Original Article

Influence of Blood Contact on the Calcification of Glutaraldehyde-pretreated Porcine Aortic Valves

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Background: The rat subcutaneous model reproduces clinically observed mineralization of bioprosthetic tissues. However, the effectiveness of antimineralization treatment can be overestimated in subcutaneous implants, since specimens using this model are not subjected to mechanical and dynamic stress or blood-surface contact. The purpose of this study was to evaluate the influence of blood contact on the calcification of bioprosthetic valves.

Methods: Glutaraldehyde-pretreated porcine aortic valves were prepared. Aortic wall and cusp discs were implanted subcutaneously in six rats for 8 weeks and were implanted within the jugular vein wall in six sheep for 3 and 6 months (blood contact model). Tissue discs were analyzed by gross inspection, radiography and light microscopy. Calcium content was determined by atomic absorption spectrometry.

Results: X-ray and light microscopic examination showed calcification in the cusps to be higher than that in the aortic wall in the rat subcutaneous model. On the other hand, in the blood contact model, the cusps were slightly calcified and calcification in the aortic wall was more pronounced. Calcium analysis in rats revealed more calcium in the cusp than in the aortic wall (71.5±9.7 μg/mg dry tissue vs. 53.7±2.6, p=0.09). Tests for calcium content of the jugular vein samples in sheep showed significantly more calcium in the aortic wall than in the cusp (3 months, 7.9±1.5 vs. 0.3±0.1, p<0.0001; 6 months, 77.2±6.1 vs. 27.2±10.2, p=0.0002). In addition, aortic wall and cusp calcification significantly increased with time.

Conclusions: These data suggest that the results from the rat subcutaneous model were completely opposite to those for the blood contact model. This study confirms the need to include blood contact as a factor in in vivo pre-clinical valve testing. (Ann Thorac Cardiovasc Surg 2003; 9: 245–52)

Key words: bioprostheses, large animal model, experimental, calcification

Introduction

Due to the expense and time commitment involved in circulatory models, subcutaneous implant models have been developed using rabbits, rats or mice.[1)] Subcutaneous implantation experiments have the advantage of being relatively inexpensive and showing evidence of mineralization over a very short time course. These studies are relatively straightforward and, because of their ease, allow investigators to use very large numbers of animals. The advantage of the subcutaneous implantation model is that it repeatedly produces a pathologic morphology of dystrophic calcification consistent with that seen in both circulatory models and clinical explants.[2)] The disadvantage, and potential criticism, of this model is that the implanted materials do not reside within the host's circulatory system and do not experience the dynamic stresses imposed by implantation into the heart. In addition, these implants are not exposed to blood-borne proteins, lipids and cells which can all potentially affect valve cusp calcification.[3)] The utility of the subcutaneous model for

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screening anticalcification therapies has been further questioned recently following reports that several anticalcification strategies that worked well in subcutaneous implants were not effective when placed into a circulatory model. Carpentier et al.[4] showed that iron pretreatment of aortic valves followed by fixation with glutaraldehyde mitigated calcification in the subcutaneous rat model. When this same treatment was tested in the sheep circulatory model, however, the tissue not only did not resist calcification, but the pretreatment appeared to accelerate the mineralization process. Thus, additional factors, potentially not present in the subcutaneous rat model, may influence calcification in the circulatory system. A further important point is that calcification is more pronounced in the cusps than in the aortic wall when the calcification characteristics of aortic wall versus cusps are tested using rat subcutaneous implants,[5] a situation contrary to the findings gained when the same bioprostheses are implanted inside the circulatory system. It was a further goal of this study to assess the influence of blood contact on valve calcification.

Materials and Methods

Rat subcutaneous model

Glutaraldehyde-preserved porcine aortic valve preparation

Porcine hearts were obtained from a slaughter-house, dissected immediately and transferred into a cold solution of phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Following transport on ice to the laboratory the aortic tissue was immersed in purified 0.2% glutaraldehyde (Sigma Co., St. Louis, MO, USA) in PBS at 4°C. After one week of fixation the treatment was completed in 0.2% glutaraldehyde in PBS containing 1% isopropanol (Merck Co., Darmstadt, Germany; 4°C; 210 min). Immediately after fixation and prior to any further treatment, all samples were rinsed in cold PBS for 24 h (4°C). Discs 1.2 cm in diameter were punched out and trimmed at the adventitial side.

