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Role of endothelial permeability hotspots and endothelial mitosis in determining age-related patterns of macromolecule uptake by the rabbit aortic wall near branch points

K. Yean Chooi [a, b], Andrew Comerford [a, b], Stephanie J. Cremers [a], Peter D. Weinberg [a, *]

[a] Department of Bioengineering, Imperial College London, London, SW7 2AZ, UK
[b] Department of Aeronautics, Imperial College London, London, SW7 2AZ, UK

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ABSTRACT

Background and aims: Transport of macromolecules between plasma and the arterial wall plays a key role in atherogenesis. Scattered hotspots of elevated endothelial permeability to macromolecules occur in the aorta; a fraction of them are associated with dividing cells. Hotspots occur particularly frequently downstream of branch points, where lesions develop in young rabbits and children. However, the pattern of lesions varies with age, and can be explained by similar variation in the pattern of macromolecule uptake. We investigated whether patterns of hotspots and mitosis also change with age.
Methods: Evans' Blue dye-labeled albumin was injected intravenously into immature or mature rabbits and its subsequent distribution in the aortic wall around intercostal branch ostia examined by confocal microscopy and automated image analysis. Mitosis was detected by immunofluorescence after adding 5-bromo-2-deoxiuridine to drinking water.
Results: Hotspots were most frequent downstream of branches in immature rabbits, but a novel distribution was observed in mature rabbits. Neither pattern was explained by mitosis. Hotspot uptake correlated spatially with the much greater non-hotspot uptake ($p < 0.05$), and the same pattern was seen when only the largest hotspots were considered.
Conclusions: The pattern of hotspots changes with age. The data are consistent with there being a continuum of local permeabilities rather than two distinct mechanisms. The distribution of the dye, which binds to elastin and collagen, was similar to that of non-binding tracers and to lesions apart from a paucity at the lateral margins of branches, that can be explained by lower levels of fibrous proteins in those regions.

1. Introduction

Elevated uptake of circulating macromolecules, particularly low density lipoprotein (LDL), by the arterial wall is seen in anatomical locations that are particularly susceptible to atherosclerosis and is thought to be a risk factor for it. The first study to show this used the intravital dye Trypan Blue [1], an isomer of Evans' Blue dye (EBD), which binds chiefly to serum albumin in the circulation [2]; the dye was preferentially taken up by the flow divider at arterial branch points, a site that was already known to be particularly prone to lesions in the cholesterol-fed rabbit [3]. Although the study was conducted using frogs, the same result was subsequently obtained in young pigs [4].

The endothelium presents a substantial resistance to macromolecule transport into the wall. Possible routes across it include vesicular pathways and intercellular junctions. Normal junctions are ≤20 nm in width so although they should allow the passage of albumin (4 × 14 nm; [5]), LDL (Stokes-Einstein diameter 23 nm; [6]) is unlikely to enter the arterial intima via this route. The cell turnover leaky junction hypothesis of Weinbaum et al. [7] proposes that intercellular junctions temporarily widen when endothelial cells divide or die, leading to foci of high permeability for macromolecules such as LDL. Typical dimensions of these leaky junctions were estimated by Chen et al. [8] to be 80–1330 nm during mitosis and 15–1000 nm for dead or dying cells. The hypothesis is consistent with earlier observations that mitosis rates are higher in

* Corresponding author. Department of Bioengineering, Imperial College London, London, SW7 2AZ, UK.
E-mail address: p.weinberg@imperial.ac.uk (P.D. Weinberg).

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areas of Evans' Blue dye-albumin (EBA) uptake by the pig aortic wall [9].

Stemerman et al. [10] observed that uptake of horseradish peroxidase (HRP, Stokes-Einstein diameter 6.4 nm; [11]) by the rabbit aorta occurred in distinct spots (here termed "hotspots") 1 min after administration. Concentrations of LDL in the HRP hotspots were up to 47 times greater than in HRP-free arterial tissue, suggesting that LDL had crossed the endothelium via routes also taken by the much smaller HRP. Subsequently, Lin et al. showed that 99% of mitotic cells [12] and 63% of dead or dying cells [13] were associated with EBA hotspots. Furthermore, these mitotic and dead or dying cells accounted for 30% and 37%, respectively, of all EBA hotspots. (EBA is particularly suitable for hotspot studies because the EBD binds preferentially to elastin and collagen on entering the wall [14], thus leaving a permanent record of its site of entry rather than dispersing in the underlying tissue.) A study using ^{125}I-LDL reported that 80% of mitotic cells were associated with LDL hotspots, and that mitotic cells were present in 45% of hotspots [15]. However, a similar study by Truskey et al. [16] found a weaker association, approximately 25% of leakage sites being associated with mitosis, and a further study from the same group [17] found that only 8% of LDL hotspots were associated with endothelial cells in S phase. Hotspots occur at a particularly high frequency downstream of branch points [18,19].

