Morphological Evidence for a Change in the Pattern of Aortic Wall Shear Stress With Age

Andrew R. Bond, Saadia Iftekhar, Anil A. Bharath and Peter D. Weinberg
Arterioscler Thromb Vasc Biol 2011;31;543-550; originally published online Jan 4, 2011;
DOI: 10.1161/ATVBAHA.110.219683
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.
7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online
ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/cgi/content/full/31/3/543

Data Supplement (unedited) at:
http://atvb.ahajournals.org/cgi/content/full/ATVBAHA.110.219683/DC1

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at
http://atvb.ahajournals.org/subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax: 410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/reprints
Morphological Evidence for a Change in the Pattern of Aortic Wall Shear Stress With Age

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

Objective—The distribution of atherosclerosis around branch sites changes with age in human and rabbit aortas. We tested whether that reflects a change in the pattern of wall shear stress by examining shear-dependent morphological features of endothelial cells.

Methods and Results—Endothelial cells and their nuclei align and elongate with applied shear. These parameters were examined in the descending thoracic aorta of immature and mature rabbits. The use of Häutchen preparations, fluorescent stains, and automated image analysis allowed nuclear morphology to be mapped reliably at high resolution over large areas. Cells and their nuclei were most elongated downstream of branch ostia in immature aortas but upstream of them in mature aortas. Elongation was generally greater in mature animals, and nuclei aligned toward the ostia more in these animals, consistent with a greater flow into the branch. Morphology away from branches was indicative of helical flow in the aorta, with greatest shear on the dorsal wall, at both ages.

Conclusion—The data are consistent with age-related changes in the pattern of shear around aortic branches. Maps of nuclear elongation closely resembled maps of lesion frequency. The association was positive, implying that lesions occur at sites of high shear stress at both ages. (Arterioscler Thromb Vasc Biol. 2011;31:543-550.)

Key Words: atherosclerosis ■ blood flow ■ endothelium ■ shear stress

The prevalence of atherosclerosis varies greatly from one region of the arterial system to another. This patchy distribution is particularly striking near branch points and in curved vessels, consistent with the development of disease depending on mechanical factors. The current consensus is that lesions occur most frequently where blood flow exerts a low, oscillatory shear stress on the wall. We have drawn attention to age-related changes in the pattern of lesions around branch ostia in the human aorta, and we have demonstrated similar changes in the pattern of spontaneous and diet-induced lesions in rabbits. These changes could reflect alteration of the mechanical stresses acting on the wall or alteration of the relationship between mechanical stress and lesion development; the latter would imply that the low shear stress hypothesis cannot be valid at all ages.

Blood flow velocity near the wall cannot be measured in vivo with sufficient spatial resolution to determine whether the pattern of wall shear stress around branch points changes with age. However, local wall shear stress can be inferred from endothelial morphology. In vitro studies have shown that endothelial cells and their nuclei align with the predominant flow direction and elongate with increasing shear stress. This relationship also seems to hold in vivo: patterns of cell morphology seen near branches correlate with the flow visualized in models of the branches, and if flow or the orientation of the endothelium is altered, cells and their nuclei adjust their shape and alignment accordingly.

In a preliminary study using endothelial morphology to determine whether the pattern of shear stress near branches changes with age, we measured nuclear orientation and elongation around the origins of intercostal arteries in aortas from immature and mature rabbits. Changes with age in the pattern of nuclear elongation, implying changes in mechanical stresses, were observed. However, morphology was measured manually, restricting the number of nuclei that could be assessed. Nuclear elongation therefore had to be averaged over areas that were too large to permit a direct comparison with maps of lesion prevalence, and the patterns of morphology near branches could not be related to the patterns occurring over the aorta as a whole. Furthermore, morphology was assessed from en face views of intact aortic segments that had their 3-dimensional geometry preserved, meaning that foreshortening of nuclei could have occurred in areas where the wall curved into the branch.