The animals

All animals received care in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institute of Health (NIH publication 85-23, revised 1985). The study was approved by the Ethics Committee of the Katholieke Universiteit Leuven. Sprague-Dawley-derived rats (body weight, 150-200 g) were used for the studies.

Surgical preparation

Male Sprague-Dawley rats were anaesthetized with an intraperitoneal injection of freshly prepared chloral hydrate (400 mg/kg; Vel Co., Leuven, Belgium). Transmural aortic wall and cusp discs were implanted subcutaneously into the abdominal wall. Four randomly assigned tissue discs, comprising two cusps and two aortic wall fragments, were implanted per animal in six rats.

Experimental protocol

After 8 weeks the tissue was retrieved for subsequent analysis.

Analysis of explanted tissue

a) X-ray assessment

To demonstrate and localize calcifications, X-ray images (60 kV, 150 mA, 2 seconds) were taken from each sample.

b) Histology

For histological examination, a longitudinal transection of the specimen through the middle of the cusp and the aortic wall respectively was embedded in paraffin. Sections 4 μm thick were routinely stained with hematoxylin and eosin (HE), Masson's trichrome stain for collagen, elastin-Van Giesson stain for elastin, phosphotungstic-acid-hematoxylin (PTAH) for fibrin, and von Kossa stain for calcium.

c) Transmission electron microscopy

Each specimen was examined by transmission electron microscopy (TEM). From each specimen, three to 10 1-mm blocks were embedded in Epon. Sections 1 μm thick were stained with toluidine blue and examined by light microscopy. The central part was selected for TEM. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Sections were treated with 2% potassium pyroantimonate to demonstrate calcium. Grids were examined in a Philips CM10 electron microscope. Random photographs were taken.

d) Quantitative calcium determination

Half of each specimen was used for quantitative calcium determination. After lyophilization, the tissue was pulverized, and desiccated to constant weight in an oven. Pulverized tissue was diluted in 20% HCl at a ratio of 10 mg dried tissue: 1 ml 20% HCl. Calcium content was measured by flame atomic absorption spectrometry, and expressed as μg per mg of dry cuspal weight.

e) Data management and statistical analysis

Replicate data were calculated and expressed as mean ±

standard error of the mean (SEM). Two-way ANOVA was performed on calcium content data with time of implantation and type of sample (cusp or wall) as independent factors and calcium content as the dependent factor. Posthoc testing with the LSD-test was performed if ANOVA yielded $P<0.05$.

Blood contact model

Glutaraldehyde-preserved porcine aortic valve preparation

The same procedure was followed as described in the rat subcutaneous model. Discs of 2 cm diameter were punched out and trimmed at the adventitial side.

The animals

This experiment was performed in six sheep. All animals were cared for by a veterinarian in accordance with the "Guide for the Care and Use of Laboratory Animals" published by NIH. The Ethics Committee of the Katholieke Universiteit Leuven approved the study. Juvenile sheep were bred especially for this study. The sheep weighed 38.3±2.1 kg (mean ± standard deviation; range, 35 to 40 kg).

