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Calcification and identification of metalloproteinases in bovine pericardium after subcutaneous implantation in rats

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The purpose of this study was to evaluate the influence of two anticalcification pre-treatments (chloroform/methanol and ethanol) and serum conditioning of glutaraldehyde-crosslinked bovine pericardium on the calcification degree and the presence of gelatinase activities in a subcutaneous implantation model in rats. Regarding calcification of the implants, glutaraldehyde control treatments showed a significatively higher calcification degree than pericardium treated with anticalcification reagents. Serum conditioning of glutaraldehyde treated tissues did not influence the calcification degree; moreover, no differences were found in these samples with the time of implantation (30 and 90 days). On the other hand, anticalcification treatments resulted in a very significant decrease in the calcium content in the implanted membranes.

Gelatinase activities were detected by gelatin zymography in almost all the implanted samples. However, control tissues with and without serum conditioning showed less gelatinase activities than those samples pre-treated with anticalcification treatments. Metalloproteinase (MMP-2) activity was detected in all the samples analyzed but a higher expression of MMP-9 was detected in those implants treated with chloroform/methanol and ethanol. Additional gelatinase activities showing lower molecular weight than MMP-2 were also detected in both anticalcification treated samples. The presence of these gelatinase activities is probably due to host cellular infiltrates and could contribute to the biomaterial degradation.
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Introduction

Cardiac biological prostheses are collagen-based biomaterials and their advantage versus metallic ones is that they do no need anticoagulant therapy that is continuously required when using the metallic ones [1]. However, biological prostheses have two main problems: degradation and calcification of the soft tissue [2, 3] . The origin of these problems is not established. Regarding calcification, it has been proposed that connective tissue cells could be involved [4–6], as well as noncollagenous extracellular proteins [7, 8], matrix vesicles [9, 10], tissue lipids [11–16], and the chemical treatments to which they were subjected in order to increase the *in vivo* stability of the bioprosthesis [17–22]. Moreover, serum proteins are known to also play an important role in the mineralization of tissues. On this idea, Santin *et al.* [23] have described that glutaraldehyde (GA) crosslinked collagen sponges adsorb serum proteins ranging from 10 to 203 kDa, specially a 10 kDa protein arising from a complement C3 fragment and fibronectin. On the other hand, after treatment of the sponges with $FeCl_3$, an anticalcification treatment, the adsorption pattern changed showing a remarkable increase in a 29 kDa protein. Gura *et al.* [7] have described the presence of several proteins, derived from calcium-binding non-collagenous proteins of bone and teeth, in mineralized bovine pericardium after a 60 days' implantation period in rats, showing a different pattern of protein adsorption in dependence of the amount of mineral deposition in calcified bovine pericardium.

Concerning the enzymatic biodegradation of soft tissue biomaterials, our group has previously determined

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the presence of metalloproteinases in bovine and porcine untreated pericardium as well as in pericardium pre-treated with different chemical crosslinking agents (unpublished results). Metalloproteinases (MMPs) are members of a family of enzymes which under normal physiological conditions contribute to tissue remodeling and reparation and play important roles in embryo development and morphogenesis and in numerous diseases [24–28]. Regarding their possible role in cardiovascular materials degradation, Simionescu *et al.* [29, 30] have reported the presence of matrix metallo-proteinases in human pathologic cardiac valves and in pericardium-derived bioprosthetic valves [29]. The aim of this study is to analyze the presence of MMPs, and other possible gelatinase activities, in calf pericardium incubated *in vitro* in the presence of bovine serum (BS) after subcutaneous implantation in rats to determine its relationship with tissue's calcification and degradation. We have also analyzed the effectiveness of two antic-alcification pre-treatments previous to GA-crosslinking, chloroform/methanol [14, 16, 31] and ethanol [32, 33] and the influence of these treatments in the presence of gelatinase activities in the materials after *in vivo* implantation.