In the present study, we developed and used methods that overcome these limitations. Endothelium was stripped from the aorta and adhered to a flat surface, and different staining techniques gave cleaner, more even images of higher contrast; these changes allowed automated analysis of average nuclear dimensions with high spatial resolution over large...
areas and eliminated the problem of foreshortening. The study confirmed that the pattern of nuclear morphology changes with age and also demonstrated agreement between patterns of nuclear morphology and the morphology of whole endothelial cells. It also revealed greater complexity in these patterns (and by implication, in the flow) than previously observed, showed that the branches modify larger-scale trends in morphology that suggest the presence of secondary flows in the descending thoracic aorta (with highest shear on the dorsal wall), and permitted the demonstration of strong correlations with maps of lesion prevalence at different ages. Although the data were consistent with age-related changes in the pattern of mechanical stresses, they did not support the low shear hypothesis in either immature or mature aortas. A preliminary report of this study has been published.

**Methods**

Full details are given in the Supplemental Methods, available online at http://atvb.ahajournals.org.

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. Descending thoracic aortas of immature (6 to 7 weeks, n=7) and mature (>6 months, n=7) male New Zealand White rabbits (Inter-fauna strain, Harlan UK) were fixed in situ at physiological pressure. For studies involving measurement of whole cell morphology, fixation was interrupted after 90 seconds to allow perfusion with a solution of silver nitrate. Aortas were dehydrated in ethanol and cut perpendicular to their longitudinal axis to give rings containing pairs of intercostal branches. Each ring was cut longitudinally along its ventral aspect and pressed, endothelial surface down, onto a strip of double-sided adhesive tape adhered to a microscope slide. The tissue was air dried and immersed in glycerol before the subendothelial ventral aspect and pressed, endothelial surface down, onto a strip of double-sided adhesive tape adhered to a microscope slide. The tissue was air dried and immersed in glycerol before the subendothelial intima and media were peeled away, leaving the endothelium attached to the tape. Nuclei were stained with propidium iodide after treatment with Triton X-100 and RNase.

Propidium iodide staining was imaged by epifluorescence microscopy. Because the area of the Häutchen preparation was larger than the field of view, a montage of images in a grid-like pattern was created (Figure 1A and 1B). Using ImageTool software (version 3.0, University of Texas Health Science Center at San Antonio), the area, perimeter, major axis length, length, minor axis length (width), angle of orientation, elongation (length-to-width ratio), and location of the center of mass of objects within the image were automatically collected.

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. Because 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.

Nuclear dimensions were averaged over 100×100-μm square regions of the Häutchen preparation. Maps of the dimensions were created by shading cells on a spreadsheet. For the areas around branches, data were combined within each age group using the center of mass of the ostium as a datum.

Silver staining was imaged using conventional transmitted light. Cell boundaries were identified manually and also by a novel automated method. In the manual method, borders were traced using Photoshop software and a graphics tablet (Volito2, Wacom) (Figure 1C). Selected regions could then be opened in ImageTool, analyzed automatically, and filtered by size as described for nuclei. The automated method was based on the class of supervised machine-learning algorithms known as Support Vector Machines. Each pixel in the image was characterized by 35 features of itself and its 8 nearest neighbors. A training set was generated by manually classifying pixels as boundary or nonboundary in 1 image of a silver-stained Häutchen preparation. A Support Vector Machines training algorithm used this data set to construct a kernel for automatically ascribing pixels to the 2 categories according to their 35-dimensional feature vectors. Cellular length-to-width ratios near branches were averaged over various regions described below.

**Results**

**Nuclear Length-to-Width Ratios Around Branch Ostia**

Maps of nuclear elongation around intercostal branch ostia are shown for immature and mature rabbits in Figure 2A and 2B, respectively. Differences in elongation greater than 0.16 (immature) and 0.29 (mature) are significant (P<0.05). In immature aortas, length-to-width ratios were lowest at the sides and upstream of the ostium and were greatest in a region displaced slightly downstream of the branch. In mature aortas, length-to-width ratios were again relatively low in regions at the sides and upstream of the ostium. Contrary to the patterns seen in immature animals, however, values were also relatively low in a triangular area downstream of the branch and were highest in a band displaced slightly upstream of the branch, especially away from the longitudinal midline. Length-to-width ratios in mature rabbits were generally greater than those in immature ones. The spatial and age-related differences in length-to-width ratios reflected reciprocal changes in length and width rather than changes in 1 of
these dimensions alone and were not obviously correlated with nuclear area (data not shown).

