Abdominal Aortic Aneurysm Rupture is not Associated with an Up-regulation of Inflammation within the Aneurysm Wall

W.R.W. Wilson a,*, J. Wills b, P.N. Furness b, I.M. Loftus c, M.M. Thompson c

Abstract  Background: The nature of the inflammatory change within ruptured AAA has not been extensively reported. The aim of this study was to compare the inflammatory response in non-ruptured and ruptured aneurysms with emphasis on the site of rupture.

Methods: Non-rupture site biopsies were taken from the anterior aneurysm sac of non-ruptured (n = 31) and ruptured AAA (n = 20). In 12 ruptured AAA, a further biopsy was taken from the rupture site. Enzyme-linked immunosorbent assay was used to quantify IL-6, IL-1beta and TNF-alpha. Quantitative immunohistochemistry was undertaken for generic lymphocytes, T-cells, and B-cells.

Results: Comparing biopsies in non-ruptured AAA versus a non-rupture site biopsy from ruptured AAA; there was no significant difference in IL-6, IL-1beta, TNF-alpha, generic lymphocytes, T-cell or B-cell content. Comparing ruptured AAA – non-rupture site with rupture site; IL-6 and TNF-alpha were unchanged. By contrast IL-1beta and lymphocytes were lower at the rupture site compared to the non-rupture site (IL-1beta 1.39 ng/mg [0.97–2.29] vs. 1.92 ng/mg [1.46–2.57], p = 0.027; generic lymphocytes 2.89% [0.51–5.51] vs. 4.73% [2.27–12.40], p = 0.018; T-cells 0.28% [0.04–1.18] vs. 0.82% [0.40–1.36], p = 0.027; B-cells 0.16% [0.04–1.14] vs. 1.30% [0.32–5.40], p = 0.021).

Conclusions: These findings suggest the biological events leading to AAA rupture may not be dependent on an up-regulation in the inflammatory process.

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Introduction

The abdominal aorta is a complex three layered structure containing supporting fibres (elastin and collagen) and cellular components of smooth muscle and mesenchymal lineage. Abdominal aortic aneurysm (AAA) formation is associated with a change in cellular composition. Cellular changes are characterised by destruction of the intima, loss of smooth muscle cells from the media, infiltration of inflammatory cells and other mesenchymal cells into the adventitia and deposition of thrombus around the expanded vessel lumen. In conjunction with these cellular changes there is a marked up-regulation of matrix metalloproteinases (MMP), a group of enzymes which degrade elastin and collagen.

Inflammation is associated with aneurysmal change in the abdominal aorta. Inflammation takes two forms; firstly, inflammatory cells (in all their facets); secondly, inflammatory mediators (interleukins and cytokines). Up-regulation of both inflammatory cells and mediators are extensively reported in aneurysm literature however, a direct analysis at the site of vessel rupture is lacking. Furthermore, whilst macrophages and generic lymphocytes are spatially orientated around neo-vessels at the rupture site, quantitative analysis of these cells fails to show an increase. Rather, generic lymphocytes may be decreased at the site of aneurysm rupture. The study presented here, observes inflammatory changes in aneurysm biopsies from three distinct sites: 1) stable non-ruptured aneurysms, 2) a non-ruptured site within the ruptured aneurysm and 3) the actual site of aortic aneurysm rupture. The objectives were to further examine lymphocyte and inflammatory mediator changes within the wall of ruptured aneurysms.

Methods

Study design

Local research ethics committee approval for the study was obtained and written consent was given by all patients. Two patient groups were studied, 31 patients with non-ruptured AAA (nrAAA) undergoing elective repair, and 20 patients with ruptured AAA (rAAA) undergoing emergency surgery (mean age; nrAAA 69 yrs +/- 4.8, rAAA 71 yrs +/- 6.7, \( p = 0.465 \)). The maximum external diameter of each non-ruptured AAA was determined from a pre-operative computed tomogram. The diameter of each ruptured AAA was measured intra-operatively (median size and IQR; nrAAA 6.5 cm (5.1–7), rAAA 8.1 cm (5.9–9.0), \( p = 0.011 \)).

Tissue collection

Non-rupture site biopsies of aortic wall were obtained intra-operatively from the surgical arteriotomy in the anterior aneurysm wall, 5 cm distal to the left renal vein, in all non-ruptured and ruptured AAA. An additional biopsy from the actual site of aneurysm rupture was taken from 12 of the ruptured AAA (the definitive site of rupture could not be determined in 8 ruptured AAA). No rupture site coincided with the non-rupture site. All biopsies were washed in saline, dissected of luminal thrombus and adipose tissue then divided in two and frozen in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin wax.

