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Inhibition of extracellular-matrix degrading proteases in pericardial-derived cardiovascular bioprostheses

D. T. SIMIONESCU, R. DEAC, A. SIMIONESCU
Cardiovascular Surgery Research Department, Public Health and Medical Research Institute, PO Box 118-4300, Tirgu Mures, Romania

The presence of intrinsic remnant extracellular-matrix degrading proteases in glutaraldehyde-treated bovine pericardium *per se* and in explanted bioprosthetic heart valves has been demonstrated recently by us. These enzymes were shown to degrade type I collagen and thus could contribute to a possible *in vivo* slow tissue degeneration in time. We have investigated the potential use of protease inhibitors for effective control of this degenerative process. Collagenase, trypsin and chondroitinase were inhibited *in vitro* by 0.5% glutaraldehyde to 10–20% of initial activity; these remnant activities could be further inhibited by their specific inhibitors suggesting that a combination of glutaraldehyde and a mixture of enzyme inhibitors could be applied to bovine pericardium. Analysis of glutaraldehyde-treated tissue extracts by gelatin zymography revealed the presence of at least four distinct enzyme species which were active towards a collagen substrate and were inhibited most effectively by ethylenediaminetetraacetic acid and thus could be included in the matrix-metallo-proteinase family. Bovine pericardial fragments that were treated with a combination of protease inhibitors and glutaraldehyde showed a reduced colagenolytic activity compared to glutaraldehyde alone; furthermore, upon implantation in the rat subcutaneous model the proteolytic activities found in these preparations were further reduced.

1. Introduction

The pericardium is a three-dimensional extracellular matrix complex that performs *in vivo* both mechanical and metabolic functions. The major protein constituents known to date are type I collagen, a low molecular weight dermatan sulphate proteoglycan (decorin) and elastin. For use as a biomaterial, i.e. in the form of bio-artificial cardiac valves, the fibrous pericardium is crosslinked in 0.2–1% buffered glutaraldehyde, a treatment that renders the tissue quite resistant biomechanically, non-thrombogenic, non-antigenic and biocompatible. However, recent data show that its performance as a biomaterial is limited by factors related to mechanical fatigue, calcium deposition and a putative ground matrix degeneration. The presence of intrinsic remnant extracellular-matrix degrading proteases in glutaraldehyde-treated bovine pericardium *per se* and in explanted ruptured bioprosthetic heart valves has been demonstrated recently by us [1]. These enzymes were shown to degrade both major biochemical components of bovine pericardium (type I collagen and small dermatan sulphate proteoglycan) [2] and thus could contribute to a possible slow tissue degeneration in time [3]. In order to be able to predict the outcome of this biomaterial *in vivo* and optimize a procedure for inhibition of these activities we have investigated three main aspects of interest: (a) the synergistic actions of glutaraldehyde and specific enzyme inhibitors on several extracellular-matrix degrading hydrolases (collagenase, trypsin and chondroitinase ABC); (b) the biochemical identification and characterization of the active proteases that could be extracted from glutaraldehyde-fixed pericardium; and (c) elaboration of a treatment that would reduce the remnant proteolytic activities for *in vivo* use.

2. Materials and methods

Collagenase form II was obtained from Advance Biofacture Corp. (Lynbrook, NY), TPCK-treated trypsin from E. Merck (Darmstadt, Germany) and chondroitinase ABC from Sigma Chemical Co. (St. Louis MO). Collagenase activity was determined by incuba tion with type I collagen or gelatine and estimating free liberated aminoacids with ninhydrin [4]; trypsin was investigated by colorimetry against tosyl-arginyl-methyl-ester (TAME) [5]; type I collagen membranes or gelatin [1] and chondroitinase ABC activity was quantitated by an Alcian Blue colorimetric assay with chondroitin sulphate as substrate [6]. All experiments were performed in the presence of increasing amounts of purified glutaraldehyde (0–1% final concentration) in combination with specific inhibitors – 50 mM ethyl-

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enediamine tetraacetic acid (EDTA) for collagenase, 0.1 mg/ml soybean trypsin inhibitor for trypsin and 1 mM Zn^{2+} for chondroitinase ABC. All substrates, inhibitors and general-use chemicals were from Sigma Chemical Co. (St. Louis, MO).