Surgical preparation

Juvenile sheep were fasted for 48 hours and premedicated with ketamine at 10-20 mg/kg intramuscularly before induction of anaesthesia. After the animal was placed in the right lateral decubitus position, a peripheral intravenous line was placed in the left lower limb using an 18-gauge angiocatheter. Ampicillinum anhydricum Albipen LA (Mycofarm Co., Brussels, Belgium) at 15 mg/kg was given intramuscularly for antibiotic prophylaxis and maintenance intravenous fluids were given with crystalloid fluid. A three-lead electrocardiogram was used to monitor heart rate and rhythm. Anaesthesia was induced with halothane-oxygen mixture. The animal was intubated and ventilated with a respirator (Engstrom 200). All ventilation parameters were adjusted to keep the blood gas values within the normal range. Anaesthesia was maintained with a mixture of 0.5% to 2% halothane and oxygen, and bolus doses of fentanyl (Durotep, Janssen Co., Beerse, Belgium) as needed. A fluid filled pressure catheter was inserted into the ear artery. The left side of the neck was shaved, prepared, and draped in sterile fashion. The left jugular vein was exposed. After heparin administration in each of the six sheep, three aortic wall discs and three cusps were implanted within the wall of the left jugular vein as shown in Fig. 1. The skin incision was closed in layers. After the animal was turned to the left lateral position, the right side of the neck was shaved, prepared, and draped in a sterile fashion. The right jugular vein was exposed. Three aortic wall discs and three cusps were similarly implanted in the right jugular vein. The skin incision was closed in layers.

Fig. 1. A representative view of the implantation of a valve piece in the jugular vein. This macroscopic view shows 3 quadrangular fragments of aortic wall (top) and 3 smaller fragments originating from the cusps (bottom) immediately after implantation.

Postoperative treatment

After extubation, the sheep were kept in the recovery room for 1 day and returned to a controlled animal facility where the general health of the animal was checked daily.

Experimental protocol

After 3 and 6 months the tissue was retrieved for subsequent analysis.

Analysis of explanted tissue

The explanted tissue was analysed in the same way as in the rat subcutaneous model, including X-ray, histological, ultrastructural examination and data management.

Pulsatile model

The animals

The experiments were carried out on six sheep. All animals were cared for by a veterinarian in accordance with the "Guide for the Care and Use of Laboratory Animals" published by NIH. The Ethics Committee of the Katholieke Universiteit Leuven approved the study. Ju-

venile sheep were especially bred for this study. The sheep weighed 51.7±2.1 kg (mean ± standard deviation; range, 35 to 63 kg).

Valves studied
Stentless porcine aortic valves, fixed in 0.5% glutaraldehyde at low fixation pressure (less than 2 mmHg) and no anticalcification treatment (Toronto SPV, St. Jude Medical, Minneapolis, MN, USA) were selected for this study.

Implantation
The valves were implanted as described previously.[6,7] The animals were premedicated with ketamine (10-20 mg/kg intramuscularly). Anaesthesia was induced and maintained with halothane and N_2O. Fentanyl (Janssen Co.) was administered in boluses as necessary. After endotracheal intubation, mechanical ventilation was instituted. A left thoracotomy was performed through an incision in the second intercostal space. The main pulmonary artery was isolated. After administration of 3 mg/kg heparin (Rhone-Poulenc Rorer Co., Brussels, Belgium) intravenously, a pneumatic right ventricular assist system (Medos-HIA VAD, 54 ml ventricle, Medos-Helmholtz Institute, Aachen, Germany) was installed with the inflow cannula in the right atrium and the outflow cannula 1 cm before the pulmonary bifurcation. Proximal and distal clamping of the pulmonary artery were carried out. After transection of the pulmonary artery, a stentless valve was interposed using continuous 4-0 prolene suture for both proximal and distal anastomoses. After removal of the clamps, the native pulmonary valve was destroyed by tearing two cusps with a clamp introduced through a pursestring suture placed at the sinuses, and afterwards the Medos system was stopped. The chest was closed in layers with a chest drain in the left pleural space. After waking up, the animal was extubated and brought to the recovery room. Feeding was allowed immediately. Intravenous fluid administration was stopped after 2 hours. The chest drain was removed after 6 hours.

The animals received analgesics (piritramide, Dipidolor, Janssen Co.) for the first two days on regular schemes and diuretics as necessary. Antibiotics (ampicillinum anhydricum, Albipen LA, Mycofarm Co.) and low molecular weight heparin (enoxaparine, 20 mg twice daily, Clexane, Rhone-Poulenc Rorer Co.) were administered for 6 days. Afterwards, the sheep returned to the controlled animal facility where their general health was checked daily.

