CARDIOVASCULAR MEDICINE

Tissue resident C reactive protein in degenerative aortic valves: correlation with serum C reactive protein concentrations and modification by statins

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Objective: To assess aortic valve probes for valvar C reactive protein (CRP) presence, the relation between valvar and serum CRP, and a possible modification of CRP by statin medication.
Setting: Tertiary referral centre.
Patients and design: End stage, degenerative valve tissue was taken from 81 patients, 57 with non-rheumatic aortic valve stenosis (AS) and 24 with degenerative aortic valve bioprosthesis (BP). Five non-stenosed valves served as controls. Tissue from four non-implanted bioprostheses was also examined. The presence and location of CRP was analysed by use of immunostaining and morphometry. Serum CRP concentrations were measured preoperatively.
Results: The majority of AS and BP valves exhibited CRP labelled cells, predominantly localised to the valvar fibrosa. The expression of CRP was much higher in BP than in AS (by a factor of 3.7, $p = 0.03$). Notably, non-stenosed aortic valves and non-implanted bioprostheses did not have CRP signalling. Serum CRP was also increased with BP (by a factor of 2.5, $p = 0.02$) and was significantly correlated with valvar CRP expression ($r = 0.54$, $p < 0.001$). The main finding in patients with (n = 26) and without statin treatment (n = 55) was that both valvar CRP expression ($p = 0.02$) and serum CRP concentrations ($p = 0.04$) were lower in the statin treated group.
Conclusions: CRP was found in a large series of degenerative aortic valves, more often in bioprostheses than in native cusps. Serum CRP concentrations may reflect inflammatory processes within the aortic valve. The association of statin treatment with decreases in both valvar and serum CRP concentrations may explain known pleiotropic effects of statins in patients with aortic stenosis.

Although calcific aortic stenosis is recognised as the most common form of valve disease among the elderly, its underlying aetiology is still poorly understood.[1] Valve degeneration has been shown to be preceded by focal endothelial damage at the fibrosa, with subsequent inflammatory cell infiltration and cellular transformation into an osteogenic phenotype, making these cells capable of initiating mineralisation.[2–6] Specifically, T lymphocytes and macrophages indicating chronic inflammation are known to aggregate in aortic valve stenosis, in many ways similar to atherosclerotic plaques.[4 5 7–13] Also, increased serum concentrations of C reactive protein (CRP) have recently been observed in patients with non-rheumatic aortic stenosis (AS) and CRP concentrations decreased six months after aortic valve replacement, suggesting that the diseased aortic valve is the site of active inflammation.[4 14 15] As yet, no data on the valvar expression of CRP have been reported, in particular in degenerative bioprostheses.

The hypothesis of an active disease process suggests that aortic stenosis may be amenable to medical treatment to prevent or slow disease progression. Indeed, four recent retrospective studies consistently showed an association between statin treatment and much lower haemodynamic progression of aortic stenosis.[16–19] Furthermore, two of these studies showed that this effect is independent of cholesterol concentrations, suggesting pleiotropic or anti-inflammatory statin effects.[18 19]

Therefore, we assessed the presence and frequency of CRP in degenerative native and bioprosthetic valves, elucidated the relation between valvar and serum CRP concentrations, and evaluated a possible modification of CRP concentrations by long term statin medication.

METHODS

Patients and valve specimens

In the present study, we assessed a series of surgically removed diseased aortic valves. Valve tissues were obtained from 57 consecutive patients with AS who underwent aortic valve replacement. All valves examined were tricuspid. In addition, 24 severely degenerative aortic valve (porcine) bioprostheses (BP) were obtained during surgical replacement (11 Carpentier-Edwards and 13 Hancock devices, mean (SD) implant time 13 (4) years). Specific exclusion criteria were evidence of rheumatic disease, other coexisting valve diseases, endocarditis, or other systemic inflammatory processes. The diagnosis of aortic valve disease was based on detailed history and physical examination of the patients, as well as echocardiographic and invasive findings. Information on the type and dosage of long term statin treatment was recorded. Non-stenotic aortic valve specimens (n = 5) were obtained as controls from surgical patients affected by aortic valve insufficiency caused by aneurysms on the ascending aorta (three men and two women; mean (SD) age 59 (4) years; two with arterial hypertension, two treated with angiotensin converting enzyme inhibitors, and two treated with aspirin). In addition, four non-implanted bioprostheses (Hancock) were examined. Written informed consent for the subsequent analysis of valve tissue was given by each patient; the local ethics committee had approved the present study.

