid, and the IEL breaks apart into thin, non-continuous fragments. Though it is clear from the data shown here that EBD fails to accumulate where ORO staining is positive, it is unclear whether albumin-bound EBD penetrates the lesion. If it does, it is not known whether it diffuses laterally until it encounters intact IEL or diffuses through the tissue, failing to bind before reaching the media. EBD-rich areas adjacent to lesions marked by ORO could be either the result of such lateral diffusion or could arise because the edges of these lesions are associated with compromised endothelial barrier function to albumin, but are not yet advanced enough to be characterized by accumulation of LDL and a fragmented IEL. We thus conclude that caution should be exercised when spatially quantifying macromolecular transport using EBD in the presence of lesions that stain positive for ORO.

REFERENCES

1. Kwon H.M., et al, 1998, Atherosclerosis, 139, pp. 283-289.
2. Friedman M.H., et al, 2000, Biorheology, 37, pp. 265-277.

ACKNOWLEDGEMENTS

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