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Collagen degradation in ischaemic rat hearts

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Myocardial extracellular matrix is organized into a complex arrangement of intercellular and pericellular fibres and fibrils that serves as a supporting framework for contracting cells. Recent evidence suggests that changes in ventricular shape and function occurring after ischaemic injury may be related to alterations of this matrix. In this report we describe the rapid and extensive loss of collagen in myocardial infarction produced by ligating the left anterior descending coronary artery of the rat for 1–3 h. The total collagen content in the myocardial infarct zones after 1, 2 and 3 h of ligation was 75 ± 8 %, 65 ± 7 % and 50 ± 10 % respectively (mean ± S.D.) of that of either the non-infarcted tissue controls or of the same regions in sex- and age-matched normal left ventricles. A marked decrease also occurred in the residual collagens which were not extractable with 6 M-guanidine hydrochloride, suggesting that rapid degradation of insoluble collagen fibres may also occur. The decreased collagen content in the 3 h myocardial infarct coincided with the appearance of several enzyme activities. Collagenase, other neutral proteinase and presumed lysosomal serine proteinase activities were increased by 3, 3 and 2 times the control values respectively. These results suggest that the increased activities of collagenase and other neutral proteinases may be responsible for the rapid degradation of extracellular matrix collagen in myocardial infarct.

INTRODUCTION

Recent studies indicate that collagen structures of myocardial connective tissue between and around myocytes serve as a skeletal framework to provide support and to modulate contraction (Robinson *et al.*, 1985). Degradation of collagen after acute myocardial infarct (MI) has been reported to occur (Cannon *et al.*, 1983; Factor *et al.*, 1987); this may cause changes in the ventricular wall shape and plasticity, leading to expansion (Eaton *et al.*, 1979; Hochman & Bulkley, 1982). The expansion of the infarcted ventricular wall appears to be related to the collagen content of the wall (Leiman *et al.*, 1983). Recent evidence also suggests that loss of connective tissue collagen occurs before myocellular death, which may lead to post-ischaemic non-infarcted myocardial dysfunction, or myocardial stunning (Zhao *et al.*, 1987). Extracellular degradation of collagen would be expected to involve proteinases, some of which would be active at near neutral pH. It is probable that only a small degree of degradation (i.e. a few proteolytic scissions) is sufficient to impair the mechanical properties of the structural macromolecules. Therefore it appears that degradative metabolism of collagen in the heart may play an important role in the pathophysiology of myocardial dysfunction following ischaemic injury. The biochemical effect of acute, early ischaemia on myocardial collagen metabolism has not yet been studied. An initial step in elucidating the molecular mechanisms involved in degradation of extracellular matrix collagen in MI is the determination of the collagen content of the tissue and of the activities of the proteinases involved. In the present study, we report that rapid decreases in total, extractable and residual collagen contents occur in MI produced in rats by ligating the left anterior descending coronary artery (LAD), and that this decrease coincides with increased collagenase, non-specific neutral proteinase and presumed lysosomal serine proteinase activities.

The collagen content of a tissue is determined by a balance of the processes of synthesis and degradation. A portion of newly synthesized collagen may be degraded intracellularly (Hurych & Chvapil, 1962; Bienkowski *et al.*, 1978). On the other hand, newly secreted collagen molecules may be degraded by a priming action of tissue collagenases (Gross & Lapiere, 1962). However, if sufficient collagenase is not present or is not activated at the proper time, the collagen molecule may be cross-linked to form polymeric fibrils (Siegel, 1979); these structures may not be so readily attacked by the collagenases, since the introduction of cross-links into reconstituted collagen fibrils markedly decreases the rate of collagen degradation by collagenase as compared with that of the control reconstituted collagen fibrils (Harris & Farrell, 1972; Vater *et al.*, 1979). The rates of degradation of insoluble collagen fibres by purified collagenases have been reported to be 1–5 % of that of the reconstituted collagen fibrils (Woolley *et al.*, 1975, 1978); others have found that the isolated collagenase alone is unable to degrade insoluble collagen fibres (Leibovich & Weiss, 1971), and proposed that a multienzyme system must