Length-to-width ratios along the longitudinal centerline through the branch (corresponding to the central column of squares in the maps) are shown more precisely and with SEMs for immature and mature aortas in Figure 3A. In both immature and mature aortas, length-to-width ratios decreased as the ostium was approached from its upstream side. In immature aortas, length-to-width ratios increased in a region displaced slightly downstream of the ostium, compared with areas further from the branch, but in mature aortas a decrease occurred. For regions within 500 μm (=1 branch diameter) of the ostium, downstream values were significantly greater than upstream values in immature rabbits (P<0.001), but the opposite trend occurred in mature rabbits (P=0.018). For the full 1000-μm range, the difference remained significant in the immature rabbits (P<0.001) but not in the mature ones (P=0.165). Within the immature and the mature age groups, each bar in the histogram was compared with its neighboring bars; significant differences (which generally occurred near the ostium) are indicated by lines in Figure 3A.

Values were higher in mature aortas than in immature aortas at every location. Adjustment was made for this trend before statistical tests were performed, so that differences in the pattern of elongation with age could be assessed: the mean elongation was calculated separately for the immature and the mature maps (Figure 2A and 2B), and the difference between the means (0.75) was then subtracted from the mature values in all regions shown in Figure 3A. After this adjustment, statistical significance was obtained for the squares 201 to 500 μm downstream of the branch (P<0.001), where ratios were higher in immature animals.

Nuclear Orientations Around Branch Ostia
Maps of nuclear orientation around intercostal branch ostia are shown for immature and mature rabbits in Figure 2C and 2D and in Supplemental Figure Ia and Ib. In Figure 2C and 2D, angles are color coded. In Supplemental Figure Ia and Ib, angles are represented graphically; the latter gives a better impression of large angles but cannot show the small angles or the small differences between angles that are apparent in Figure 2C and 2D. Differences in orientation greater than 5.5° (immature) and 4.2° (mature) are significant (P<0.05).

In the immature and the mature animals, nuclei at the upstream left and right margins of the ostium were oriented with their long axes deviating toward the branch. These deviations averaged >20° in many squares and >40° in some. (They can be seen in Figure 1B.) The trend was more pronounced in the mature than the immature animals: values were more negative on the anatomic right side, and the areas...
over which the deviations occurred extended further upstream.

Orientations along an aortic circumference corresponding to the 11th row of squares from the top of the maps in Figure 2C and 2D (ie, the most upstream row passing through the ostium) are shown more precisely and with SEMs for immature and mature aortas in Figure 3B. Values on the left were significantly different from those on the right in both age groups (\(P < 0.001\)). Within the immature and the mature age groups, each bar in the histogram was compared with its neighboring bars; significant differences (which occurred more frequently in the mature group) are indicated by lines in Figure 3B. Differences in orientation between age groups, rather than within them, were statistically significant in the squares within 200 \(\mu m\) of the ostium on the anatomic right side (\(P \leq 0.0012\)), and in the square 101 to 200 \(\mu m\) from the ostium on the anatomic left side (\(P = 0.0191\)); in each case, the magnitude of the angle was greater in the mature group, consistent with the trend noted above.