Explantation and analysis
Three out of the six valves were explanted after 3 months and the remaining 3 after 6 months. Sheep were premedicated and anaesthetized as described above. The left thoracotomy was reopened and the heart dissected free. The valve function was studied using echocardiography. Heparin at 3 mg/kg was administered, and after exsanguination, the valve was excised together with the proximal and distal parts of the pulmonary artery.
a) Examination of macroscopically identifiable features
An overall inspection was made of the valves, and photographs were taken. The valve was longitudinally transected through the commissures. Each of the three specimens thus includes a pre- and post-valvular part of the sheep pulmonary artery, together with a part of the porcine aortic wall (wall of the stentless valve), and respectively a right coronary cusp (RCC), a non-coronary cusp (NCC), or a left coronary cusp (LCC). Another set of photographs was taken.
b) X-ray assessment
X-ray examination (face, profile) was performed as described above to demonstrate and localize overall calcification.
c) Histology
Histology was performed as described above.
d) Quantitative calcium determination
Half of each specimen (RCC, NCC, LCC) was used for quantitative calcium determination. The cusps were divided into three subsegments: the commissural area, basal part, and free edge. The procedure for calcium determination was the same as described above.
e) Data management and statistical analysis
The data management was performed in the same way as in the rat subcutaneous model.

Results

Epicardial echocardiographic study
At the time of valve explantation, echocardiography was performed in the six animals with Toronto SPV stentless valves. All valves at 3 and 6 months showed well functioning without evidence of valve regurgitation.

X-ray examination
Two months after subcutaneous implantation in the rat, calcification was clearly evident both in the aortic wall samples and in the cusps. The degree of calcification in

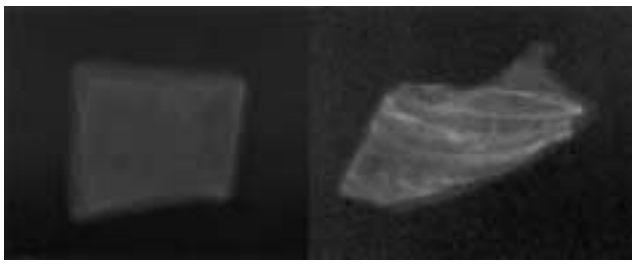

Fig. 2. A representative example of an X-ray taken of an aortic wall portion (left) and of a cusp (right) explanted after 2 months in the rat subcutaneous model. The degree of calcification in the cusps is higher than that in the aortic wall.

the cusps was higher than that in the aortic wall (Fig. 2). On the other hand, after 3 months in the blood contact model in sheep, the cusps were slightly calcified and calcification in the aortic wall was more pronounced (Fig. 3). At 6 months after implantation, in the blood contact model, the cuspal calcification increased somewhat and calcification in the aortic wall became severe (Fig. 4). These results in the blood contact model resemble that of stentless porcine aortic valves fixed using glutaraldehyde without anticalcification treatment in the pulsatile model. Calcification in both the cusps and in the aortic wall increased with time. In addition, calcification in the aortic wall occurred earlier than in the cusps in the blood contact model.

Light microscopy

In the rat subcutaneous model, the calcified areas, which stain darkly with von Kossa stain, were seen in the centre of the cusps (Fig. 5). On the other hand, aortic wall calcification was limited to two bands along the intima and the adventitia surfaces, with no to very little sign of calcification in most of the media (Fig. 6). In general, cusps were more calcified than aortic wall tissue. In the blood contact model, no cuspal calcification was seen after 3 months (Fig. 7). However, half of the cusps had calcific nodules after 6 months (Fig. 8). In the aortic wall, calcification was limited to two dense bands along the intima and the adventitia surfaces (Fig. 9). The features of aortic wall calcification were similar to those in the rat subcutaneous model. However, aortic wall calcification was denser in the blood contact model than in the rat subcutaneous model. After 6 months, aortic wall calcification increased and extended more towards the media (Fig. 10). There was more calcification in the aortic wall than in the cusps in the blood contact model.