Active proteases were extracted from glutaraldehyde-treated bovine pericardium as described earlier [1], concentrated on Amicon Centriprep and subjected to unreduced, sodium dodecyl sulphate, 5–15% gradient polyacrylamide gel electrophoresis (SDSPAGE) in 1 mg/ml gelatine-containing vertical slab gels (zymography), followed by incubation in substrate buffer and Coomassie Blue staining [7]. Identical strips were incubated in the presence of specific inhibitors – 50 mM EDTA, 10 mM N-ethylmaleimide (NEM), 10 μg/ml leupeptin and 1 mM phenylmethylsulphonyl fluoride (PMSF), respectively. This technique facilitated the identification and molecular weight characterization of several gelatinolytic bands and their specific inhibition.

For *in vivo* studies, 1 × 1 cm^2 selected bovine pericardial fragments were incubated in a mixture of

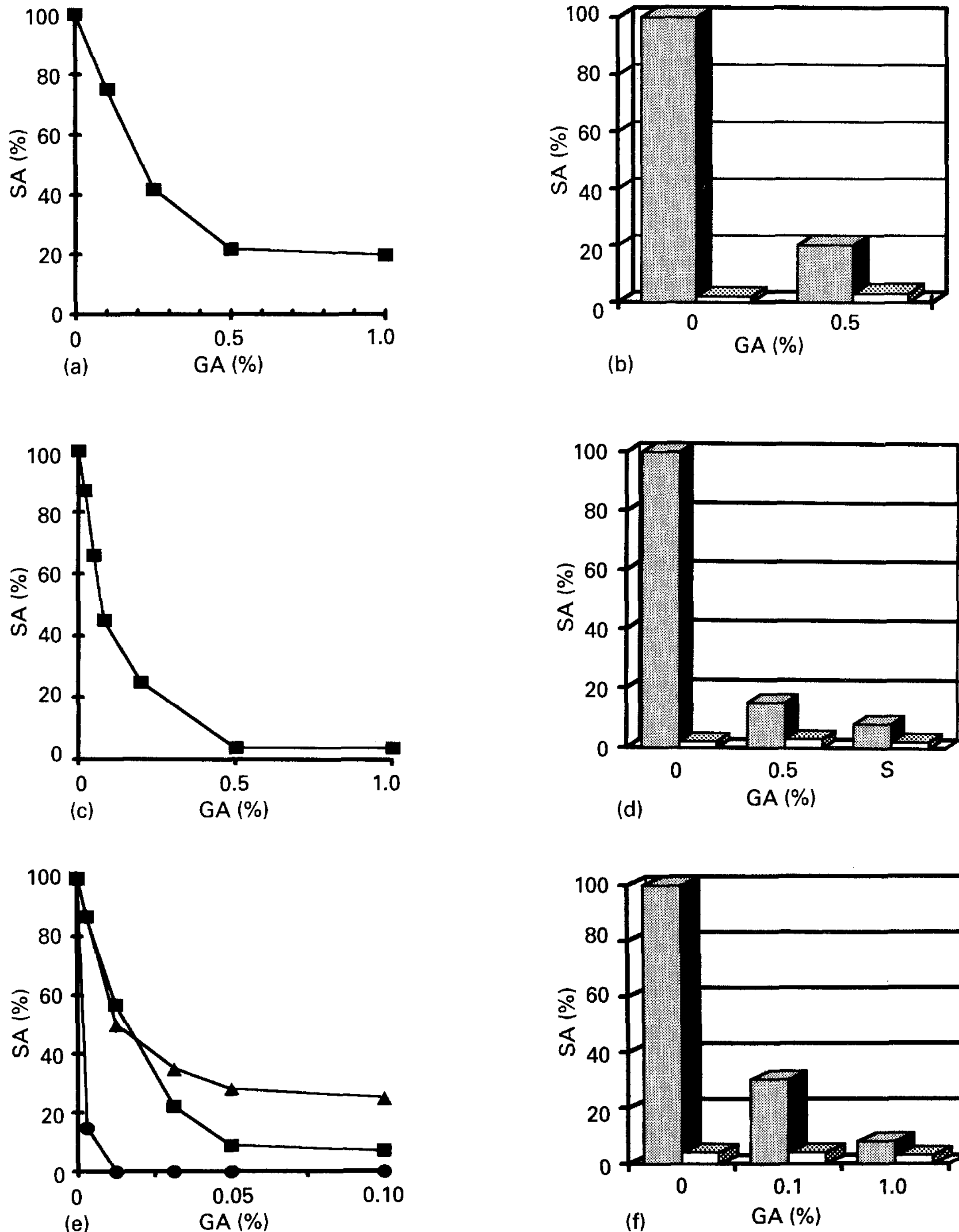

Figure 1 *In vitro* inhibition of extracellular-matrix degrading hydrolases by glutaraldehyde (GA) and specific inhibitors. Specific activity (SA%) = IU/mg enzyme is expressed as percentage of original activity. (a), (b) chondroitinase ABC using chondroitin sulphate as substrate; (c), (d) collagenase using type I collagen as substrate; (e), (f) trypsin using either gelatin (●), TAME (■) or collagen membranes (▲) as substrates. (a), (c), (e) depict the influence of increasing concentrations of GA on the SA while (b), (d), (f) show the inhibition of GA-reacted hydrolases by their specific inhibitors (Zn^{2+} for chondroitinase (■ (–); □ (+)), EDTA for collagenase (■ (–); □ (+)) and soybean trypsin inhibitor for trypsin (■ (–); □ (+))).

protease inhibitors (10 μg/ml pepstatin A, 10 mM NEM, 100 μM elastatinal, 10 mM benzamidine HCl, 10 μg/ml leupeptin, 1 mM PMSF and 50 mM EDTA) for 4 h, followed by a 0.5% buffered glutaraldehyde post-treatment; after thorough washing, more than 100 fragments were implanted in small ventral subcutaneous pockets of 50 white albino rats (80–100 g each) together with control fragments that were not pre-treated with inhibitors. After 8 weeks the fragments were explanted and proteolytic activities de tected either by using TAME as substrate [5] or by incubation of extracted enzymes with type I collagen substrate and reduced SDSPAGE analysis of the digestion products, as described previously [1].