Material and methods

Tissue chemical treatments

Pericardium from young calves was obtained directly from a local slaughterhouse and transported to our laboratory in sterile saline solution 0.9% (w/v) NaCl. The tissue was cleaned to remove fat and portions were selected for biochemical studies and for subcutaneous implantation in rats.

The bovine pericardium membranes were subjected to the chemical treatments, as follows. A group of control specimens was treated for 24 h with 0.625% GA in 0.1 M sodium phosphate, pH 7.4, prepared from a commercial 25% (v/v) solution (Merck, Darmstadt, Germany). Pieces of tissue were also treated with a 1 : 4 (v/v) chloroform/methanol (CM) solution in a weight/volume ratio of 1/30 (g/ml) for 24 h [14, 34]. After the CM-treatment, the tissue was exhaustively washed with saline solution and subjected to GA-treatment as described for the control group. Other group of samples was pre-treated with ethanol (ET) by immersion in 80% ethanol in 50 mM HEPES, pH 7.4 at room temperature for 24 h, as described elsewhere [32, 33], followed by an additional treatment with GA as in the control group.

Serum conditioning of the tissue

Chemically modified pericardium specimens were incubated with antibiotic-enriched (100 U/ml penicillin and 10 µg/ml streptomycin) neonatal bovine serum (BS) (Sigma, St. Louis, MO, USA) for 48 h at 37 °C in static conditions using a weight/volume ratio of 1 mg of tissue/ 100 µl of BS. Afterwards, the tissue portions were washed three times for 10 min in 40 ml phosphate buffered saline, pH 7.4, at room temperature.

Subcutaneous implantation in rats

Wistar rats weighing 110–130 g (5–6 weeks old) were used for the subcutaneous implantation of chemically modified pericardium specimens. Six pericardium disks measuring 1 cm in diameter were implanted close to the abdominal wall muscle of each of the 20 animals employed in the study. Eight rats were used exclusively for the control group of GA-treated pericardium without serum conditioning; four of them were sacrificed 30 days after the implantation and the other four, 90 days after the implantation. On the other hand, the different serum-conditioned chemically-modified pericardium specimens were implanted into the same rats. Thus, two GA + BS discs were sutured to the upper position of the abdominal wall, two CM + BS disks were placed in the middle part, and the two remaining ET + BS disks were implanted in the inferior part of the rat's abdominal wall. Twelve rats were used for these mixed implantation; six of them were sacrificed 30 days after the implantation and six after 90 days.

At the end of the implantation periods, the rats were killed by CO_2 asphyxiation and the different implants were surgically excised, carefully freed of any sur-rounding host tissue, and washed in sterile saline solution. From the six pieces of tissue implanted in each rat, the three from the right part of the abdominal wall were cut in two halves; one for biochemical studies (SDS-PAGE and gelatin zymography) and the other half and the left side disks, for calcium quantification. The calcium accumulated on the different implants was quantified by atomic absorption as previously described [16, 34], and was expressed as µg of calcium/mg of dry tissue ($\pm$ SD).

Electrophoretic analysis and gelatin zymography

Portions of the surgically excised pericardium mem-branes were minced manually and suspended in extraction buffer (50 mM Tris, pH 7.4, containing 0.25% (v/v) Triton X-100 and 0.5% (w/v) SDS) at 1.25% (w/v). Samples were incubated 48 h at 4 °C and, afterwards, the tissue fragments were homogenized for 5 min using a glass mortar and pestle. After centrifuga-tion for 10 min at 14 000 rpm, the supernatants were collected and the protein concentration analyzed by the Amido Black method [35] and by the method described by Bradford [36] using bovine serum albumin as standard.

Supernatants were prepared for electrophoretic ana-lysis by addition of three-fold-concentrated loading buffer [1× loading buffer: 62.5 mM Tris, pH 6.8, containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.02% (w/v) bromophenol blue] followed by heat-denaturation 5 min at 90 °C . Samples (15 µg protein) were analyzed by SDS-PAGE according to Laemmli (1970) using 4% stacking gels and 10% resolving gels in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA, USA) at 25 mA/gel. The gels were afterwards routinely stained with Coomassie brilliant Blue R250 (Sigma). Pre-stained molecular weight standards from Bio-Rad were used for molecular weight determination.