Transmission electron microscopy

In the rat subcutaneous model, calcific nodules are most frequently associated with collagen fibres and substrate and occasionally with elastin fibres, and could be readily identified in the centre of the aortic wall. Several partially confluent calcific nodules in the cusps were also frequently observed in association with collagen fibres.

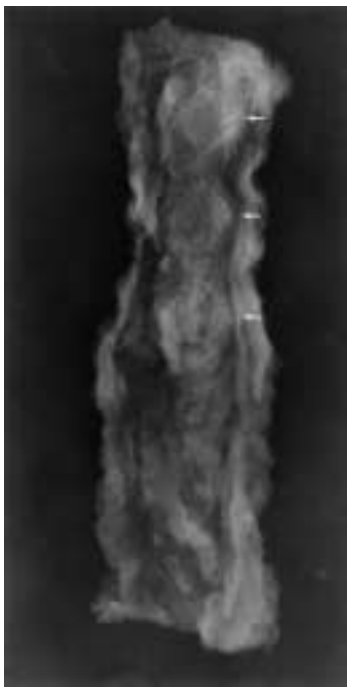

Fig. 3. A representative example of an X-ray taken of aortic wall samples and cusps explanted after 3 months in the blood contact model. The calcification (arrows) within the 3 quadrangular fragments of aortic wall (top) is clearly visible. Cusps (bottom) show virtually no calcification and cannot be discerned with certainty at this moment (compare to Fig. 4).

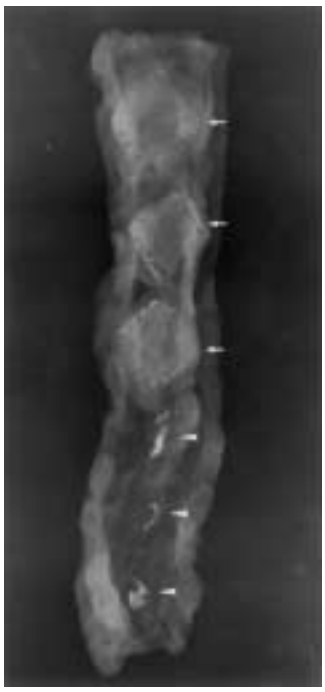

Fig. 4. A representative example of an X-ray taken of the aortic wall samples and the cusps explanted after 6 months in the blood contact model. Compared to Fig. 3, the cuspal calcification (arrowheads) is now more pronounced and can be clearly identified (bottom). The calcification (arrows) in the aortic wall quadrates remains severe (top).

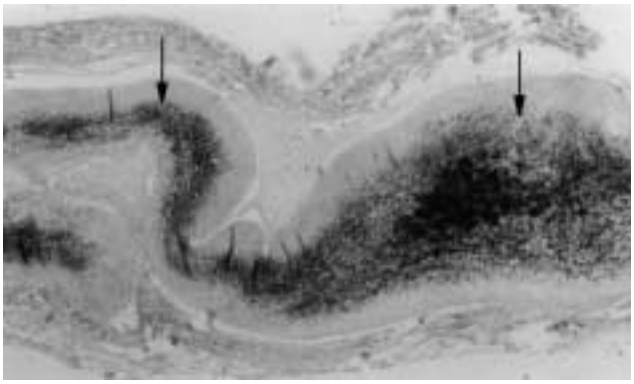

Fig. 5. A representative example of the light microscopy general view using cusps explanted after 2 months in the rat subcutaneous model. Calcification (arrows) is seen in the centre of the cusps. (von Kossa stain, original magnification ×40)

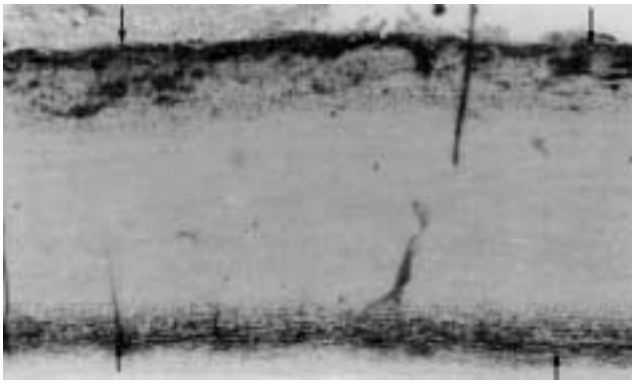

Fig. 6. A representative example of the appearance under light microscopy of the aortic wall explanted after 2 months in the rat subcutaneous model. Aortic wall calcification (arrows) is limited to two bands along the intima and the adventitia surfaces. (von Kossa stain, original magnification ×40)