Cytokine quantification

A protein extract was prepared from the tissues by an established methodology. In brief, frozen aortic biopsies were thawed then homogenized in a volume of buffer solution directly proportional to the wet weight of the aortic biopsy. Following centrifugation (12,000 rpm, 60 min, 4 °C) the protein extracted supernatant was dialyzed (overnight, 4 °C). The concentration of each protein extract was then standardized to 1 mg/ml by spectrophotometry. Enzyme-linked immunosorbent assay kits (Amer sham Pharmacia Biotech, Buckinghamshire, UK) employing the conventional "sandwich" formats were used to quantify the cytokines; Interleukin-6 (IL-6), Interleukin-1beta (IL-1β) and Tumour Necrosis Factor-alpha (TNF-α). The final concentration of each cytokine was expressed as nanograms of target protein per milligram of protein extract.

Histological analysis

Immunohistochemistry

Anterior aneurysm wall biopsies from non-ruptured (\( n = 31 \)) and ruptured AAA (\( n = 20 \)) were analysed along with site of rupture biopsies (\( n = 12 \)). Five histological sections from each biopsy underwent immunohistochemistry for generic lymphocytes (CD-45), T-lymphocytes (CD-3) and B-lymphocytes (CD-20). Histological sections were dewaxed in xylene and rehydrated through graded alcohols to water. Antigen retrieval was achieved with 0.1% trypsin (mean age; nrAAA 69 yrs / C0 4.8, rAAA 71 yrs / C0 6.7, \( p = 0.011 \)). The sections were then washed in phosphate-buffered saline. Diaminobenzidine (DAB) was used according to the manufacturers instructions with exception of the primary antibody for 1 h at room temperature (either Monoclonal CD-45\(^{\text{TM}}\) [1:50], Monoclonal CD-3\(^{\text{TM}}\) [1:50] R&D Systems, Minneapolis, Minnesota, USA, or Monoclonal CD-20\(^{\text{TM}}\) [1:100] Novocastra Laboratories, Newcastle upon Tyne, UK), the EnVision Detection System (EnVision\(^{\text{TM}}\), Dako, Glostrup, Denmark) was used according to the manufacturers instructions with a DAB solution in 0.1% calcium chloride, buffered to pH 7.8 (37 °C, 12 min). Following addition of the primary antibody for 1 h at room temperature (either Monoclonal CD-45\(^{\text{TM}}\) [1:50], Monoclonal CD-3\(^{\text{TM}}\) [1:50] R&D Systems, Minneapolis, Minnesota, USA, or Monoclonal CD-20\(^{\text{TM}}\) [1:100] Novocastra Laboratories, Newcastle upon Tyne, UK), the EnVision Detection System (EnVision\(^{\text{TM}}\), Dako, Glostrup, Denmark) was used according to the manufacturers instructions with washes between each step using phosphate-buffered saline. Diaminobenzidine (DAB\(^{\text{TM}}\), Dako, Glostrup, Denmark) at a concentration of 0.05% was added to each slide (room temperature, 10 min). The sections were then washed in distilled water, dehydrated in absolute alcohol and mounted, from clean xylene in mounting medium. Microscopically, areas of immuno-reactivity were visualised as dark brown staining, demonstrated by the DAB being oxidised. Negative controls involved exception of the primary antibody stage. Fig. 1 demonstrates examples of haematoxylin and eosin staining of aortic wall tissue (a), generic lymphocyte immunohistochemistry (b, arrow) and negative control (c), in serial sections cut from the same tissue block (magnification \( \times 200 \)).

Histological quantification

Quantification of the immunohistochemistry (IHC) staining was undertaken using a Nikon E800 microscope with an attached JVC KYF50 3 chip color video camera linked to an Apple G3 computer through a Scion CG-7 frame grabber. Images were analysed using the freeware package ‘NIH..."
Image', automated by the use of in-house macros. In each case the aortic adventitia was distinguished. Background illumination was digitally subtracted from each image before thresholding at a pre-set level which distinguished consistently between stained and non-stained areas. A percentage area fraction of immunostaining per field area was calculated for each aneurysm biopsy from the mean of 10 images taken over 5 stained sections.

Statistical analysis

Statistical analysis used GraphPad Prism 5. The continuous variable, age was normally distributed, presented as a mean (and standard deviation) and compared using the unpaired t-test. Other continuous variables were non-normally distributed, reported as a median and interquartile range (AAA diameter, inflammatory mediator levels and cellular quantification data) and compared using the Mann–Whitney U-test or Wilcoxon Paired test. Statistical significance was assumed at the p < 0.05 level.