Samples for gelatin zymography were redissolved as described above, but in absence of thiol reducing agents and without thermal denaturation, as previously described [37]; 15 μg of protein were loaded into 10% SDS-containing polyacrylamide resolving gels copolymerized with 1 mg/ml gelatine (Sigma). After electrophoresis, the gels were washed in 50 mM Tris, pH 7.4 containing 2.5% (v/v) Triton-X-100 for 30 min, and two times with 50 mM Tris, pH 7.4, in order to remove SDS and to allow the refolding and reactivation of the gelatinase activities. The gels were then incubated for three days in 50 mM Tris, pH 7.5, 0.15 M NaCl, 10 mM $CaCl_2$, 0.1% (v/v) Triton X-100 and 0.02% (w/v) sodium azide and were stained with Coomassie brilliant Blue R250 and washed afterwards with 7.5% (v/v) acetic acid containing 20% (v/v) methanol. A lane with fibroblast conditioned medium is normally included as a standard for MMP-2 and MMP-9 mobility [37].

Statistical analysis

To study the calcium accumulation during subcutaneous implantation in rats, first the Kolmogorov-Smirnov test was performed to ensure that the normality hypothesis was not rejected in either case. Then, intragroup comparison was carried out using analysis of variance (ANOVA) and the Newman–Keuls multiple comparisons test and intergroup comparison by means of a Student's test.

Results and discussion

We have used a total number of 20 rats, each of them with six implants, in order to check the induction of tissue calcification and the presence of gelatinase activities after 30 or 90 days of subcutaneous implantation into the abdominal wall of different chemically modified pericardium membranes with or without serum conditioning.

Calcification of implanted pericardium membranes after subcutaneous implantation in rats

After the different implantation times, the pericardium implants were surgically excised and the calcium content was determined by atomic absorption (Table I). In general, a high variability in the calcification degree of the implants was detected, mainly affecting to GA-treated pericardium with or without serum conditioning. These samples presented a high degree of calcification that ranged from 7.0 to 71.5 mg Ca/g of dry tissue in GA-treated pericardium and from 4.7 to 45.3 mg Ca/g in the GA + BS group, both after 30 days implantation. Results after a three months implantation period in these two groups were quite similar, also showing a relatively high standard deviation. This high variability in the calcification of GA-treated pericardium is quite normal after *in vivo* implantation and has been also previously reported [18]. Statistical analysis of the data from all the GA-treated groups revealed that there were no significant differences in the calcification degree between GA and GA + BS neither at 30 nor at 90 days subcutaneous implantation in rats. Thus, serum proteins adsorbed by GA-treated pericardium seem not to influence the calcium uptake by the implant.

On the other hand, the anticalcification treatments with CM or ET followed by serum conditioning of the chemically modified pericardium membranes resulted in a significantly lower degree of calcification ($p < 0.001$) when these groups were compared with both GA and GA + BS implants at both times of implantation (Table I). However, no significant differences between both anticalcification treatments were detected; only a slight increase in the calcium content was observed in both groups with time of implantation (1.2 ± 4.0 to 6.8 ± 11.8 mg Ca/g and 0.2 ± 1.0 to 4.0 ± 7.3 mg Ca/g in CM + BS and ET + BS groups, respectively, from 30 to 90 days implantation). If these results are compared with those previously reported by our group and other groups regarding the anticalcification effectiveness of the CM and ET treatments of pericardium [14, 16, 32, 33], it can be concluded that serum conditioning of the chemically modified membranes does not induce any significant variation.