**Comparison Between Nuclear and Cellular Morphology Near Branches**

Three sets of comparisons were made. In the first, the shapes of silver-stained endothelial cell boundaries were manually traced in 600\(\times\)600 \(\mu m\) regions upstream and downstream of intercostal branch ostia. The length-to-width ratios of the cells in immature rabbits were 7.16 \(\pm 0.31\) (mean \(\pm 1\) SEM) upstream and 7.75 \(\pm 0.16\) downstream (8\% higher downstream; \(P = 0.09\), \(n = 7\) branches), whereas in mature rabbits they were 9.22 \(\pm 0.58\) upstream and 8.72 \(\pm 0.67\) downstream (6\% higher upstream; \(P = 0.35\), \(n = 7\) branches). Nuclear length-to-width ratios were analyzed in similar-sized (500\(\times\)500 \(\mu m\)) regions. In immature rabbits they were 2.42 \(\pm 0.025\) upstream and 2.69 \(\pm 0.039\) downstream (11\% higher downstream, \(P < 0.001\), \(n = 34\) branches), whereas in mature rabbits they were 3.32 \(\pm 0.074\) upstream and 3.19 \(\pm 0.074\) downstream (4\% higher upstream, \(P = 0.032\), \(n = 38\)). Thus, the percentage differences were essentially the same for cell and nuclear length-to-width ratios (although
only the latter attained statistical significance, presumably because of the smaller sample sizes attainable for cell boundaries).

In the second, a direct cell-by-cell comparison was made of nuclear and manually traced cell morphology in Häutchen preparations from 2 immature aortas and 1 mature aorta stained with both silver and propidium iodide. There was more scatter in the plot of nuclear versus cellular length-to-width ratio than the plot of nuclear versus cellular orientation ($r^2 = 0.38$ versus $r^2 = 0.88$; Supplemental Figure IIa and IIb), but there was a highly significant positive correlation between cells and their nuclei for both these measures, as well as for length, width, and area (all $P<0.005$, $n=350$ cells).

In the third, nuclear morphology was compared with cell morphology obtained using the automated technique. Data were obtained for 8 600-μm regions around each of 7 branches. There was a good agreement between the mean nuclear and mean cellular measurements in each region (see Supplemental Figure IIIa and IVb), the scatter once again being less for orientation than for alignment. Comparing the data on a cell-by-cell basis gave $r^2$ values of 0.34 for length-to-width ratio and 0.81 for orientation, essentially identical to the values obtained by manual analysis.

**Nuclear Morphology Away From Branches**

The periostial regions described above occupied only part of the area of each Häutchen preparation. Figure 4 shows maps of nuclear elongation and nuclear orientation in whole Häutchen preparations from an immature and a mature aorta. (These were the aortas in each age group that yielded the largest number of preparations.) Figure 5 shows nuclear elongation and orientation as a function of circumferential location. Data, obtained from the 3 rabbits in each age group yielding ≥4 Häutchen preparations, were averaged along the length of the aorta. The near-branch regions mapped in Figure 2 were excluded from this analysis (and from Figure 4), as nuclear morphology was locally altered in these areas and would have countered the larger-scale trends.

For every aorta, longitudinal features were seen in the maps of nuclear elongation; that is, areas of high or low length-to-width ratio occurred as stripes running lengthwise along the aorta rather than as random patches. In 3 of the 4 immature aortas, there was a clear trend for nuclei to be more elongated in a broad dorsal stripe. In the mature aortas, this trend was seen in some preparations, but in others the stripes were narrower and not confined to a single circumferential region. Nevertheless, Figure 5A shows that, when averaged along the length of all the preparations, length-to-width ratios were elevated in the dorsal region in mature as well as immature aortas, although SEMs were larger in the older group. The maps and Figure 5A also demonstrate that the tendency for length-to-width ratios to be higher in mature rabbits holds away from branches, as well as near them.

