Abstract. Ruptured abdominal aortic aneurysm (AAA) contributes largely to aneurysm-related morbidity and mortality. An inflammatory gene, COX-2, was found to be widely expressed in AAA. However, the involvement of COX-2 metabolites and other inflammatory mediators in the disease and particularly in AAA rupture still needs elucidation. The purpose of the present study was to evaluate the secretion of inflammatory mediators and the expression of macrophages in aneurysms and determine their significance in ruptured AAA. Aortic tissue was harvested at time of aortic reconstructive surgery for the group of intact AAA (n=20) and ruptured AAA (n=10) or at time of organ harvest for normal aortic tissue (n=4). Aortic explant cultures were immediately established and the culture medium was collected after 72 h. Specific enzyme-linked immunoassorbent assays were used to quantify COX-2 metabolites and inflammatory cytokines. Inflammatory macrophage cells were also quantified in the corresponding aortic walls immunohistochemically. Differences in the secretory levels of inflammatory metabolites and the macrophage quantity in all groups were assessed. All three explant culture groups secreted detectable levels of studied COX-2 metabolites, including PGE₂, PGF₂α, PGI₂ and TxB₂ and inflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-8 and IL-10. The secretory levels of PGE₂, TXB₂ and IL-6 were highest in the ruptured AAA explant cultures and statistically higher than those in intact AAA cultures (p<0.05). The secretion of those inflammatory mediators and the local expression of macrophages in ruptured aneurysm probably reflects the active inflammatory processes in the aortic lesions. A means of modifying the inflammatory process in the wall of AAAs might play an important role in preventing aneurysm rupture.

Introduction

Ruptured abdominal aortic aneurysm (AAA) is frequently lethal, and leads to high morbidity and mortality (1). In the last decade, inflammatory cyclooxygenase-2 (COX-2) has been found to be widely expressed in the aneurysmal wall with concomitant synthesis of prostaglandin E₂ (a metabolite of COX-2) (2,3). Nevertheless, knowledge of the etiology of AAA and the risk of aneurysm rupture is still scarce, yet considerable progress is being made through the application of cellular and molecular biological approaches to human aortic aneurysm tissue.

Increasing evidence has suggested that the size of the aneurysm is probably not the sole useful determinant for the risk of rupture (4,5). The potential role of infective agents in AAA development and possibly rupture was also implicated in a study which C. pneumoniae DNA was found in significantly higher frequency in the walls of ruptured AAAs than in those of intact AAAs (6). The importance of inflammation as a factor contributing to the expansion of AAA has also been emphasized (7). Studies have shown that the magnitude of inflammation and the serum concentration of inflammatory cytokines appear to be related to aneurysm diameter and growth (7,8). More recent histological findings showed that there are more prominent inflammatory infiltrates consisting mainly of T lymphocytes and macrophages in ruptured aneurysms than in intact aneurysms (9). Those inflammatory cells can produce matrix metalloproteinases (MMPs) with the ability to degrade the extracellular matrix protein in the aortic wall (7) and may lead to aneurysm rupture ultimately.

Theoretically, a more drastic inflammatory process might be expected in the walls of ruptured aneurysms, where the destructive process has progressed extensively. Investigating the inflammation occurring in intact and ruptured AAA may discover factors promoting rupture. However, there is still no comprehensive study to show the correlation or contribution of all profound inflammatory mediators to this human aortic disease, particularly in the case of rupture.

The aim of the present study was to develop an explant culture using human aneurysm biopsies for investigating the secretion of all profound inflammatory mediators. The correlation of these mediators and inflammatory macrophages, which are responsible for the inflammatory processes occurring in the aneurysm with rupture will be explored.
Materials and methods

Patients and specimens. From January 2004 to March 2006, 30 patients who underwent aneurysmectomy and graft replacement for abdominal aortic aneurysms (AAAs) were recruited. The patients were divided into two groups based on the presentation of the aneurysm. Full-thickness aortic specimens from the central part of the anterior wall of all aneurysms were excised at surgery. The normal infrarenal aortas were obtained from organ donors. All specimens were divided into 2 pieces. One half was washed in saline, and dissected of luminal thrombus and adipose tissue for the aortic explant cultures. The remaining aortic walls were then fixed in 10% formalin for histological examination. Patients with Marfan syndrome and other known connective tissue disorders were excluded from this study. All studies were performed with approval from the local research committee and all organ donors or their relatives gave their informed consent.

