Supplemental Material

Morphological evidence for a change in the pattern of aortic wall shear stress with age

Andrew R. Bond, Saadia Iftikhar, Anil A. Bharath and Peter D. Weinberg

Methods

Animals

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 or Imperial College London. Immature (6-7 weeks old) or mature (>6 months old) male New Zealand White rabbits (Interfauna strain; Harlan UK) were fed a standard laboratory diet without supplementary fat. They were given heparin (2000 USP units) and an overdose of pentobarbitone (approx. 200 mg/kg) iv before a midline thoracotomy and laparotomy were performed to allow retrograde insertion of an aortic cannula at the level of the diaphragm.

Fixation and silver staining

For studies of nuclear morphology alone, the descending thoracic aortas of 4 immature and 4 mature rabbits were fixed via the cannula with 10% formalin or 10% formalin + 4% glutaraldehyde (Sigma) at physiological pressure for 30 minutes. For studies involving measurement of whole cell morphology, an additional 3 immature and 3 mature rabbits were treated in the same way except that fixation was interrupted after 90 s to allow perfusion with 20 mL of a 0.25% w/v solution of AgNO₃ [1].

Aortas were excised and stored in the same fixative at least overnight.

Häutchen preparation

Fixative was removed by placing the aortas in phosphate buffered saline (PBS) for ≥1 h and adventitial tissue was dissected away. The intercostal arteries were cut so that
only short stubs remained. The aortas were then dehydrated in ascending concentrations of ethanol, left in 100% ethanol for ≥16 h and cut perpendicular to their longitudinal axis to give rings containing pairs of intercostal branches. Each ring was cut longitudinally along its ventral aspect, opened out and pressed, endothelial surface down, on to a strip of double-sided adhesive tape (Scotch pressure sensitive, 3M) that had been adhered to a microscope slide. The tissue was air dried for 5 minutes to remove excess alcohol and immersed in 10% v/v glycerol for 8-10 minutes. The subendothelial intima and media were then peeled away, leaving the endothelium attached to the tape. Occasional patches of smooth muscle cells that remained were removed using fine forceps. Further details are given elsewhere [2].

Nuclear staining

Häutchen preparations were rinsed to remove glycerol, and were immersed in 0.2% Triton X-100 (Sigma, 30 s) followed by PBS (15 s) before being incubated in RNase (Sigma, 10 minutes at 37 C) to remove cytoplasmic nucleic acids. They were then rinsed in PBS, stained for nucleic acid with propidium iodide (PI, Molecular Probes, 1 mg/mL), rinsed again and mounted in Fluorsave (Calbiochem).

Imaging

PI staining was imaged by epifluorescence microscopy using a x20/0.45 NA objective, a standard rhodamine filter set with 546 nm excitation (Zeiss) and a cooled CCD camera (Apogee) with Kodak KAF 1600 chip, driven by Maxim DL software (Diffraction Limited, Canada). Silver staining was imaged using conventional transmitted light; it was occasionally necessary to combine images taken from different focal planes, as previously described [3]. Since the area of the Häutchen preparation was larger than the field of view, images were acquired in a grid-like pattern, ensuring each image overlapped slightly with its neighbours.

Processing and analysis of images of nuclei

Using Photoshop software (Version 7.0, Adobe Systems Incorporated), the range of pixel intensity values was compressed, noise was removed by median filtering, the individual images for each Häutchen preparation were stitched together (Fig 1a) and histological artefacts were removed. A sharpen filter and a high-pass filter (10-pixel radius) were applied to improve contrast and remove variation in intensity caused by uneven illumination and sensitivity across the microscope field of view (Fig 1b).

Using ImageTool software (Version 3.0, UTHSCSA), montages were binarised and the area, perimeter, major axis length ("length"), minor axis length ("width"), angle of
orientation, elongation ("length-to-width ratio") and the location of the centre of mass of objects within the image were automatically collected.

Data analysis

Results were transferred to a spreadsheet (Excel 2003, Microsoft) and filtered by area and perimeter to remove objects that were too small or large to represent nuclei. The former objects were typically artefacts due to image noise or small fluorescent particles, and the latter were typically nuclei that were too close to their neighbours to be resolved as discrete objects.

Nuclear dimensions were then averaged over 100µm x 100µm square regions of the Häutchen preparation. Squares at the edges of the Häutchen preparation and at the margins of branch ostia contained data from truncated nuclei and were removed. Since it was impossible to obtain perfect alignment of the longitudinal axis of each aortic segment with the longitudinal axis of the camera, individual nuclear orientations were redefined relative to the average orientation of all the nuclei within the Häutchen preparation, rather than to the image axis. Data for 36 immature branches (4-12 per animal) and for 38 mature branches (4-15 per animal) were combined within each age group using the centre of mass of the ostium as a datum. Maps of the object dimensions in different regions were created by shading cells on a spreadsheet that had the same topography as the area analysed. The custom macros used for these procedures are presented elsewhere [2].