Nuclear orientations were more negative on the anatomic left side and more positive on anatomic right side in many segments at both ages. There was also a horizontal banding in many segments. Although this banding could be an artifact caused by buckling of the tissue, the effect was not seen in the maps of elongation. Both trends can be seen in Figure 4C and 4D. Figure 5B shows the circumferential pattern more pre-
Circumferential variation in nuclear elongation (A) and orientation (B), averaged in the axial direction. Means±1 SEM are shown for 3 immature and 3 mature rabbits. Negative distances from the dorsal midline correspond to the anatomic right of the aorta, and positive values correspond to the left. Graphs were truncated at the distance from the dorsal midline where data ceased to be available for all aortas (4.3 mm, roughly equivalent to the width of the top Häutchen preparation in Figure 4B and 4D). Despite this truncation, values are the same at the left and right ends in both graphs, as required if the 2 ends of the unwrapped preparations represent regions close to the ventral midline in the intact aorta.

Figure 5. Circumferential variation in nuclear elongation (A) and orientation (B), averaged in the axial direction. Means±1 SEM are shown for 3 immature and 3 mature rabbits. Negative distances from the dorsal midline correspond to the anatomic right of the aorta, and positive values correspond to the left. Graphs were truncated at the distance from the dorsal midline where data ceased to be available for all aortas (4.3 mm, roughly equivalent to the width of the top Häutchen preparation in Figure 4B and 4D). Despite this truncation, values are the same at the left and right ends in both graphs, as required if the 2 ends of the unwrapped preparations represent regions close to the ventral midline in the intact aorta.

There was a tendency for nuclear elongation and orientation to vary down the aorta (for example, elongation was often greater in the more proximal segments, and the pattern of orientation often reversed near the middle of the thoracic segment), but many more aortas would be required to assess the consistency of these trends rigorously.

Discussion
Nuclear morphology has been used less frequently than the morphology of the entire endothelial cell to assess hemodynamic shear stress, but it has a number of advantages. Nuclei can be stained reliably and affordably over large areas, whereas cell boundaries require staining either by silver, which is temperamental, or by antibodies raised against junctional proteins, which are costly. Furthermore, nuclei are discrete objects and hence easy to recognize by simple automated techniques, whereas cell boundaries constitute a network of lines, each of which has to be attributed to 2 cells, a process that is complicated by the existence of breaks in stained boundaries. In earlier studies, nuclei were stained with hematoxylin, but the use of fluorescent dyes gives greater and more even contrast, again facilitating the automated analysis that is essential to creating high resolution maps over large areas.

We used fluorescent nuclear stains in a previous study. However, whole aortas were viewed en face, so manual analysis was still required to distinguish between endothelial and smooth muscle nuclei. Here, we used Häutchen preparations to obviate this problem. The technique also avoided the foreshortening of nuclei that can occur when applying en face methods to regions of high curvature, although we cannot discount the possibility that nuclear orientations were altered close to the branch when forcing the vessel onto a flat surface. Häutchen preparations have been used in many previous studies. We modified the technique of Hirsch et al.24 to enable reliable production of preparations up to 1 cm in length and encompassing the whole circumference of the aorta; each preparation contained >10⁶ endothelial cells and was uncontaminated by smooth muscle cells. It was possible to analyze the whole descending thoracic aorta (≈1 million endothelial cells) in this way. These methods might be of value for studying spatial variation in other endothelial properties (eg, gene expression, assessed using fluorescence in situ hybridization).

Whole endothelial cells have been shown in several well-controlled in vitro studies to align and elongate with flow; equivalent data have recently been obtained for endothelial cell nuclei.11 We have reviewed elsewhere evidence that the time-averaged magnitude and direction of hemodynamic wall shear stress are likely to be the dominant determinants of the morphology of the whole cell in vivo; shear has a greater effect than cyclic strain, and mean shear dominates over unsteady components. In the present study, we obtained evidence for parallel behavior of endothelial cells and their nuclei, implying that the same assumption can be made for cell nuclei. However, the relationship was less strong for length-to-width ratios than for alignment, so other influences on elongation, such as pulsatility or wall stiffness (which varies around branches and changes with age) cannot be entirely dismissed. Surrogate indicators of shear are required because it is not currently feasible to make high-resolution measurements of near-wall flow or to obtain the precise boundary conditions required for unequivocal computational simulations; for the same reason, it remains unproven that nuclear elongation is an accurate surrogate at all arterial locations and ages, as assumed here.