It has emerged that the pattern of lesions in cholesterol-fed rabbits changes with age: although lesions occur downstream of aortic side branches in young animals, they occur more frequently at the sides and upstream of branches at later ages [20,21]. A similar switch is seen in the spontaneous lesions that occasionally affect rabbit aortas [22] and it parallels a comparable change with age in human aortas [23–25]. Furthermore, the pattern of macromolecule uptake by the rabbit aortic wall also changes with age, and in the same way [26–30]. This concordance is important because it resolves inconsistencies between patterns of lesions and uptake that were apparent between earlier studies and provides additional evidence for a key role of transport properties in atherogenesis [31]. However, the effect of age on the pattern of hotspots and its dependence on mitosis has not been examined.

The present study investigated the hypothesis that patterns of EBA hotspots and mitosis change with age in rabbits in the same way as lesions, and examined the proportion of total uptake that occurs via hotspots. The study employed a range of technical innovations: EBD was detected from its fluorescence rather than its absorbance in order to increase sensitivity; *en face* confocal microscopy was used in conjunction with a maximum intensity projection in order to preferentially detect EBD bound to elastin in the inner wall; hotspots were identified and their area and intensity were quantified by an objective, automated method of image segmentation; mitosis occurring over several days rather than solely at the time of death was identified by adding a DNA synthesis marker to drinking water; and comparisons between patterns of hotspots, mitosis and lesions were made by rigorous statistical methods [32] that account for autocorrelation and avoid assumptions of linearity.

2. Materials and methods

Methods and their validation are given in the on-line Supplementary data. All animal procedures complied with the Animals (Scientific Procedures) Act 1986 and were approved by the Local Ethical Review Panel of the University of Reading.

3. Results

3.1. Hotspots

The number of hotspots and their area, and the amount of EBD fluorescence in hotspots, outside hotspots and in both compartments combined, averaged for each grid square, are mapped for regions of aortic wall around intercostal branch ostia in immature and mature rabbits in Fig. 1. (Note that every maps in this and subsequent figures use a color bar that ranges from the lowest to the highest value for that map.) Within each age group, the patterns for all 5 metrics were broadly similar, but there was a strong effect of age: in the immature group, high values for each metric tended to occur downstream of the branch ostium whereas in the mature group they occurred in four patches located at the corners of the map.

At both ages, the averaged number of spots reached a maximum of approximately one per grid square (area 56,644 μm^2). Overall, however, mature maps had significantly more spots than immature maps (mature = 49.6 ± 5.3 spots per branch; immature = 26.2 ± 3.8 spots per branch, mean ± SEM, $p = 0.0005$, Student's t-test). For each branch, the area mapped was 2.4 × 2.4 mm; we have previously shown that endothelial cells areas, measured in 600 × 600 μm regions upstream and downstream of rabbit intercostal branches, are: immature upstream, 336 ± 19 (μm^2, mean ± SEM); immature downstream, 359 ± 8; mature upstream, 304 ± 18; and mature downstream, 453 ± 32 [33].

The peak value for the average area of hotspots within each grid square differed substantially, being around 250 pixels in immature animals but only 80 in mature ones. However, equivalent mean rather than peak values for spot areas at the two ages were 139.6 ± 8.2 pixels for immature and 125.2 ± 2.6 for mature rabbits, and were not significantly different. At both ages, uptake in those areas of the map defined as hotspots was approximately an order of magnitude lower than uptake in the larger portion of the map that fell below the hotspot intensity threshold, and non-hotspot uptake therefore accounted for the great majority of total uptake.

3.2. Mitosis

One immature rabbit had more than 8 times as many BrdU-positive nuclei as the average for the group, and nearly 5 times as many as the rabbit with the next highest number. It was determined to be an outlier by Chauvenet's criterion and excluded from all further analysis.

Maps of the mean number of BrdU-positive nuclei in each grid square are shown for immature and mature animals in Fig. 2. The data were more scattered than the hotspot metrics and although the maps suggest that there were slightly more labeled nuclei downstream of the branch in immature animals, and slightly more upstream of the branch in mature ones, these differences were not statistically significant (immature, $p = 0.15$; mature, $p = 0.14$; 1-tailed t-test comparing the top and bottom halves of the maps).

3.3. Lesions

Maps of the frequency of lesion occurrence around intercostal branches in immature and mature cholesterol-fed rabbits are also shown in Fig. 2. As discussed in detail elsewhere [21], the highest frequencies are seen in an arrowhead-shaped region surrounding the downstream half of the ostium in immature branches, and at the lateral margins of branches in mature animals.