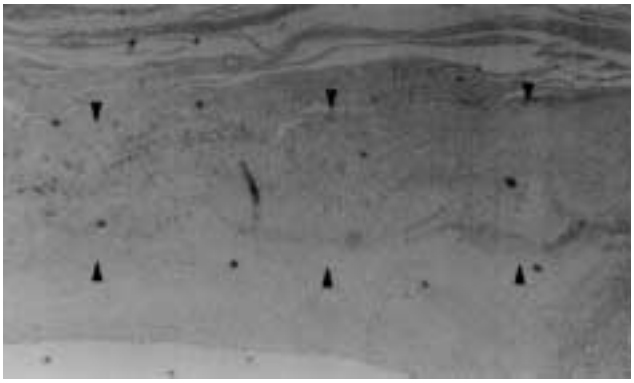

Fig. 7. A representative example of the appearance under light microscopy of the cusp (delineated by arrowheads) explanted after 3 months in the blood contact model. After 3 months, no calcification is seen. (von Kossa stain, original magnification ×40)

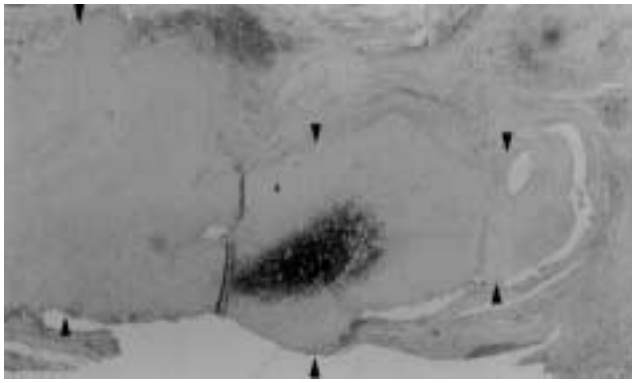

Fig. 8. A representative overview of appearance under light microscopy is shown in a cusp (arrowheads) explanted after 6 months in the blood contact model. A single focus of calcification is seen within the cusps. (von Kossa stain, original magnification ×40)

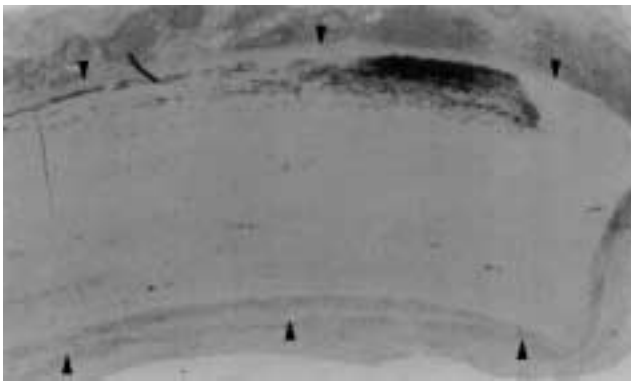

Fig. 9. A representative example of the appearance under light microscopy of the aortic wall (arrowheads) explanted after 3 months in the blood contact model. Aortic wall calcification increases and extends more toward the media. (von Kossa stain, original magnification ×40)

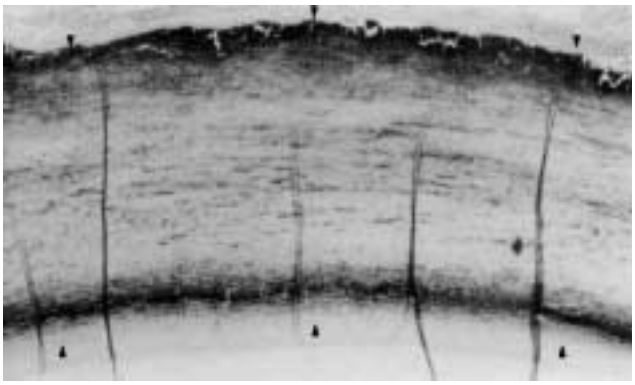

Fig. 10. A representative general view under light microscopy is shown from the aortic wall (arrowheads) after 6 months in the blood contact model. Massive calcification extends from the outer areas into the centre of the media. (von Kossa stain, original magnification ×40)