Abbreviations: AS, non-rheumatic aortic valve stenosis; BP, degenerative aortic valve bioprosthesis; CRP, C reactive protein

Immunohistochemistry

Valve probes were fixed in 4.5% buffered formaldehyde and embedded in paraffin. Serial sections (4 μm) were cut from diseased tissue adjacent to calcified areas for immunohistochemical analysis. First the paraffin embedded tissue was dewaxed and rehydrated, followed by saponin (2%; Sigma, Deisenhofen, Germany) retrieval for 20 minutes at room temperature. Then the tissue was incubated for 30 minutes at room temperature in fetal bovine serum (1:25) to block non-specific binding sites. In the next step, the polyclonal antibody specific for CRP (1:250; ICN, Eschwege, Germany) was added and the tissue sections were incubated overnight at 4℃. After addition of mouse anti-rabbit IgG (1:75, 30 minutes at room temperature) the colour reaction was done with alkaline phosphatase antialkaline phosphatase marking (Dianova, Hamburg, Germany) according to a standard protocol.[10 13 20] The colour substrate of alkaline phosphatase was fast red (Sigma). Lastly, nuclear counterstaining was done by haematoxylin. Negative controls were omission of the antibodies (mouse monoclonal IgG1, clone NCG01, Dianova) and staining with unspecific antibody (ChromPure rabbit IgG, code number 011-000-003, Dianova) in known concentrations for primary antibodies.

Histological analysis

Haematoxylin stained histological sections allowed for the detection of cellular nuclei. Cell density was assessed with a computer assisted morphometric system (VFG 1 graphic card, VIBAM 0.0. software) to count nuclei per area (0.04 mm^2) and to calculate cell density.[10 13 20] Pictures were obtained by an Optiphot-2 microscope (Nikon, Düsseldorf, Germany) and a downstream KP-C 553 charge coupled device video camera (Hitachi, Rodgau, Germany). Five randomly selected areas were assessed for each distinct leaflet layer—that is, fibrosa, spongiosa, and ventricularis. The percentage of immunostained cells was determined as the proportion of positive cells in the total number of cells in each layer. Two independent examiners evaluated all morphometric data.

Serum CRP

In 72 patients high sensitive CRP was measured with a commercially available kit (N high sensitivity CRP; Dade Behring, Marburg, Germany). From all patients, serum was collected at admission before operative valve replacement.

Statistical analysis

Data are presented as the percentage (mean (SEM)) of positively stained cells among five randomly chosen high power fields. We assessed group differences by use of the χ^2 test for categorical variables. Probability was calculated with the Mann-Whitney U test for differences in cell density and expression of valvar determinants. Values of $p < 0.05$ were considered to be significant.

Table 1 Characteristics of patients (n = 81) undergoing aortic valve replacement

Variable	Patients with AS	Patients with BP	p Value
Number	57	24	
Age (years)	68 (10)	68 (6)	0.9
Men	34 (60%)	13 (54%)	0.6
CAD	30 (53%)	11 (46%)	0.5
Hypertension	33 (58%)	11 (46%)	0.2
Diabetes	9 (16%)	3 (13%)	0.7
Smoker	25 (44%)	11 (46%)	0.9
Hyperlipidaemia	30 (53%)	10 (42%)	0.3
Obesity	37 (65%)	13 (54%)	0.4
Family history	12 (21%)	5 (21%)	0.9
Aspirin	34 (60%)	13 (54%)	0.6
Statin	20 (35%)	6 (25%)	0.4
ACE inhibitor	32 (56%)	12 (50%)	0.6

Age is mean (SD).
ACE, angiotensin converting enzyme; AS, non-rheumatic aortic valve stenosis; BP, degenerative aortic valve bioprosthesis; CAD, coronary

RESULTS

Diseased valve probes from 81 patients undergoing aortic valve replacement were examined for the presence and location of CRP. Fifty seven valves were from patients with AS and 24 from those with BP. Table 1 presents patient characteristics such as age, sex, cardiovascular risk factors, and medication, which did not differ between groups.

CRP was found in the majority of degenerative valves. Specifically, CRP was detected in 34 of 57 (60%) AS and 16 of 24 (67%) BP. Stained cells were predominantly localised to the fibrosa of AS and the superficial regions of BP (fig 1). Figure 2 summarises quantitative immunohistochemical data for these compartments. Mean expression of CRP was 3.8% and expression was maximal in 65% of valve cells. Remarkably, the percentage of CRP labelled cells was increased by a factor 3.7 in BP compared with AS ($p = 0.03$). Non-degenerative native valves and non-implanted bioprostheses did not show any signalling and served as negative controls.

Of note, a subgroup of 72 patients were analysed for serum CRP concentrations. Mean serum CRP was 10.7 mg/l. Serum CRP was increased in patients with BP compared with those with AS (factor 2.5, $p = 0.02$) (fig 2). Furthermore, a significant positive correlation between tissue resident CRP and circulating CRP was found ($r = 0.54$, $p < 0.001$). Comparison of patients with (n = 50) versus without (n = 31) tissue resident CRP found serum CRP concentrations to be higher in the first group (17.7 (2.6) *v* 4.4. (1.1) mg/l, $p < 0.001$). In this first group, 12 of 50 (24%) patients were treated with statins versus 14 of 31 (45%) in the second group ($p = 0.047$).