Results

Inflammatory mediator concentrations in non-rupture site biopsies — non-ruptured AAA versus ruptured AAA

There were no statistical differences in the median levels of IL-6, IL-1β or TNF-α within non-rupture site biopsies from ruptured and non-ruptured AAA (Table 1). These data indicated there was no global increase in inflammatory mediator concentration within the aneurysm sac prior to rupture. A further analysis was therefore performed to investigate any local differences within ruptured AAA comparing the non-rupture site with the site of rupture.

Inflammatory mediator concentrations in ruptured AAA — non-rupture site versus site of rupture

Table 2 details the biopsies of the non-rupture site and rupture site within paired ruptured AAA. There were no statistical differences in the concentrations of IL-6 and TNF-α between the paired biopsies. However, mean levels of IL-1β were lower in biopsies from the site of aortic rupture compared to the non-rupture site (IL-1β, 1.39ng/mg [0.97–2.29] vs. 1.92ng/mg [1.46–2.57], p = 0.027). These differences suggested that there was no localised up-regulation of inflammation at the actual site of aortic rupture.

Histological analysis

Morphometric quantification of cellular content

Table 1 details the quantitative morphological data taken from non-rupture site biopsies of non-ruptured and ruptured AAA. Overall there was no difference in any observed parameter with the percentage area fraction of immunostaining for generic lymphocytes (CD-45), T-lymphocytes (CD-3) and B-lymphocytes (CD-20) being comparable between the groups. Table 2 shows the quantitative morphological data from ruptured AAA comparing site of rupture versus non-rupture site biopsies from the same ruptured AAA. The percentage area fraction of CD-45, CD-3 and CD-20 were lower at the rupture site when compared to non-rupture site biopsies from the same AAA (generic lymphocytes 2.89% [0.51–5.51] vs. 4.73% [2.27–12.40], p = 0.018; T-cells 0.28% [0.04–1.18] vs. 0.82% [0.40–1.36], p = 0.027; B-cells 0.16% [0.04–1.36] vs. 1.30% [0.32–5.40], p = 0.021).

Discussion

The present investigation demonstrated no elevation of inflammatory cell and mediator concentrations between the site of aneurysm rupture and a non-rupture area in the same ruptured aneurysm or stable non-ruptured aneurysms. Indeed, rather than a simple equilibrium, lymphocytes and IL-1β appeared to be decreased at the site of rupture. The exact role of lymphocytes in aneurysm rupture is poorly understood. Our group previously demonstrated comparable macrophage and neutrophil content but reduced lymphocyte (CD-45) levels at the rupture site compared with a non-rupture site in ruptured aneurysms.4 Choke et al. detailed increased neovascularisation at the site of aneurysm rupture compared to a non-rupture site in the ruptured aneurysm. Macrophages and lymphocytes were spatially orientated around neo-vessels but there was no qualitative increase in inflammatory cell content at the rupture site.3 Kazi et al. reported increased T-cell (CD-3) and B-cell (CD-20) content in thrombus covered versus thrombus-free aneurysmal wall. However, they could not show a difference...
in macrophage infiltration. They concluded that greatest inflammation may occur at aneurysm inception but this decreases in larger aneurysms. Duftner et al. described higher levels of a specific subgroup of circulating T-cells (CD-28(-)) in smaller aneurysms than larger aneurysms. Humana et al. found greater macrophage expression in small aneurysms than larger aneurysms.

Animal models that rely on a preliminary chemical or inflammatory stimulus for aneurysm growth are typified by aneurysms smaller than larger aneurysms. Duftner et al. described inflammation may occur at aneurysm inception but this slow progression human AAA. Resolution of these issues limit what is feasible in human research. Studies report contrasting results with increased, decreased or equivocal levels of most inflammatory mediators including IL-1β and IL-6 in aneurysmal tissue compared with atherosclerotic aortic samples. The most consistently elevated factors in larger aneurysms were TNF-α and Interferon Gamma.