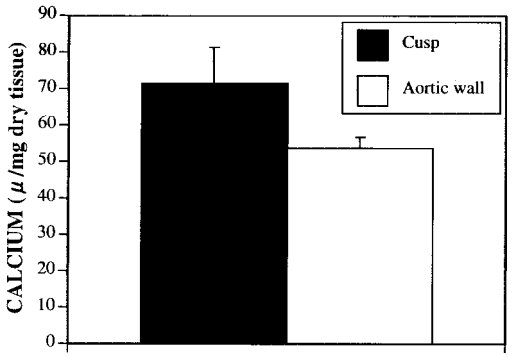

Fig. 11. Calcium content analysis of samples implanted for 2 months in the rats. There is more calcium in the cusp than in the aortic wall.

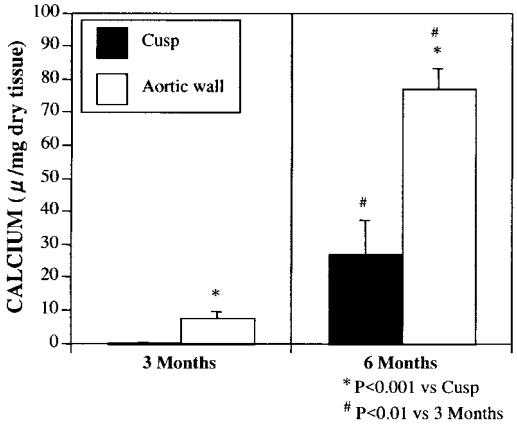

Fig. 12. Calcium content analysis of samples implanted in the jugular vein in sheep (blood contact model). There is significantly more calcium in the aortic wall than in the cusp.

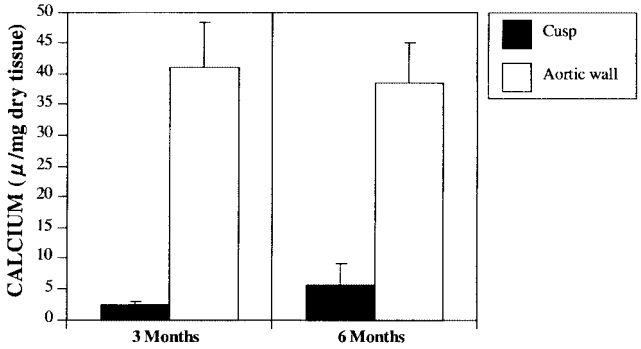

Fig. 13. Calcium content analysis of stentless porcine aortic valves, fixed in glutaraldehyde at low fixation pressure and no anticalcification treatment in the pulsatile model. The results from the pulsatile model are very similar to those from the blood contact model. In the blood contact model, aortic wall calcification significantly increases with time. On the other hand, aortic wall calcification in the pulsatile mode is already seen at 3 months.

In the blood contact model, the findings were qualitatively similar to the rat subcutaneous model with respect to calcification of the aortic wall. However, large calcified bodies were more frequently identified than in the rat subcutaneous model. Calcification was mainly visible between collagen bundles in the cusps. In addition, aortic wall and cusp calcification tended to increase with time.