Aortic organ explant cultures. All aortic walls were washed free of blood and transported to a sterile tissue culture hood in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% bovine serum albumin and antibiotics. The aortic tissues were divided into 2-mm² segments of full-thickness aortic wall, and were placed into six separate wells of tissue culture plates. After 1 h of incubation to allow adherence to the tissue culture plate, each well was supplemented with 1.5 ml of DMEM containing 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The plates were incubated at 37˚C in a humidified 5% CO₂ atmosphere. After 72 hours, before the cultured cells reached confluence, the conditioned media were collected and stored at -20˚C until the cells were used for enzyme-linked immunosorbent assay (ELISA).

Quantification of COX-2 metabolites and cytokines using ELISA. The content of PGE₂, PGF₂α, PGI₂ and cytokines including IL-1β, IL-6, IL-8 and IL-10 in the culture medium from aortic explant culture samples were quantified using enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, USA) according to the manufacturer's recommendation, except TXB₂ (a stable metabolite of TxA₂). TXB₂ was quantified by a specific immunoassay kit (Amersham, Canada). All ELISAs were performed in duplicate and were specific for the corresponding COX metabolites and cytokines, with no known cross-reactivity with other inflammatory metabolites. Standard curves were included in each assay.

Quantification of macrophage cells. The fixed aortic walls were then processed and embedded in paraffin wax. Paraffin embedded sections (3 μm thick) were cleared and rehydrated by sequential immersions in three changes of xylene, followed by graded ethanol and distilled water. For antigen retrieval, tissues were heated by microwave treatment in 0.01 M citrate buffer for 90 sec. After cooling, specimens were incubated with human anti-macrophage (Dako, Glostrup, Denmark) for 1.5 h at 37˚C and detected by the Dako EnVision system (Dako) with dianaminoenzidine as the chromogenic substrate. Staining development was stopped by immersion in tap water and was counterstained with Lillie-Mayer hematoxylin (Merck, Darmstadt, Germany). All sections were then dehydrated and cleared by sequential immersion in gradient ethanol and xylene. Negative control was always performed by omitting primary antibody. Representative microscopic fields of the aortic media were chosen under low-power light microscopy. To avoid underestimating the medial quantity of macrophages in aortic specimens with considerable regional variation, the areas chosen were restricted to microscopic fields with the largest number of macrophages. The number of macrophage-positive cells was measured for each 10 contiguous x200 high-power fields. The overall mean of macrophage cells for each specimen was determined as macrophages per high-power field.

Reagents. Cell culture medium and reagents were obtained from Gibco BRL (Grand Island, New York, USA) and plasticware for cell culture was obtained from Falcon (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. Relative inflammatory mediators' secretory levels are shown as the mean ± standard error mean (SEM). Intergroup differences were detected with the use of one-way analysis of variance. A Bonferroni-adjusted p value of <0.05 was considered significant. Independent Student t-test was used for ruptured and intact AAA comparison. Statistical analysis was performed with the use of SPSS 13.0 for Windows (SPSS Inc, Chicago, III).

Figure 1. Representative images of the appearance of 3-day explant cultures from control aorta (A), intact AAA (B) and ruptured AAA (C).
Results

Demographic characteristics of all AAA patients. At operation, all abdominal aortic aneurysms (AAAs) were macroscopically non-inflammatory. Group I (n=25) consisted of patients undergoing elective resection of intact AAAs. Only 20 in vitro cultures of vascular smooth muscle cells were obtained from those patients successfully with an AAA mean diameter of 7.5 cm (range, 5-10 cm). The mean age of the patients was 77.3 years old. There were 16 men and 4 women. Group II (n=20) comprised of patients undergoing emergency repair for rupture. Half of the ruptured AAAs can be cultured for the following secretory study. The average diameter of the ruptured AAAs was 7 cm (range, 5-8 cm) and the mean age was 75.8 years old. There were 9 men and 1 woman. A third control group consisting of 4 healthy organ transplantation donors were chosen with a mean age of 60.5 years old. There were 2 men and 2 women. In vitro cultures from all organ donors were successfully performed.

Morphology of aortic explant cultures. All aortic explants were well maintained in culture with little sign of tissue deterioration for 6 days. The appearance of control aorta, and intact and ruptured AAA explant cultures under a microscope is shown in Fig. 1A, B and C respectively.