We consider first the nuclear length-to-width ratios near branches (Figures 2A, 2B, and 3A). The most striking features in immature aortas were low values at the sides and
upstream of the ostium and high values downstream of it. In mature aortas, low rather than high values occurred downstream; the highest values were seen in a band upstream of the branch. The relationship between nuclear elongation and shear has not been quantified in vitro for rabbit endothelial cells, but our recent computational fluid dynamic studies of steady flow in simplified geometries suggest that shear varies by a factor of approximately 2 in near-branch regions.

Unlike the maps of nuclear elongation, the maps of nuclear orientation around immature and mature branches (Figure 2C and 2D) were broadly similar, suggesting that the pattern of flow direction near the wall stayed approximately constant with age. The only change concerned the tendency for nuclei in regions near the upstream left and upstream right margins of the ostium to deviate toward the branch; this trend was greater in mature than immature aortas (Figure 3B). According to our computational studies, this observation is consistent with mature animals having higher flow rates into the side branch, a change that would also elevate wall shear stress upstream of the branch. However, there is no evidence for a change with age in the impedance of the intercostal vasculature, and there are other plausible explanations, such as higher Reynolds numbers, altered branch geometry, or changes to flow characteristics in the descending aorta as a whole.

Patterns of nuclear morphology and, by implication, near wall flow in the descending thoracic aorta as a whole changed much less than the patterns near branches. There was a general tendency for nuclear elongation to be greater in mature animals, which has been extensively discussed elsewhere; here we comment only on the relative patterns. Longitudinal features were seen in maps of nuclear elongation for both age groups; there was a tendency in immature and mature animals for higher length-to-width ratios to occur in a broad dorsal stripe, but greater variability in this trend was seen in the mature animals. A dorsal stripe of high shear has been detected in studies using physical models of the aorta; it was abolished by preventing flow into the large vessels branching off the aortic arch. The longitudinal features and the observation that nuclear orientations were more negative on the anatomic left side and more positive on anatomic right side of many aortic segments are consistent with the presence of 2 vortices, 1 on the right side rotating anticlockwise when viewed from the upstream end of the segment, and 1 on the left rotating clockwise. There are parallels with longitudinal features in maps of aortic endothelial permeability and with the near-axial alignment of human aortic fatty streaks; some but not all of the published maps of lesion prevalence in the descending rabbit thoracic aorta appear to show a predilection for the dorsal wall.

In the present study, nuclear morphology was averaged in patches of endothelium that were 30-fold smaller than those assessed in our preliminary work. This improvement in spatial resolution allows a direct comparison with existing high-resolution maps of lesion frequency around branches. Figure 6 shows maps of the mild lipid deposition that is occasionally seen around intercostal branch ostia in normocholesterolemic weanling and aged rabbits. There is a good spatial concordance between Figure 6 and the maps of nuclear elongation in Figure 2, remarkably so for the mature animals. (Lesions in immature hypercholesterolemic rabbits show the same pattern as Figure 6A, but lesions in mature hypercholesterolemic rabbits have a somewhat different pattern than the one in Figure 6B, occurring most frequently at the sides of branches.) More quantitatively, in the 600×600-μm regions considered above, lesions frequencies averaged 3.6% downstream and 0.0% upstream of immature branches, and 0.1% downstream and 0.9% upstream of mature branches; the tendency for frequencies to be greater downstream in immature animals and upstream in mature ones is similar to the trends in cell and nuclear length-to-width ratios. Importantly, the correlation is positive rather than negative; contrary to the current consensus, higher lesion frequencies are associated with greater nuclear elongation, consistent with lipid deposition occurring in regions experiencing high wall shear stress, at both ages. The relevance to human lesions, which also switch from a downstream to a lateral or upstream location around intercostal branch ostia with age, remains to be determined.

Sources of Funding

This study was funded by the British Heart Foundation.

Disclosures

None.

References

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