Calcium content

The calcium analysis of samples implanted for 2 months in the rats suggests that there was more calcium in the cusp than in the aortic wall although the difference is not statistically significant (71.5±9.7 μg/mg dry tissue vs. 53.7±2.6, p=0.09) (Fig. 11). The calcium content of samples implanted in the jugular vein in sheep demonstrated that there was significantly more calcium in the aortic wall than in the cusp (3 months, 7.9±1.5 vs. 0.3±0.1, p<0.0001; 6 months, 77.2±6.1 vs. 27.2±10.2, p=0.0002) (Fig. 12). In addition, aortic wall and cusp calcification significantly increased with time (aortic wall: p<0.0001, cusp: p=0.008). Quantitative calcium content analysis showed that calcification of the aortic wall was markedly more pronounced than that of the cusps in the pulsatile model (3 months, 41.2±7.1 vs. 2.4±0.7; 6 months, 38.5±6.5 vs. 5.6±3.5) (Fig. 13). None of these values differ significantly from each other (p>0.05). Calcium content in the aortic wall from the pulsatile model is already high at 3 months.

Discussion

In this study, we can confirm the necessity of blood contact as a factor of in vivo pre-clinical valve testing. Indeed, the results from the rat subcutaneous model were completely opposite to those in the blood contact model. Calcium content in the blood contact model demonstrated that there was significantly more calcium in the aortic wall than in the cusp at any time. Furthermore, calcification characteristics in the blood contact model were very similar to those in the pulsatile model that we tested. The differences among those three models are intriguing.

Differences between the rat subcutaneous model and the blood contact model

The advantage of the rat subcutaneous implantation model is that it reproduces clinically noted bioprosthetic tissue mineralization and provides a well-characterized calcifi-

cation model that is economical, quantitative, and rapid. The disadvantage and potential criticism of this model is that the implanted tissue does not reside in the host's circulatory system and therefore does not experience the stresses imposed by implantation into the heart. Moreover, there is no blood-surface contact, thereby preventing assessment of interactions with blood-borne substances, particularly platelets, coagulation factors, lipoproteins, and lipids.[2,3] Therefore, the utility of the rat subcutaneous model for in vivo valve testing before clinical use has been further questioned. Carpentier et al.[4] reported that large discrepancies were found between the results from the rat subcutaneous model and those from implantation in the sheep circulatory model. In the circulating blood environment, contrary to the subcutaneous environment, iron treatment of aortic valves not only failed to mitigate calcification, but actually accelerated it, and calcification occurred much earlier than in the untreated control valves. Thus, additional factors, potentially not present in the rat subcutaneous model, may influence calcification in the circulation.

Differences between the blood contact model and the circulatory model

We have already demonstrated that there was significantly more calcium in the aortic wall than in the cusp at any time in the pulsatile model (Fig. 13). At first glance, these results from the pulsatile model were very similar to those obtained in the blood contact model. However, in the blood contact model, aortic wall calcification significantly increased with time, while in our pulsatile model it was already high at 3 months and remained at this level at 6 months. The mechanism of early aortic wall calcification is not yet understood, and its elucidation would shed some light on the general mechanism by which it occurs. The question now becomes as to why such early aortic wall calcification occurs in the pulmonary position but not in the jugular vein environment. The important difference that exists between these two environments is that dynamic stress is present only in the pulmonary position. Our hypothesis is that dynamic stress enhances the preliminary ionic diffusion such as that of Ca^{2+} and PO_4^{3-}.[8] We consider bioprosthetic valve mineralization to be a process based on the formation of hydroxyapatite nuclei, which accompanies preliminary diffusion phenomena. The initial mineralization occurs when Ca and PO_4 ions diffuse through the interstitial space and pores of the collagen pores present in the unmineralized matrix. Thus, the concentration of these ions is increased locally, and they bind with collagen to form hydroxyapatite crystal nuclei. Therefore, the diffusion mechanism can be enhanced by dynamically expanding the diffusion paths of Ca and PO_4 ions. Accordingly, it is assumed that diffusion is largely affected by the dynamic effect of creating diffusion paths by applying tensile stress in the biaxial and triaxial directions. This mechanism was proposed by Yokobori et al.[8]

We conclude that (1) blood contact is a prerequisite factor in initial mineralization, and dynamic stress enhances the diffusion paths of Ca and PO_4 ions; (2) blood contact and dynamic stress play an important role in aortic wall calcification; and (3) this may explain in part the mechanism of aortic wall calcification.

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