Secretory levels of inflammatory mediators by aortic explant cultures. After 72 h of incubation, culture media were collected for ELISA analysis as per the aneurysmal cultural study of Walton et al (3). The ELISA data for different groups of aortic specimens are depicted in Table I. All three groups of aortic explant cultures (intact AAAs, ruptured AAAs and control aortas) secreted similar detectable levels of studied COX-2 metabolites and inflammatory cytokines except PGE2, TXB2 and IL-6. The highest level of those mediators was detected in the cultures of ruptured AAAs. The secretory levels of these three mediators were significantly higher in aneurysmal cultures as compared with control (p<0.05). More PGE2, TXB2 and IL-6 were secreted in ruptured AAAs than in intact AAAs (p<0.05).

Quantification of macrophages. More pronounced inflammatory macrophages were found within the medial layer of ruptured AAAs than in the intact AAAs (Fig. 2). In contrast, no macrophage was detected within the normal aortic walls. Morphometric analysis demonstrated that there were

<table>
<thead>
<tr>
<th>Inflammatory mediators</th>
<th>Group I, intact AAAs (n=20)</th>
<th>Group II, ruptured AAAs (n=10)</th>
<th>Group III, control (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandins (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGE2</td>
<td>1020.1±286.4</td>
<td>2310.2±580.7a</td>
<td>350±39.7b</td>
</tr>
<tr>
<td>PGF2α</td>
<td>248.4±113.6</td>
<td>253.6±93.9</td>
<td>260.6±65.6</td>
</tr>
<tr>
<td>Prostacyclin (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGI2</td>
<td>299.3±86</td>
<td>263.6±116.7</td>
<td>358.9±109.1</td>
</tr>
<tr>
<td>Thromboxanes (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TxB2</td>
<td>26.8±9.9</td>
<td>93.7±38.2a</td>
<td>7.7±2.3a</td>
</tr>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>91±23.6</td>
<td>88.8±25.4</td>
<td>60.3±12.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>90.3±31.7</td>
<td>245.4±122.4a</td>
<td>28.1±5.9a</td>
</tr>
<tr>
<td>IL-8</td>
<td>141.9±58.5</td>
<td>108.4±31.6</td>
<td>127.8±25.8</td>
</tr>
<tr>
<td>IL-10</td>
<td>217.9±92.9</td>
<td>158.4±45.8</td>
<td>207.3±206.2</td>
</tr>
</tbody>
</table>

There were 4 control aortas, 20 intact AAAs and 10 ruptured AAAs. PGE2, TxB2 and IL-6 levels in culture media of intact AAAs, particular in ruptured AAAs were several orders of magnitude greater than in control groups. Statistical comparisons among all groups are also indicated: a p<0.05 vs intact AAA groups, and b p<0.05 vs intact AAA groups and c ruptured AAA groups.
Table II. Quantification of macrophages in both types of aneurysms.

<table>
<thead>
<tr>
<th>Type of aortic tissue</th>
<th>n</th>
<th>No. of macrophages</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact AAAs</td>
<td>20</td>
<td>30±5.5</td>
<td>-</td>
</tr>
<tr>
<td>Ruptured AAAs</td>
<td>10</td>
<td>60.5±19</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

significantly more macrophages in ruptured AAAs compared with intact AAAs (Table II, p<0.05).

Discussion

We reported the first successful primary cultures from human ruptured aneurysmal lesions. Augmented secretions of three inflammatory mediators were found in the explant cultures of ruptured aneurysms compared to that of intact aneurysms, suggesting that the inflammatory process may play a significant role in the process leading to aneurysm rupture. The results of immunohistochemical studies, including the present study found that the inflammatory cells were most pronounced in medial layers of the end-stage ruptured aneurysms than in the intact aneurysms (9). This phenomenon may correlate with the higher secretion of inflammatory mediators in this type of aneurysm. The cause of the inflammatory process is still unknown, and possibilities include autoimmune reaction (10) or an infection agent such as Chlamydia pneumonia (11). Soluble peptide fragments derived from the degradation of extracellular matrix components such as elastin, laminin and fibronectin may serve as a chemotactic agent for infiltrating macrophages (12). It is also likely that inflammatory cells in the atheromatous lesions release a cascade of pro-inflammatory mediators that results in the activation of proteolytic enzymes, which are responsible for degradation of the extracellular matrix (13,14). Thus, the accumulation of inflammatory cells may perturb the structural integrity and stability of the vessel wall and thereby increase the risk for aneurysm rupture.