Abbreviations used: MI, myocardial infarct; TIMP, tissue inhibitor of metalloproteinase; LAD, left anterior descending coronary artery; Suc-$(Ala)_2$-Pro-Phe-pNA, succinylalanylalanylprolylphenylalanyl-*p*-nitroanilide; Suc-$(Ala)_3$-pNA, succinylalanylalanylalanyl-*p*-nitroanilide; MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, methoxysuccinylalanylalanylprolylvalyl-chloromethane; Z-Gly-Leu-Phe-CH_2Cl, Z-glycylleucylphenylalanyl-chloromethane; LV, left ventricle; PBS, phosphate-buffered saline (1.5 mM-KH_2PO_4/8.2 mM-Na_2HPO_4/2.7 mM-KCl/137 mM-NaCl); RV, right ventricle; PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide-gel electrophoresis; DIFP, di-isopropylphosphofluoridate; GdnHCl, guanidine hydrochloride; EHS mouse, Engelbreth–Holm–Swarm mouse.

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operate. The rate of collagen degradation may be determined, in part, by the types of collagen present, e.g. type III collagen may be degraded at a greater rate than type I (Harper, 1980), and it may also be degraded by other proteinases (Miller *et al.*, 1976). In addition, lysosomal enzymes may facilitate the breakdown of highly cross-linked collagens (Burleigh *et al.*, 1974). The major neutral-acting proteinases of human neutrophilic polymorphonuclear leukocytes (neutrophil leukocytes) are the specific collagenases (Lazarus *et al.*, 1968) and the lysosomal serine proteinases such as elastase and cathepsin G (Starkey & Barrett, 1976*a*,*b*). The latter two proteinases have been shown to solubilize proteoglycans (Roughley & Barrett, 1977) and to attack the terminal peptides of mature collagen molecules which are essential for the integrity of the intermolecular cross-links (Rauterberg, 1973). Certain cells are capable of releasing these lysosomal proteinases extracellularly; these include neutrophil leukocytes (Cohn & Weiner, 1963; Goldstein & Weissmann, 1974), macrophages (Werb & Gordon, 1975) and tumour cells (Gilfillan, 1968; Zeydel *et al.*, 1986).

A tissue collagenase (EC 3.4.24.7) is one of a family of metalloproteinases that specifically cleaves native interstitial collagen molecules at one site in each of the three constituent polypeptide chains; this cleavage produces characteristic fragments of $\frac{1}{4}$ and $\frac{3}{4}$ of the length of native molecules. Collagenases are released as an inactive form of procollagenase or zymogen (Harper *et al.*, 1971; Vaes, 1971). There is as yet no consensus about the mechanisms of activation. Proposed mechanisms include proteolysis, autoactivation with or without loss of M_r or removal of an inhibitor from a complex (Harper, 1980). General agreement exists among investigators that collagenases in tissues are not stored intracellularly (Valle & Bauer, 1980), with the exception of leukocyte collagenase (Lazarus *et al.*, 1968). Several mechanisms for the control of extracellular collagenase activity have been described. The α_2-macroglobulin (M_r 720000), a non-specific proteinase inhibitor with broad specificity, accounts for over 90% of the collagenase-inhibitory activity found in serum (Woolley *et al.*, 1976). Serum also contains β_1-collagenase inhibitor (M_r 30000) (Woolley *et al.*, 1976). In addition, tissue inhibitor of metalloproteinase (TIMP; M_r 28000) has been proposed to be an inhibitor of tissue collagenases (Causton *et al.*, 1981) and to control the local extracellular activity of these enzymes (Galloway *et al.*, 1983). An acidic protein of M_r 10000, extracted from various tissues, also inhibits collagenase activity (Kuettner *et al.*, 1976).

MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated sources: Ca^{2+}- and Mg^{2+}-free Hepes buffer from Gibco Laboratories (Grand Island, NY, U.S.A.); Suc-(Ala)$_2$-Pro-Phe-pNA and Suc-(Ala)$_3$-pNA from Vega Biotechnologies Inc. (Tucson, AR, U.S.A.); MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl and Z-Gly-Leu-Phe-CH_2Cl from Enzyme Systems Products (Livermore, CA, U.S.A.); ^{125}I (100 mCi, 3.7 GBq) from Amersham Co. (Chicago, IL, U.S.A.) and Protein A from Calbiochem Corp. (La Jolla, CA, U.S.A.). Female Wistar rats were purchased from Charles River Laboratory Inc. (Wilmington, MA, U.S.A.) and housed until used. Fischer rats were provided by Dr. Sam Seifter and Dr. Olga O. Blumenfeld, Department of Biochemistry, Albert Einstein College of Medicine, and mouse liver type III collagen was a gift from Dr. Marcos Rojkind, Department of Medicine, Albert Einstein College of Medicine. We have previously produced rabbit antibody to purified acid-soluble mouse skin type I collagen (Biempica *et al.*, 1983; Takahashi & Koda, 1984). We also have purified acid-soluble rat-tail tendon type I collagen as described (Takahashi *et al.*, 1973, 1980). Other biochemical reagents were as in previous publications (Takahashi & Simpser, 1981; Zeydel *et al.*, 1986; Takahashi & Shefer, 1987).

Production of MI in rats

MI was produced in Wistar rats according to published techniques (Fishbein *et al.*, 1978*a*), under ether anaesthesia, by ligating the LAD for 1, 2 or 3 h. At the end of ligation, the heart was perfused with Evan's Blue dye through the root of the aorta; the dye stains the non-infarcted area of the left ventricle (LV), but not the area of infarction. After the rat was killed, the heart was removed and rinsed with phosphate-buffered saline (PBS). The LV was separated from the right ventricle (RV), and the infarcted area was dissected from the non-infarcted area; the latter was used as a non-infarcted tissue control. Areas that stained diffusely were eliminated, since the contents of Hyp or enzymes were intermediate between those in MI tissue and non-infarcted tissue control. Sex- and age-matched normal LV were obtained from two groups of rats: one group was perfused with Evan's Blue dye and the other with PBS. The tissues were stored at −70 °C.

Preparation of assay samples

A sample of tissue was used for determination of dry weight of tissue and total Hyp content. Another sample was cut into small pieces and suspended (5%, w/v) in 50 mM-Tris/HCl/5 mM-$CaCl_2$ buffer, pH 7.5 (Tris/$CaCl_2$ buffer) containing 0.02% NaN_3, and sonicated at 4 °C (for 15 s with 45 s intervals) until the suspension became homogeneous. Subsequently, the homogenate was distributed into tubes for the determination of protein content and serine-proteinase activity.

Extraction of collagen

Extraction of collagen from the tissue was performed as described (Takahashi & Shefer, 1987), with a slight modification. In brief, the tissue (1.5 g) was cut into small pieces, suspended in 10% (w/v, initial volume) PBS containing proteinase inhibitors (2 mM-PMSF/10 mM-NEM/10 mM-EDTA) and 0.02% NaN_3, and sonicated as above. A portion was used for determination of total extractable collagen with 6 M-guanidine hydrochloride (GdnHCl), and the rest was used for the more extensive extraction procedure. The latter was modified by extracting twice each with 0.5 M-acetic acid and 6 M-GdnHCl and by combining the acetic acid extract with the supernatant of the original homogenate. For each of the four fractions [total extractable collagen (homogenate), combined supernatant plus acetic acid extract (acid fraction), GdnHCl extract and residue], total collagen was determined by measuring the Hyp content and the type I collagen by e.l.i.s.a. In the latter assay, the reaction mixture contained 0.6 M-GdnHCl to keep collagen in solution.

Preparation of samples for collagenase and serine proteinase assays

The following procedures were performed at 4 °C; centrifugations were at 27000 *g* for 30 min and the buffer contained 0.02 % NaN_3. The homogenate prepared as above (from 1 g of tissue) was centrifuged and the resulting supernatant was brought to 25 %-satd. ammonium sulphate by addition of solid salt. The precipitate formed was collected by centrifugation and saved; the supernatant was then brought to 60 % ammonium sulphate. The precipitate was collected. Both precipitates were dissolved separately in Tris/$CaCl_2$ buffer, and dialysed against the same buffer (48 h, with four changes). Heparin–Sepharose Cl 6B (10 ml), pre-equilibrated in the same buffer, was added to each diffusate, then stirred for 2 h at 4 °C; the mixture was poured into a column (1.5 cm × 6 cm) and eluted with the same buffer until the eluate contained no detectable protein at A_{280}. The column was then eluted using the following stepwise NaCl gradient: 20-fold column volumes of the same buffer containing 0.2 M-, 0.4 M- and 0.8 M-NaCl. