

Loss of Anti-Aggregatory Effects of Aortic Valve Tissue in Patients with Aortic Stenosis

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Background and aim of the study: Patients with aortic stenosis (AS) exhibit increased platelet aggregability, and thrombus formation has been documented on calcific and severely stenosed valves. Isolated porcine and canine aortic valves (AV) release nitric oxide (NO) and prostacyclin, which exert local anti-thrombotic effects; to date, this has not been studied in humans. In the present study the possible interaction of AV tissue with platelet aggregation was examined, using fragments of AV obtained from patients with AS and aortic regurgitation (AR).

Methods: Fragments of AV tissue, excised from patients undergoing AV replacement, were co-incubated with blood samples obtained from normal subjects. The direct effects of valve tissue from patients with AS (n = 14) or with predominant AR (n = 13) on ADP-induced platelet aggregation and intraplatelet cGMP and cAMP content were compared.

Results: In whole blood, non-calcified AV fragments from AR patients inhibited platelet aggregation by $57 \pm 6\%$ ($p < 0.01$); in platelet-rich plasma results were

analogous. In order to determine whether this anti-aggregatory effect could be attributed to the valvular release of NO or prostacyclin, intraplatelet cGMP and cAMP formation was assessed, respectively. While there were no significant changes in cGMP content, cAMP increased by $26 \pm 4\%$ ($p < 0.02$). Both, anti-aggregatory and cAMP-stimulating effects were similar to those produced by 10 nM prostaglandin E₁, a prostacyclin mimetic. Fragments from stenotic valves did not inhibit aggregation and did not affect cGMP or cAMP. Furthermore, fragments from heavily calcified regions potentiated aggregation and, in some cases, induced spontaneous aggregation.

Conclusion: Minimally calcified aortic valves (i.e., AR) and, therefore, presumably also normal valves, exert anti-aggregatory effects, most likely via prostacyclin release. AS is associated with a loss of this effect, thus potentially contributing to thrombotic risk.

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Progressive disorganization (sclerosis) followed by narrowing (stenosis) of the aortic valve, termed aortic stenosis (AS), occurs frequently in the elderly (1,2). Epidemiological studies have indicated that factors predicting the occurrence and rapid progression of AS (renal insufficiency, diabetes, hypercholesterolemia, hypertension and male gender) are similar to those of atherosclerosis and vascular thrombosis (3-6). Indeed, aortic sclerosis is an independent predictor of death from cardiovascular causes, including myocardial infarction and stroke (5,7). Histological studies of valves excised from patients with AS reveal evidence of changes similar to those occurring within atheroma-

tous plaques: atheroma, inflammatory change and later fibrosis, calcification and ossification (for a review, see reference (8)). Calcific aortic valve stenosis involves an atherosclerotic process (8), and itself has been described as a 'window to atherosclerosis' (6). Narrowing of the aortic valve orifice in AS, together with deformation of leaflets and increasingly rough surface of the valve, contributes to local turbulence in blood flow which creates shear stress, affecting both valve endothelium and passing platelets (9). Patients with AS exhibit increased platelet reactivity (9,10), and thrombus formation has been documented on calcific and severely stenosed valves (11,12). Furthermore, loss of the aortic valve endothelium predisposes towards calcification of the aortic valve leaflets (13). Potentially, the aortic valve endothelium plays a critical role in maintaining normal aortic valve function. In isolated porcine and canine aortic valves, it was shown that the aortic valve endothelium releases nitric oxide (NO)

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and prostacyclin, which exert local anti-thrombotic effects and reduce leaflet tone (14-16). It has also been shown that aortic valve sclerosis is associated with systemic endothelial dysfunction (17). However, the relationship between the development of AS and either endothelial dysfunction of the aortic valve, or factors predisposing to aortic valve endothelial dysfunction have not been explored to date.

Platelet aggregation *in vivo* is under negative control of the endogenous NO, endothelium-derived relaxing factor, via the NO/cGMP signaling pathway, and prostacyclin via the cAMP signaling pathway (for a review, see reference (18)). In the present study, the hypothesis was tested that AS, relative to AR (with minimally calcified aortic valves), is associated with impairment of release of NO and/or prostacyclin from the valve. Utilizing inhibition of ADP-induced platelet aggregation as a bioassay for diffusible anti-aggregatory materials (i.e., NO and prostacyclin) released from co-incubated aortic valve tissue, the interaction between fragments of aortic valves (obtained from patients with predominant AS and predominant/pure AR) and the process of platelet aggregation in blood samples obtained from normal subjects was examined. The effects of aortic valve fragments on intraplatelet cGMP and cAMP formation were also assessed.