3. Results

Fig. 1 depicts the specific activity (IU/mg protein) of chondroitinase ABC towards chondroitin sulfate in the presence of increasing amounts of glutaraldehyde (a) and followed by the specific inhibitor Zn^{2+} (b). Similar results were obtained with collagenase in the presence of glutaraldehyde (c), followed by its specific inhibitor EDTA (d). Trypsin activity towards either artificial or natural substrates was reduced by glutaraldehyde (e), and further reduced by soybean trypsin inhibitor (f).

Proteases were extracted from glutaraldehyde-treated pericardium and subjected to unreduced SDSPAGE gelatin zymography (Fig. 2) in which gelatin-degrading proteases are identified by light bands against a dark background. Four major gelatinolytic bands could be identified as having a Mr of about 33, 40, 100 and 185 kDa judged by comparison with comigrated unreduced standards. In the presence of EDTA these enzymes were inhibited and no light bands could be seen (lane 3 in Fig. 2).

Explanted pericardial fragments that were treated with a combination of seven protease inhibitors and glutaraldehyde were analysed for trypsin-like activity in parallel with the corresponding controls (Fig. 3). Unimplanted, inhibitor-treated pericardium showed a reduced proteolytic activity compared to glutaraldehyde alone; furthermore, upon implantation in the rat subcutaneous model the proteolytic activities found in these preparations were further reduced. Analysis of these preparations by zymography revealed the presence of the same four major enzyme species as in Fig. 2, which appeared in smaller amounts in the inhibitor-treated-unimplanted group, and practically undetectable in the inhibitor-treated-implanted group (not shown).