Of the 81 patients, 26 were treated with a statin (eight with simvastatin, eight atorvastatin, four cerivastatin, four pravastatin, and two fluvastatin). A central finding when patients were categorised with (n = 26) versus without statin treatment (n = 55) was that both valvar CRP expression ($p = 0.02$) and serum CRP concentrations ($p = 0.04$) were lower in the statin group (fig 3). Beyond this, we observed no significant relation between CRP concentrations, different statins, or different doses of statins.

DISCUSSION

The present study documented the ex vivo presence of CRP in degenerative aortic valves, showing, firstly, that increasing valvar CRP is associated with increasing serum CRP concentrations; secondly, that valvar and serum CRP significantly increased in degenerative prostheses compared with their native counterparts; and thirdly, that statin treatment is associated with notable decreases in both valvar CRP expression and serum CRP concentrations.

Our study showed, for the first time, the presence of tissue resident CRP in degenerative aortic valves (figs 1 and 2). Recently, others reported increased serum CRP concentrations in patients with degenerative aortic stenosis.[4 14] Although those authors favoured local actions at the valve tissue level to be responsible for their observation, they did not report the tissue level data.[14] In addition, the increased CRP concentrations in patients with AS were found to have decreased after valve replacement, suggesting also that the aortic valve is the key site of active inflammation.[15] Beyond confirming this, the present study extends these previous observations with evidence of valvar CRP expression and of a significant association of the staining intensity of valvar and

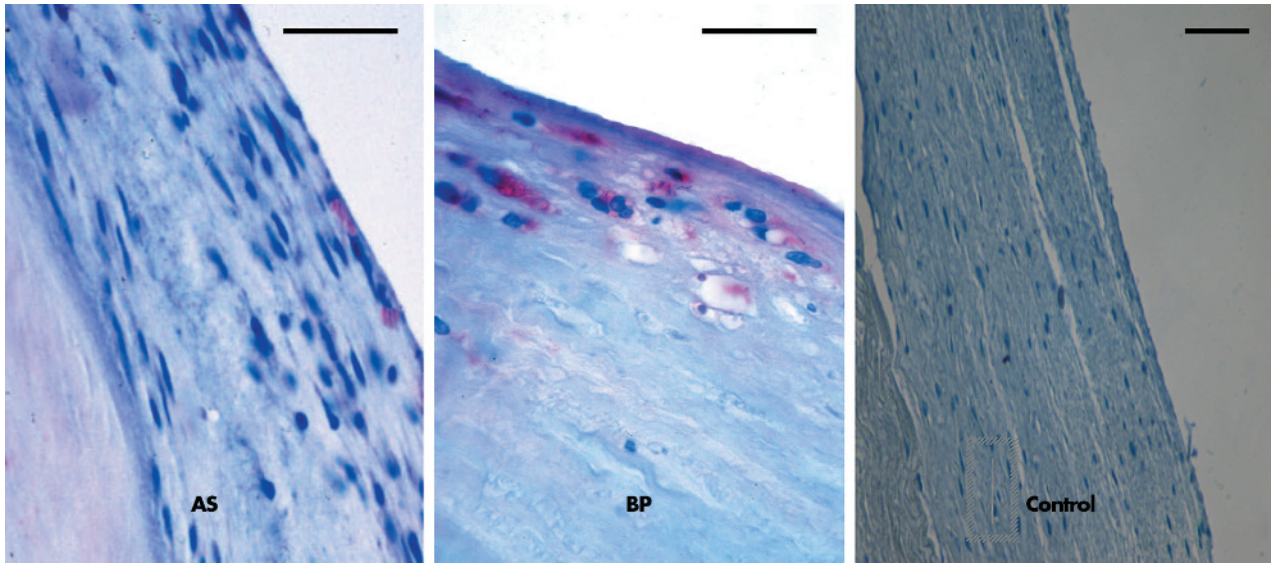

Figure 1 Photomicrographs of representative valve lesions showing detection of C reactive protein (CRP) in degenerative aortic valves by immunohistochemistry. Signals occur in valvar fibrosa but not in the valvar spongiosa, with a higher percentage of cell bound signals in degenerative aortic valve bioprostheses (BP) with a typically decreased cellularity than in degenerative native valves (non-rheumatic aortic valve stenosis (AS)). Non-degenerative native valves did not show any signalling and served as negative controls. Bar = 25 μm.

serum CRP concentrations. This finding is in accordance with recent postmortem data from sudden death coronary lesions that found correlations between intimal immunostaining intensity and serum CRP concentrations.[21] Our present data show maximal local CRP expression in 65% of all valve cells. These high values do not support the suggestion that serum CRP concentrations are increased by continuous hepatic CRP synthesis but rather they suggest local intravalvar CRP generation. This hypothesis has recently been strengthened by studies that detected CRP mRNA within atherosclerotic plaques and aneurysmal tissue.[22–24] CRP mRNA content was sevenfold higher in atheroma than in the liver and 10-fold higher than in undiseased arteries.[22] Possibly, the majority of high serum CRP found in patients with AS may be attributed to the direct release of CRP from the diseased valve, thereby reflecting the degree of individual valve inflammation.