Clinical material and methods

Patients

All patients had either severe AS or AR; patient characteristics are summarized in Table I. Patients were

Table I: Patient characteristics.

Parameter	Aortic stenosis (n = 14)	Aortic regurgitation (n = 13)
Gender ratio (M:F)	9:5	11:2
Age (years)*	66 ± 4	63 ± 3
Bicuspid valve	3 (21)	3 (23)
CAD	2 (14)	4 (31)
Drugs used		
Aspirin	8 (57)	4 (31)
ACE inhibitors	3 (21)	6 (46)
Statins	7 (50)	3 (23)
Perhexiline	3 (21)	-
Ca ²⁺ -antagonists	5 (36)	2 (15)
β-blockers	5 (36)	3 (23)

*Values are mean ± SEM.

Values in parentheses are percentages.

ACE: Angiotensin-converting enzyme; CAD: Coronary artery disease.

No significant differences were identified between groups for any parameter.

classified as having AS on the basis of: (i) demonstration on echocardiography and/or cardiac catheterization of hemodynamically significant stenosis, corresponding to an aortic valve area <1.0 cm²; and (ii) a lack of severe associated AR. However, patients with mild/moderate associated AR were included in the AS group. Patients with AR had either 'pure' regurgitation (no systolic pressure gradient across the aortic valve) or predominant regurgitation. Among those patients with AR, nine (70%) had 'pure' AR. All patients also underwent diagnostic coronary angiography prior to valve replacement; stenoses of >50% in major vessels were designated as a hemodynamically significant coronary disease. Only a small minority of patients had significant coronary disease. Although a greater proportion of patients in the AS group were receiving aspirin compared to the AR group, this difference was not statistically significant.

Patients with AS and AR underwent surgical aortic valve replacement. The excised aortic valves were immediately delivered in ice-cold Krebs' physiological solution to the research laboratory and assessed for direct effects on platelet aggregation in experiments with whole blood and platelet-rich plasma obtained from healthy male subjects (n = 14, mean age 30 ± 3 years) who were not taking any medication.

The protocol was approved by the Ethics of Research Committee of The Queen Elizabeth Hospital, and informed consent was obtained from all patients prior to study entry.

Blood sampling and platelet preparation

Blood samples from normal subjects (see above) were withdrawn from an antecubital vein and collected into plastic tubes containing 1:10 volume of acid citrate (two parts 0.1 M citric acid to three parts 0.1 M trisodium citrate). Blood was centrifuged at 250 g for 10 min at room temperature to obtain platelet-rich plasma. Platelet-poor plasma was prepared by further centrifugation of the remaining blood at 2,500 g for 20 min. Platelet counts were performed on a STKS Coulter Counter (Coulter Electronics Inc., Hialeah, FL, USA) and the platelet-rich plasma was adjusted with platelet-poor plasma to a constant count of 250,000 per μl.

Platelet aggregation studies

Platelet aggregation in whole blood and platelet-rich plasma was examined using a dual-channel impedance aggregometer (Model 560; Chrono-Log, Haverstown, PA, USA) as described previously (19). In brief, tests were performed at 37°C and a stirring speed of 900 rpm. Samples of whole blood and platelet-rich plasma were diluted two-fold with normal saline (final volume 1 ml) and prewarmed for 5 min at 37°C. Aggregation was induced with adenosine 5'-diphos-

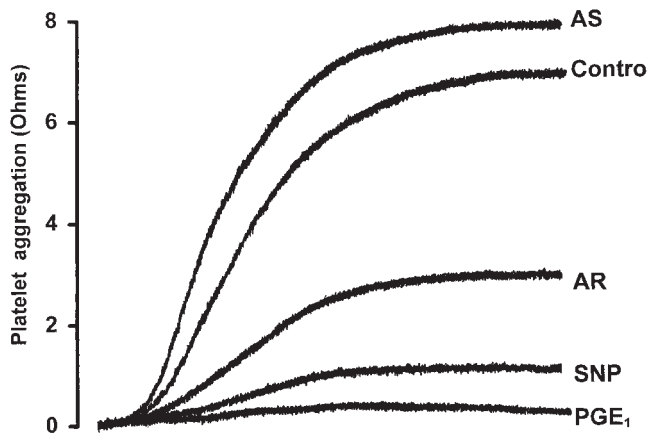


Figure 1: Representative tracings for whole-blood platelet aggregation induced by 1 μM ADP alone (Control) and after preincubation with 10 μM sodium nitroprusside (SNP), 1 μM prostaglandin E_1 (PGE_1) or fragments of aortic valves from patients with aortic stenosis (AS) or aortic regurgitation (AR).