A similar extent of PGE2 secretion in intact aneurysms has also been reported previously (2). PGE2 has a wide range of pathophysiologic effects, including contraction and relaxation of vascular smooth muscle, modulation of inflammatory processes, influencing matrix metalloproteinase expression (15,16) and thus connective tissue turnover (17,18). Another COX-2 metabolite, TxA2 has been suggested to promote the formation of atherosclerosis and restenosis in lesions associated with vascular injury (19). It has also been found to induce platelet aggregation, hypertrophy and extracellular matrix formation in cultured VSMCs (20,21). Therefore, the possibility that PGE2 and TxA2 have a pivotal role in the pathogenesis of aneurysm can be accentuated by our finding.

There is also some indirect evidence suggesting that PGE2 and other cyclooxygenase metabolites contribute to the expansion of abdominal aortic aneurysm (AAA). First, indomethacin inhibited the expansion of experimental AAA with a resultant decrease in PGE2 and matrix metalloproteinase-9 (MMP-9) (22). Second, a case control study using nonsteroidal anti-inflammatory drugs was associated with slower aneurysm growth rates, which implies an important role of COX-2 metabolites in the dilatation of AAAs (3). As rapidly expanding aneurysms are more susceptible to rupture (5,23), the present finding implied that the large quantity of COX-2 metabolite production may be a factor influencing the expansion rate as well as eventual aneurysm rupture. Nevertheless, the relationship between the secretory levels of PGE2 and TxA2 and the size of aneurysm and expansion rate is still a mystery.

Our study suggests that IL-6 may be involved in the inflammatory process underlying the pathogenesis of AAA. Interleukin (IL)-6 is an inflammatory cytokine responsible for acute-phase response (24) and elevated levels of IL-6 have been associated with worse prognosis in unstable angina (25). Accumulating evidence suggests that cytokine-induced tissue inflammation may participate in the pathogenesis of AAAs (14,26,27). An earlier study by Juvonen et al found that AAA patients had significantly higher serum concentrations of IL-18 and IL-6 than either coronary heart disease patients or control subjects (7). IL-6 also works as a chemoattractant for inflammatory infiltrates and activates integrin expression (14), which are thought to be important in the pathogenesis of AAA (28,29).

Nevertheless, circulating IL-6 concentration was found to be similar for patients with small and large AAAs (7) and plasma interferon-γ, but not IL-6, predicted aneurysm growth (27). Therefore, we deduce that IL-6 may play an important role in AAA formation, but not dilatation.

By comparing the extent of increased cytokine levels of the three-day explant cultures of intact and ruptured aneurysms, it seems probable that the highest inflammatory response present in the ruptured aneurysms is the result of a long-standing and progressive inflammatory response in the wall of aneurysms, rather than the acute elevation of cytokines in response to rupture or the injury of surgery. A previous study of the serial sampling of arterial blood pre- to post-operatively for 13 days in patients with ruptured aneurysms and intact aneurysms suggested that there were insignificant differences between the acute systemic inflammatory responses upon aneurysm rupture or surgical procedure. The authors found that the serum concentrations of tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 followed similar time course patterns in both types of patients (30). A large literature review suggested that cytokines play an essential role in regulating the amplitude and duration of the inflammatory response; the time peak levels of most cytokines released upon elective surgery ranged from 0-6 h and declined rapidly (31).

Aortic explants remain viable for up to 1 week in culture, and provide an opportunity to study the complex architecture of the aortic wall (32). In the present study, the appearance of the explants was well maintained in culture with little sign of tissue deterioration until 6 days and so the aortic tissue structure can be partly preserved. This system allows the paracrine interactions among different cell types that are important in vivo to persist in culture. The only disadvantage of explant culture is that its behaviour may not exactly mimic the situation in the living organism. Explant in vitro is also not subject to haemodynamic stress or surrounded by normal physiological conditions.

Our results showed that the abnormally high production of PGE2, TxA2 and IL-6 in aneurysms, particularly in
ruptured cases, supports the hypothesis that these inflammatory mediators are important mediators of AAA development and may be implicated in the risk of aneurysmal rupture. These results also suggest that the risk of aneurysmal rupture may depend on the degree of inflammation. As our understanding of the inflammatory process associated with ruptured AAA becomes clearer, developing ways to control inflammation may be a promising therapy by depressing inflammation within the aneurysm wall, leading to reduced matrix degradation and slower rates of dilatation.

References