Comparisons of inflammatory mediator levels in anterior aneurysm sac biopsies of non-ruptured and ruptured aneurysms have been conducted. However, there are no previous reports describing the protein levels of inflammatory mediators in tissue samples from the actual site of rupture. Cheuk et al. described significantly elevated IL-6 levels in anterior wall biopsies from ruptured AAA compared with non-ruptured AAA but no difference in IL-1β, IL-8 and IL-10 concentrations. Treska et al. reported increased IL-6, IL-8 and TNF-α levels in anterior aneurysm sac biopsies of ruptured AAA compared with non-ruptured AAA but no difference in IL-1β concentrations. The present study failed to show higher IL-6 levels in anterior aneurysm sac biopsies of ruptured versus non-ruptured aneurysms since a statistical difference could not be proved. By contrast, IL-1β and TNF-α expression were comparable between sample groups. Messenger RNA levels of IL-6 and IL-8 have been assayed in rupture site tissue and demonstrated an elevation compared to non-ruptured site levels, but the failure of this current study to observe an increase in IL-6 at protein level serves to re-enforce the difference between mRNA and their biological effectors, the protein.

<table>
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<tr>
<th>Table 1</th>
<th>Comparison of inflammatory mediator and lymphocyte concentrations in non-rupture site biopsies from non-ruptured and ruptured abdominal aortic aneurysms (AAA). (Values shown as median and interquartile range, % = percentage area fraction of immunostaining per field, comparison used Mann–Whitney U-test).</th>
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<tbody>
<tr>
<td></td>
<td>Non-ruptured site biopsies</td>
</tr>
<tr>
<td><strong>Inflammatory mediators</strong></td>
<td></td>
</tr>
<tr>
<td>IL-6 (ng/mg)</td>
<td>278.5 (237.9–421.9)</td>
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<tr>
<td>IL-1β (ng/mg)</td>
<td>2.16 (1.84–2.77)</td>
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<tr>
<td>TNF-α (ng/mg)</td>
<td>2.11 (1.56–2.99)</td>
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<tr>
<td><strong>Lymphocytes</strong></td>
<td></td>
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<tr>
<td>Generic lymphocytes CD-45 (%)</td>
<td>4.46 (1.23–5.94)</td>
</tr>
<tr>
<td>T-cells CD-3 (%)</td>
<td>0.32 (0.03–0.87)</td>
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<tr>
<td>B-cells CD-20 (%)</td>
<td>0.89 (0.27–2.71)</td>
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<tr>
<th>Table 2</th>
<th>Comparison of inflammatory mediator and lymphocyte concentrations in ruptured abdominal aortic aneurysms (AAA) in paired non-rupture and ruptured site biopsies. (Values shown as median and interquartile range, % = percentage area fraction of immunostaining per field, comparison used Wilcoxon paired test, p &lt; 0.05, * = significance).</th>
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<tr>
<td></td>
<td>Non-rupture site (n = 12)</td>
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<tr>
<td><strong>Inflammatory mediators</strong></td>
<td></td>
</tr>
<tr>
<td>IL-6 (ng/mg)</td>
<td>274.3 (192.2–504.6)</td>
</tr>
<tr>
<td>IL-1β (ng/mg)</td>
<td>1.92 (1.46–2.57)</td>
</tr>
<tr>
<td>TNF-α (ng/mg)</td>
<td>2.37 (1.64–3.15)</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td></td>
</tr>
<tr>
<td>Generic lymphocytes CD-45 (%)</td>
<td>4.73 (2.27–12.40)</td>
</tr>
<tr>
<td>T-cells CD-3 (%)</td>
<td>0.82 (0.40–1.36)</td>
</tr>
<tr>
<td>B-cells CD-20 (%)</td>
<td>1.30 (0.32–5.40)</td>
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Infiltration and inflammatory cell expression. Rather we analysed whole populations of T-cells (CD-3) and B-cells (CD-20) in order to observe general inflammatory trends rather than specific changes in cellular subsets. Secondly, aneurysm specimens were only available from large AAA. All observational studies are limited by the use of end-stage disease tissue to extrapolate early biological changes. Thirdly, the difference in aneurysm size observed between the study groups may have confounded some comparisons. However, the use of paired aortic biopsies from the same patient reduced many of the disadvantages of using clinical material.

The absolute significance of our findings is unclear. This study serves only to document observations rather than offer a mechanistic explanation for these changes. When comparing a non-ruptured area to the actual site of rupture in the same aneurysm, levels of MMP8 and MMP9 are disproportionately elevated at the site of aneurysm rupture. Similar changes are noted with neovascularisation, with the site of aneurysm rupture having greater numbers of immature neo-vessels. Logically one might also suspect inflammation to be up-regulated at the site of rupture. However, our consistent failure to observe such an increase, both in this study and in previous work, may suggest otherwise.

In conclusion, this study poses many questions regarding the role of inflammation in aneurysm biology. The findings suggest the biological events leading to rupture may be independent of an up-regulation in both inflammatory cell infiltration and inflammatory cell expression.

**Conflict of interest**

None.

**Funding**

Peel medical foundation.

**References**