phate (ADP, final concentration 1 μM ; Sigma, St. Louis, MO, USA). Aggregation was monitored continually for 7 min, and responses were recorded for electrical impedance (in Ω). Fragments (surface area 6 mm^2) of aortic valve tissue were co-incubated (for 5 min before the induction of aggregation) with whole blood or platelet-rich plasma obtained from healthy male subjects, and were present during the aggregation test, in order to assess the effects of diffusible materials released from aortic valve tissue on ADP-induced platelet aggregation. Sodium nitroprusside (SNP; Sigma) or prostaglandin E_1 (Sigma) were added to blood samples 1 min before ADP. Inhibition of aggregation was evaluated as a percentage comparing the extent of maximal aggregation in the presence and absence of the added material.

cGMP and cAMP studies

Intraplatelet cGMP and cAMP content was determined by radioimmunoassay, as described previously (19). In brief, platelet-rich plasma (0.5 ml) was incubated at 37°C with SNP or prostaglandin E_1 for 1 min. After incubation, plasma was filtered through GF/C Glass Microfibre Filters (Whatman, UK) to harvest the platelets. Filters with absorbed platelets were rinsed with physiological saline and placed into 0.5 ml 4 mM EDTA for further extraction of cGMP in a boiling water bath for 5 min. After centrifugation of samples at 3,000 g for 10 min, cGMP and cAMP concentrations in the supernatant were estimated using 'cGMP [^{125}I] assay system' or 'cAMP [^{125}I] assay system', respectively (Amersham Biosciences, UK). Results were expressed as pmol cGMP (or cAMP) per 10^9 platelets.

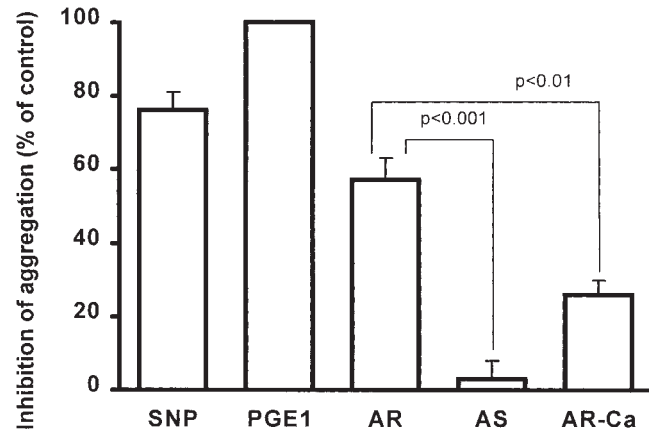


Figure 2: Inhibitory effects of aortic valve fragments obtained from patients with aortic stenosis (AS, $n = 14$) or aortic regurgitation (non-calcified fragments: AR, $n = 13$; calcified fragments: AR-Ca, $n = 5$) on platelet aggregation in whole blood. Effects of 10 μM sodium nitroprusside (SNP, $n = 21$) and 1 μM prostaglandin E_1 (PGE_1 , $n = 5$) are shown for comparison.

Histology

Valve histology was evaluated in 10 patients with AS and in three with AR utilizing a combination of light and electron microscopy, in order to examine the condition of the aortic valve endothelium. Immunohistochemistry involved staining with endothelial cell markers: *Ulex europaeus* agglutinin-1, factor VIII-related antigen, and CD 34. Histological studies were performed by the Histopathology Department of the Queen Elizabeth Hospital.

Data analysis

Results were expressed as mean \pm SEM. Patient characteristics for AS and AR were compared using Fisher's exact test for proportional values and Student's non-paired t -test for patient age. The effects of aortic valve fragments on platelet aggregation and intraplatelet cGMP and cAMP content were compared between patients with AS and AR, using a non-paired t -test. A p -value < 0.05 was considered to be statistically significant.

Results

Representative tracings for ADP-induced platelet aggregation in a whole-blood sample from a normal subject are shown in Figure 1. Within the entire cohort of normal donors ($n = 14$), platelet aggregation in response to 1 μM ADP was $6.8 \pm 0.7 \Omega$ in whole blood and $16.2 \pm 0.6 \Omega$ in platelet-rich plasma. In accordance with previous observations (19), the NO donor SNP (final concentration 10 μM) inhibited aggregation by $76 \pm 5\%$ in whole blood (Fig. 2) and by $68 \pm 9\%$ in platelet-

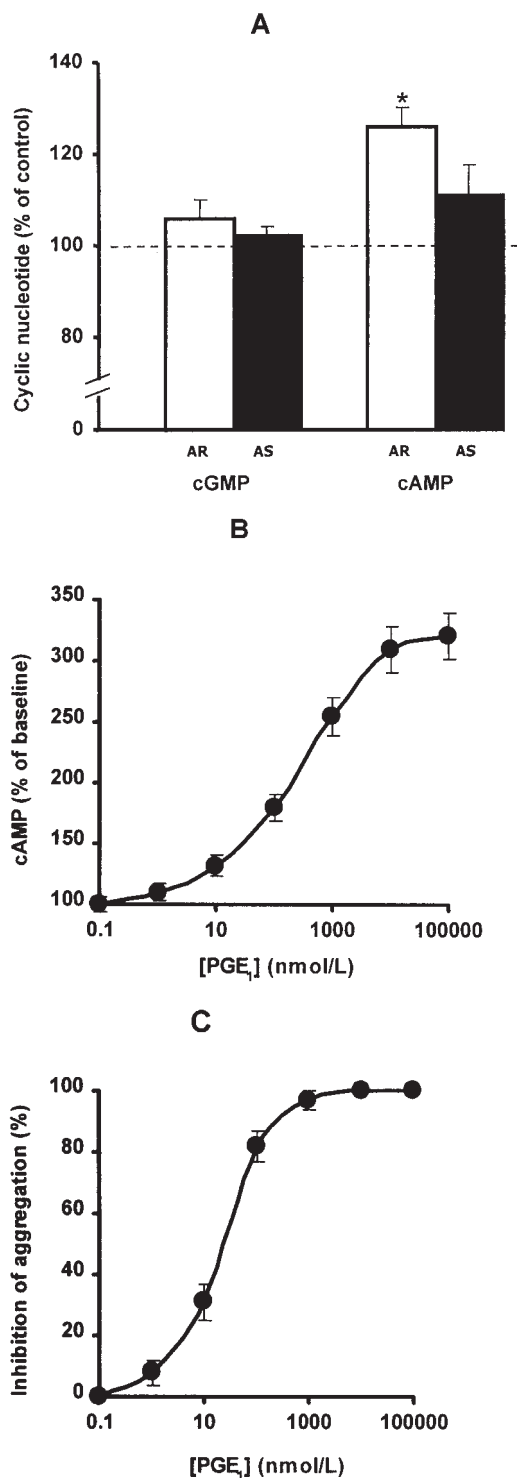


Figure 3: A) Effects of aortic valve fragments obtained from patients with aortic regurgitation (AR, n = 8) or aortic stenosis (AS, n = 8) on intraplatelet cGMP and cAMP content. Effects of prostaglandin E1 (PGE₁) on (B) intraplatelet cAMP content (n = 5) and (C) ADP-induced platelet aggregation (n = 5) are shown for comparison. *p<0.02, AR versus control.

rich plasma. Prostaglandin E1 (prostacyclin mimetic, 1 μ M) completely inhibited aggregation in both preparations. In order to demonstrate the capacity of this bioassay system to detect putative ex-vivo release of anti-aggregatory materials (NO and/or prostacyclin) from the aortic valve tissue, platelet aggregation was examined in the presence of fragments of rat aorta and rat skeletal muscle, the latter as an example of a non-endothelial tissue. Rat aorta fragments completely inhibited ADP-induced aggregation, while skeletal muscle tissue was without effect (data not shown).

Fragments of aortic valve obtained from AS and AR patients were co-incubated with whole blood or platelet-rich plasma obtained from healthy male subjects in order to assess the direct anti-aggregatory effects of diffusible materials released from aortic valve on ADP-induced platelet aggregation. In whole blood (Fig. 2), inhibition of aggregation by valve fragments was $57 \pm 6\%$ for AR versus $3 \pm 5\%$ for AS ($p < 0.001$); AS fragments had no inhibitory effect on aggregation. Furthermore, fragments from heavily calcified regions of the AS valves tended to potentiate platelet aggregation and, in some cases (n = 4), induced spontaneous aggregation (data not shown). Similarly, calcified fragments of AR valves induced less inhibition of aggregation than non-calcified fragments (Fig. 2). Experiments with platelet-rich plasma produced analogous results (data not shown).