Electrophoretic analysis of proteins present in pericardium implants

We have analyzed the different chemically modified pericardium implants by SDS-PAGE in order to establish whether there is any specific protein pattern associated with calcification or if the efficacy of the anticalcification treatments is associated with the adsorption of any particular protein. Samples from all the rats used in the study were analyzed: twelve controls (GA group) and six samples from the rest of the groups (GA + BS, CM + BS and ET + BS) were analyzed at each time point. Fig. 1 shows a representative gel with samples obtained after 30 days implantation (the tissue serum conditioned samples were obtained from the same rat). No significant differences in the protein band pattern between the different groups or implantation times were obtained when the same amount of protein was loaded into each lane, even though the yield of protein extraction among

TABLE I Calcium content in chemically modified implanted pericardium membranes

Time of implantation	Calcium content (mg/g dry tissue)			
	GA	GA + BS	CM + BS	ET + BS
30 days	35.0 ± 21.3	24.6 ± 13.4	1.2 ± 4.0	0.2 ± 1.0
90 days	30.1 ± 8.5	24.3 ± 17.3	6.8 ± 11.8	4.0 ± 7.3

Pericardium membranes were treated as described in Materials and Methods and were implanted into the abdominal wall of Wistar rats for 30 or 90 days. After these periods of time, the membranes were surgically excized, cleaned from host tissue adherences and calcium content was determined by atomic absorption.

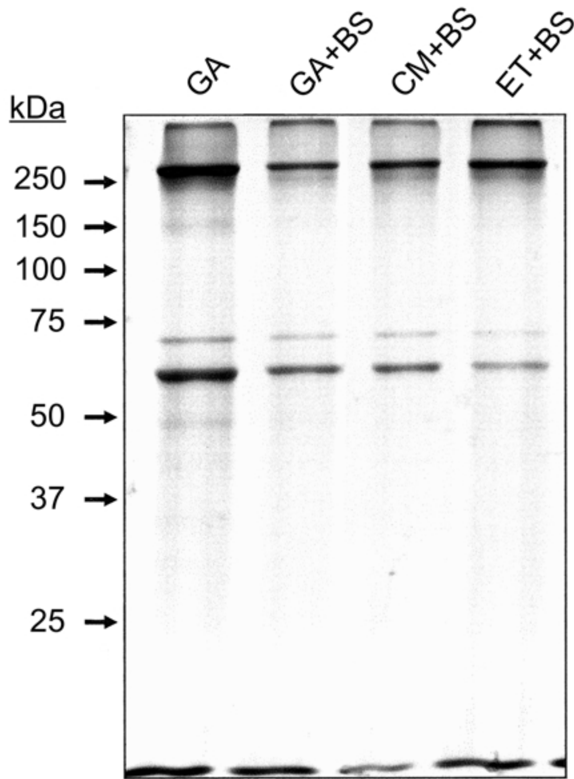

Figure 1 Electrophoretic analysis of proteins extracted from homogenized pericardium implants. Surgically excised implants were homogenized as described in Materials and Methods, and the supernatants were analyzed by SDS-PAGE using 10% polyacrylamide resolving gels. GA: glutaraldehyde-treated implant; GA + BS: serum conditioned GA-treated implant; CM + BS: serum conditioned chloroform/methanol pre-treated implant; ET + BS: serum conditioned ethanol pre-treated implant. Molecular weight standards are shown.

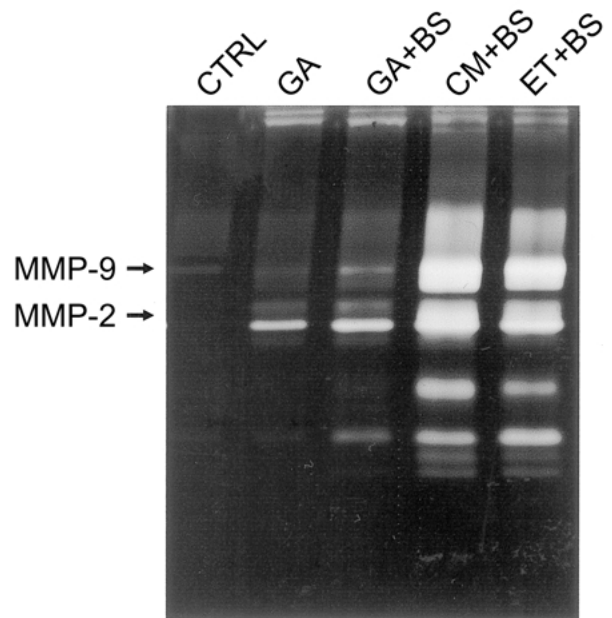

Figure 2 Gelatin zymography of proteins extracted from homogenized pericardium implants. Samples were obtained as described for the SDS-PAGE analysis and were loaded into gelatin-polyacrylamide gels without thermal denaturation. CTRL: control unimplanted GA-treated pericardium; GA: glutaraldehyde-treated implant; GA + BS: serum conditioned GA-treated implant; CM + BS: serum conditioned chloroform/methanol pre-treated implant; ET + BS: serum conditioned ethanol pre-treated implant. The mobilities of MMP-2 and MMP-9 from fibroblast cultures are shown.

the different groups varied. As observed in Fig. 1, two major proteins were extracted with apparent molecular masses of 64 and 330 kDa; a fainter band appeared at 73 kDa and much fainter ones at around 50 and 150 kDa. No correlation was found between the calcification of pericardium membranes and the presence or absence of major protein bands extracted from the implanted materials. Moreover, these proteins probably are host proteins adsorbed to the different chemically modified pericardium membranes, since we have previously reported that after exhaustive GA treatment of pericardium almost no proteins can be extracted by the procedure herein described (unpublished results).

Gelatin zymography

Zymography was used to identify the major gelatinase activities present in the implanted membranes. The same number of samples used for the electrophoretic analyzes were used for zymography. Fig. 2 shows a representative gel with one sample from each group after 30 days implantation and a control with unimplanted GA-treated pericardium. Since the same amount of total protein (15 μg) was loaded into each lane, differences in the gelatinolytic activity bands can be directly correlated with the amount of gelatinase present in the implants. Almost no gelatinase activity was detected in the unimplanted GA-crosslinked tissue, but several activity bands were detected in the implanted materials. In general, no significant differences were detected between samples implanted 30 or 90 days. Calcified samples from the GA and GA + BS groups showed low gelatinase activity (negligible in some GA samples) compared to the non-calcified membranes from the CM + BS and ET + BS groups. In all the samples, except in the already mentioned few cases were no activity was detected, MMP-2 activity was detected as a doublet around 70 kDa. In contrast to the normal finding in calf pericardium, were the predominant band is normally the pro-enzyme form of higher molecular mass, we have found that in all the implanted pericardium membranes, the major MMP-2 form is the active one showing a higher electrophoretic mobility. MMP-9 is almost not detected in GA or GA + BS membranes, while in non-calcified tissues from the CM + BS and ET + BS groups, this activity is similar or stronger than MMP-2.

Strong gelatinase activity bands were also detected around 37 and 44 kDa in all CM + BS and ET + BS samples; two fainter bands with higher mobility were detected in some of these samples (shown in Fig. 2). In the GA group, these additional gelatinase bands were not detected, but the 37 kDa band appeared in some of the GA + BS samples (Fig. 2).

Since MMP and gelatinase activities are almost null in unimplanted GA-treated bovine pericardium, the presence of gelatinase activities in the implanted materials is probably originated by cellular infiltration from the host surrounding tissue. The higher levels detected in those samples from the CM + BS and ET + BS groups could result in a lower in vivo persistence of such materials. Recently, Vyavahare [32, 33] have reported that the pretreatment of pericardium with ethanol yields significant structural changes in the tissue. Moreover, Simionescu

et al. [29, 30], have described that ruptured cardiac bioprosthetic valves show dissociated collagen bundles and tissue ''crevices''. All these changes that affect the structure of pericardium, could facilitate host cell infiltration and lead to a faster biodegradation. If mechanical stress is added to this process, all these chemical treatments which render irreversible structural damage, will reduce enormously the bioprostheses half-life.

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