Processing and analysis of images of cells

Cell boundaries were identified manually and also by a novel automated method. In the manual method, the borders were traced in a transparent layer overlying the montage, using Photoshop software and a graphics tablet (Volito2, Wacom). Selected regions could then be opened in ImageTool, binarised, and analysed automatically as described for nuclei. The resulting cellular length-to-width ratios and orientations were averaged over various near-branch regions described below.

To detect boundaries automatically, a new technique based on the class of supervised machine-learning algorithms known as Support Vector Machines (SVMs) [4] was developed. Each pixel in the image was characterised by 35 features of itself and its 8 nearest neighbours. Nine of the extracted features were the pixel intensities, 18 defined the complex orientation dominance field used to indicate anisotropy [5] and 8 were statistical values generated from the 9 intensity values (such as mean, median, second order moments, etc.). A training set was generated by manually classifying pixels as boundary or non-boundary in a 60 x 60 pixel region of one image of a silver-stained Häutchen preparation. An SVM training algorithm used this data set to construct models for automatically ascribing pixels to the two categories according to their 35-dimensional feature vectors. The algorithm was written in Matlab R2009b using the LIBSVM package of Chang and Lin [6]. The performance of four trained SVM kernels was evaluated on six images also containing manually traced boundaries. The
fraction of the 3.9 million pixels correctly classified by the best-performing kernel ranged from 81% to 93%.

This kernel was then applied to images of 8 regions around each of 7 branches from 3 different animals. The regions were those defined by Al-Musawi et al. [7]. The algorithm detected boundaries in 43 of the 56 images. The remaining 13 images were heavily corrupted or noisy and the boundaries were difficult to discern even by eye. Once boundary pixels had been identified, standard shape analysis techniques were applied to determine the orientation and elongation of the cells. (Further details of the SVM and shape analysis methods will be provided elsewhere).

Statistics
For Fig 2, differences in length-to-width ratios and in orientations between grid squares were assessed by Fisher’s Least Significant Difference method, in which the smallest difference that would be significant between a pair-wise comparison is calculated, and can then be used to examine particular pairs of interest [8]. This approach avoids type 1 errors when making multiple comparisons. The least significant difference depends on n, which varies between grid squares. The 5th percentile of n (i.e. the value equalled or exceeded in 95% of grid squares) was used.

For Fig 3, differences between upstream and downstream regions were statistically assessed within each age group by taking the average of measurements in the upstream region and the average in the downstream region for each branch, and then comparing these averages across all branches using Student’s paired t-test. (The same method was used for assessing the difference in cell rather than nuclear morphology in upstream and downstream regions). Additionally, within the upstream and the downstream region, each bar in the histogram was compared with its neighbouring bars, again using Student’s paired t-test. Differences between rather than within ages were assessed by Student’s unpaired t-test.

Associations between nuclear and cellular elongation and between nuclear and cellular orientation were assessed by correlation coefficient.

The criterion of statistical significance was p<0.05.
Results

Fig I. Maps showing aortic endothelial nuclear orientation around (A) 36 immature and (B) 38 mature intercostal branch ostia. The maps represent an en face view of the endothelium, each small square being equivalent to 100 x 100 µm, with mean aortic flow from top to bottom. Mean nuclear orientation within each square (defined relative to the average nuclear alignment for the segment) is represented graphically. White squares are areas in and around the ostium where data were available from <15 branches. Arrowheads have not been placed on the lines since nuclear staining with PI cannot indicate which is the upstream and which the downstream end of the nucleus; the direction of flow along the line is a matter of conjecture.
Fig II. Cell-by-cell comparison of cell and nuclear morphology. Cell boundaries were identified manually. Points show the length-to-width ratio (A) and orientation (B) of 350 cells and their nuclei from 3 rabbits. A straight line through the origin would represent perfect agreement between cell and nuclear properties. Note that for length-to-width ratios, co-ordinate 1,1 (corresponding to a circular cell and nucleus) represents the origin.
Fig III. Region-by-region comparison of cell and nuclear morphology. Cell boundaries were identified by an automated technique. Points show the mean ± 1 SEM of length-to-width ratio (A) and orientation (B) in 8 regions around 7 branch ostia from 3 rabbits. The regions were those defined by Al-Musawi et al. [7]. A straight line through the
origin would represent perfect agreement between cell and nuclear properties. Note that for length-to-width ratios, co-ordinate 1,1 (corresponding to a circular cell and nucleus) represents the origin. The apparent absence of SEMs for some points arises where the error bar lies within the area of the marker. For the two outliers in the plot of length-to-width ratios, only 14 and 17 cell boundaries could be identified, whereas all other points have n≥30.

References