Of course, the present study cannot definitively prove whether CRP is an active player in the inflammatory, degenerative process in the valvar fibrosa or is induced by the disease itself. The concept of direct deleterious effects of CRP on valve tissue is supported by several experimental and in vitro studies on atherosclerosis.[22–29] CRP leads to induction of the adhesion molecules intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and monocyte chemoattractant protein 1 in endothelial cells and macrophages, exerts chemotactic effects on monocytes/macrophages, propagates inflammation by release of the cytokines interleukin 1β, interleukin 6, and tumour necrosis factor α from monocytes, and, recently, was reported to cause accelerated aortic atherosclerosis in apolipoprotein $E^{-/-}$ mice.[26–29] Whereas undiseased control valves in the present study were not found to have signals of CRP, tissue expression and serum concentrations of CRP are more extensive in patients with degenerative bioprostheses than in those with degenerative native valves. Thus, CRP may be an active participant in both types of valve degeneration and, thereby, may have an even more important role in prosthetic degeneration.

A central finding of the present study was that both valvar CRP expression and serum CRP concentrations were sig-

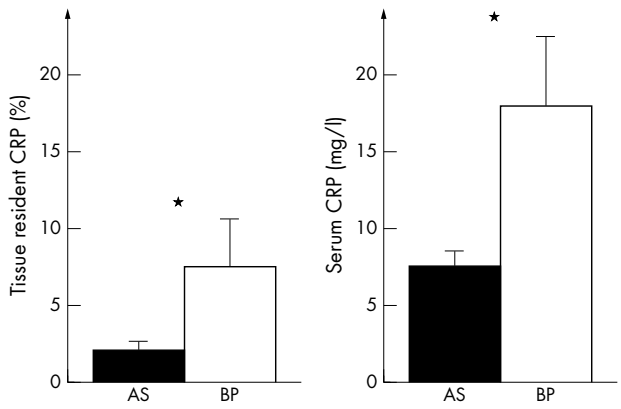

Figure 2 Quantitative analysis of valvar CRP expression and serum CRP concentrations comparing both groups of aortic valve degeneration. Expression of CRP at the tissue level and systemic CRP were significantly increased in BP versus AS. *$p < 0.05$.

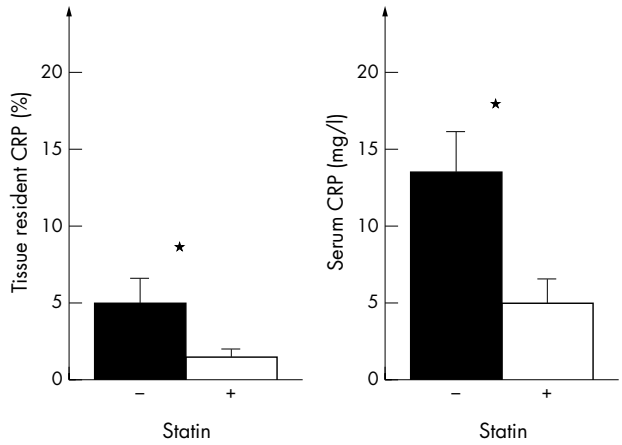

Figure 3 Expression of valvar CRP and serum CRP concentrations dependent on statin treatment. In the statin treated group (+), valvar expression of CRP and serum CRP concentrations were significantly decreased compared with the non-statin treated group (−). *$p < 0.05$.

nificantly lower in patients with valve disease who were treated with a statin. Analogous to our present data, several clinical studies on patients with atherosclerosis have shown that statins reduce CRP concentrations rapidly and for extended periods, associated with a better clinical outcome.[30] Likewise, statins had already been shown to slow the progression of aortic stenosis in retrospective studies but failed to do so in a prospective trial.[16–19 31] It was suggested that this effect is independent of cholesterol lowering.[18 19] Indeed, our findings further support the concept that these effects of statins work at the valve tissue level and may be caused by pleiotropic or anti-inflammatory properties, or both.

In conclusion, the presence of local and systemic CRP provides further evidence that inflammation has an important role in valve degeneration that is more striking in bioprosthetic valves than in native valves. Our findings may suggest the generous use of statins in particular to prevent degeneration of bioprostheses. Future clinical trials are mandatory to prove this attractive concept.

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