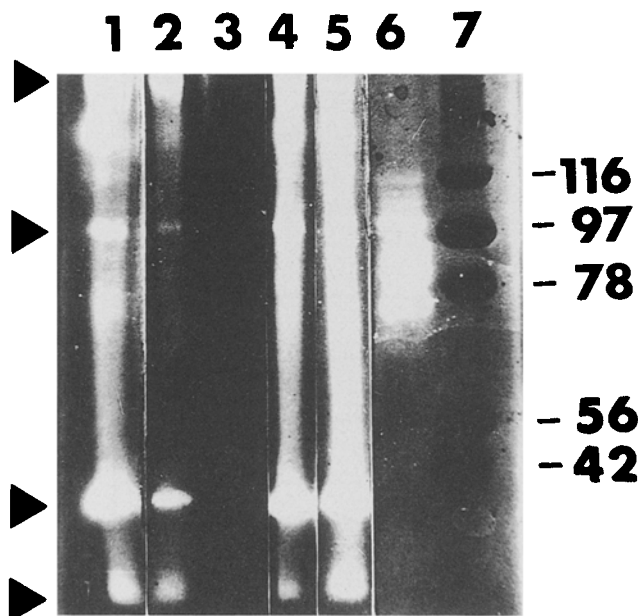

Figure 2 Gelatin zymography of proteases extracted from glutaraldehyde-treated bovine pericardium. Following electrophoresis in gelatin-containing, unreduced SDSPAGE gels, identical strips were incubated in substrate buffer containing either pepstatin A (lane 1), PMSF (lane 2), EDTA (lane 3), NEM (lane 4) or no inhibitor (lane 5). Lane 6 contains a control collagenase sample and lane 7, unreduced Mr protein standards whose values in kDa are shown at right. Note the presence of at least four gelatinolytic bands (arrowheads) and their complete inhibition by EDTA.

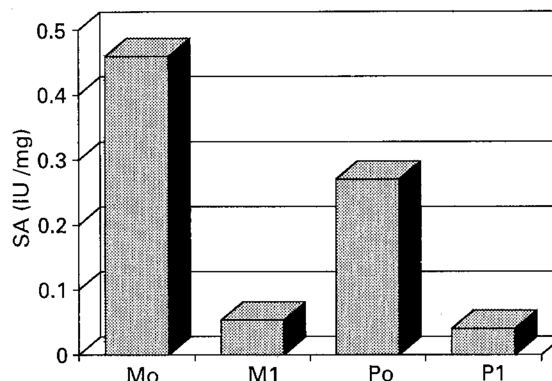

Figure 3 Specific trypsin-like activities, expressed as IU/mg protein (SA), of the extracts obtained from the four groups of glutaraldehyde-treated pericardial fragments. Mo-inhibitor untreated, unimplanted; M1-inhibitor untreated, implanted; Po-inhibitor treated, unimplanted and P1-inhibitor treated and implanted.

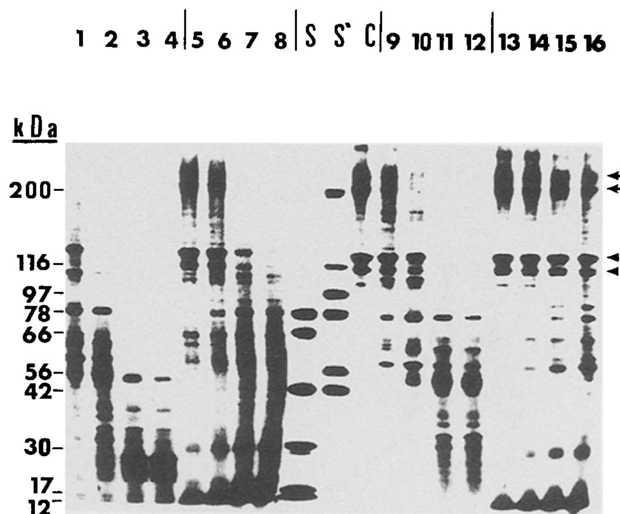

Figure 4 Reduced 5–15% linear gradient SDSPAGE kinetic analysis of the digestion products resulted from tissue extracts obtained from the four *in vivo* groups after incubation with a type I collagen substrate. Lanes 1–4, inhibitor untreated, unimplanted; lanes 5–8, inhibitor untreated, implanted; lanes 9–12, inhibitor treated, unimplanted and lanes 13–16, inhibitor treated and implanted. Samples were withdrawn at specific time intervals (lanes 1, 5, 9 and 13 at 30 min., lanes 2, 6, 10 and 14 at 3 h, lanes 3, 7, 11 and 15 at 18 h, lanes 4, 8, 12 and 16 at 26 h). Type I collagen substrate (lane C) and its typical alpha (arrowheads) and beta bands (arrows) are depicted together with Mr standards (S and S′) whose values in kDa are shown at left.