In order to determine whether the anti-aggregatory effects of aortic valve could be attributed to the release of NO or prostacyclin, the effects of valve tissue exposure on intraplatelet cGMP and cAMP formation were assessed, respectively (Fig. 3, panel A). While there were no significant changes in cGMP content, intraplatelet cAMP increased by $26 \pm 4\%$ ($p < 0.02$) after 1 min co-incubation with aortic valve fragments from AR patients. By comparison, 10 nM prostaglandin E1 induced a $22 \pm 5\%$ increase in cAMP content and $31 \pm 5\%$ inhibition of aggregation (Fig. 3, panels B and C). With AS valves, there were no significant effects on the intraplatelet content of either cGMP or cAMP (Fig. 3, panel A).

There was no significant impact of concomitant low-dose aspirin therapy on either platelet aggregation or intraplatelet cyclic nucleotide levels (data not shown). Bicuspid aortic valves elicited similar responses as tricuspid valves.

Histoimmunocytochemistry studies detected aortic valve endothelium in eight of the 10 AS patients and in all three of the AR patients. However, electron microscopy demonstrated extensive obliteration of endothelium by calcium deposits, especially in heavily calcified regions of valves.

Discussion

Although previous studies have documented platelet hyperaggregability and thrombus formation in patients with AS (9-12), the present study is the first to report: (i) an anti-aggregatory effect of human aortic valve tissue; and (ii) impairment/loss of this effect in AS. Aortic valves from patients with AR were used as a comparator in the absence of a means of studying normal human valves. Nevertheless, it is recognized that AR valves frequently were partially calcified, with minor degrees of AS in one-third of cases: thus, they represent only relative 'normality'. The finding that calcified regions of AR valves exerted impaired anti-aggregatory effects suggests that even early aortic valve calcification is associated with some attenuation of endothelial function. Similarity between the results obtained with whole blood and platelet-rich plasma implies a direct interaction of diffusible materials, released from the aortic valve, with aggregating platelets - an effect that is independent of the other blood elements.

The current demonstration of attenuation of anti-aggregatory effects of valve tissue may reflect the aortic valve endothelium obliteration/dysfunction in patients with advanced AS (Figs. 2 and 3). This impairment is likely to appear quite early during the clinical course of AS. For example, post-mortem studies of aortic 'sclerotic' valves have revealed the presence of inflammatory infiltrates of the valve matrix (8), suggesting vulnerability of the endothelium to monocyte transmigration. However, it is not logistically possible to test valvular endothelial function in humans with aortic sclerosis. Furthermore, AS is associated with variable degrees of physical loss of endothelium, observed in the present study and documented previously by others (8,13). The potential contribution of this process to perceived endothelium dysfunction is also difficult to assess. A decrease in prostacyclin release from aortic valve in AS, reflected by a decrease in intraplatelet cAMP formation during the co-incubation of aortic valve fragments with platelets, could be a further possible explanation. The lack of significant effects of AR aortic valves on intraplatelet cGMP may be associated with a deficit of NO release even in minimally diseased aortic valves. This issue cannot be resolved conveniently in human studies, and would best be addressed in an animal model of aortic valve disease (20).

Study limitations

The current study had certain limitations, notably that NO and prostacyclin release was detected on the basis of biochemical effects rather than directly, in the absence of sufficiently sensitive assays for these mate-

rials in whole blood. It also cannot be excluded that perioperative endothelial damage and/or loss might have contributed to the observed findings. Conversely, the study population was too small to permit evaluation of possible effects of 'endothelial-protective' agents such as statins and ACE inhibitors. These implications of pharmacotherapy on valve function deserve further study.

In conclusion, the main implication of the present results was that the stenotic aortic valve constitutes a pro-aggregatory milieu. The clinical consequences of the loss any valve anti-aggregatory function must be considered together with the previous documentation of platelet hyperaggregability and platelet resistance to NO in AS (10). There may be a direct interaction between the stenotic aortic valve and circulating platelets, resulting in incremental platelet activation (9). All of these factors may contribute to the pathogenesis of valve thromboembolism in patients with AS (8,11,12). Thus, aortic valve tissue of minimally diseased aortic valves (from patients with AR) exerts anti-aggregatory effects, most probably via prostacyclin release. Progressive aortic valve calcification in AS is associated with a decrease in this anti-aggregatory effect, potentially contributing to thrombotic risk. It remains to be established to what extent this reflects dysfunction rather than loss of the aortic valve endothelium.

Acknowledgements

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