Proteases extracted from the four explanted groups were incubated with a type I collagen substrate and analysis of the digestion products was performed by subjecting the reduced samples to SDSPAGE in a kinetic experiment (Fig. 4). Lanes 1–4 show a time-dependent degradation of type I collagen (as revealed by disappearance of characteristic alpha and beta bands) by inhibitor-untreated-unimplanted tissue extract with almost complete degradation after 26 h incubation time; a delay of this degradation pattern is seen in the extract from inhibitor-untreated-implanted pericardium (lanes 5–8 in Fig. 4) while inhibitor-treated-unimplanted (lanes 9–12) and especially inhibitor-treated and implanted tissue extract (lanes 13–16) exhibit low to practically undetectable colagenolytic activities. These results correlate well with the colorimetric detection in the same preparations (Fig. 3).

4. Discussion

The *in vitro* studies revealed that glutaraldehyde, a bifunctional crosslinking dialdehyde, upon interaction with the extracellular-matrix degrading hydrolases tested in this study reduced the enzymatic activity by 80–90% without obtaining complete inhibition; furthermore, these modified enzymes were still susceptible to their specific inhibitors suggesting that glutaraldehyde induced some intra- or intermolecular structural modifications such that the affinity towards the substrate is reduced but that for the inhibitors remains unchanged. These results prompted us to investigate the use of inhibitor–glutaraldehyde combinations for tissue treatments.

The biochemical identity of the proteolytic activities previously found by us in glutaraldehyde-treated pericardium was shown in this study to consist mainly of enzymes that could degrade gelatine and type I collagen and that were inhibited by the calcium chelator ethylenediaminetetraacetic acid. Based on these findings, the pericardial proteases could be included in the matrix metallo proteinase family (MMP) [8]. It is not yet possible to positively correlate them with previously described MMPs because of different experimental conditions and difficulties in estimating exact Mr in unreduced gels.

Based on the above-mentioned results a pre-treatment with protease inhibitors was designed in order to inhibit the remnant pericardial proteases. The specific prerequisites were as follows: a low molecular weight to facilitate diffusion into the pericardial matrix, high specificity, rapidity and irreversibility of the enzyme–inhibitor reaction, stability throughout the incubation period, easy removal of the unreacted excess, efficiency at low concentration and low toxicity. Pericardial fragments that were treated with a purpose-designed inhibitor glutaraldehyde procedure showed a decreased proteolytic activity as judged by both colorimetric (Fig. 3) and SDSPAGE analysis (Fig. 4); upon subcutaneous implantation for 8 weeks the activity was further reduced, suggesting that effective reduction of these activities for clinical use is possible. From the colorimetric analysis (Fig. 3) proteolytic activities in unimplanted tissues were reduced upon exposure to the inhibitor mixture to about 50% of original; while implantation alone reduced the activities 6 to 7 times; the combination of inhibitor treatment and subcutaneous implantation resulted in an inhibition factor of about 11. These data suggest that significant proteolytic inhibition could occur also in the subcutaneous *milieu*, possibly by acquisition of subdermal extracellular inhibitors (tissue inhibitor of metalloproteinase) or other factors. Since we have shown earlier that the proteolytic activity could be found in both soluble and insoluble fractions (attached to the crosslinked collagen fibres) it remains to be investigated exactly what proportion of the proteases that populate the pericardium matrix was extracted by the procedure we have used in the present study.

5. Conclusions

In *in vitro* studies glutaraldehyde inhibits hydrolases that degrade collagen, gelatine and chondroitin sulphate to about 10 to 20% of original activities; these remnant activities were further reduced (practically down to the detection limit) by their specific inhibitors.

The pericardium is populated by extractable extracellular matrix degrading proteases that, judged by their action on various connective tissue components and their susceptibility to various inhibitors, could be considered matrix metallo proteinases. These enzymes are only partially inhibited by glutaraldehyde but the remnant activities could be further reduced by the use of a purpose-designed inhibitor mixture.

Pericardial fragments that have been prepared with an inhibitor–glutaraldehyde procedure contain very low levels of extractable active proteases after 8 weeks subcutaneous implantation thus possibly increasing the durability of this biomaterial *in vivo*. It remains to be determined whether these modifications could be applied to bioprosthetic cardiac valves for clinical use.

Acknowledgements

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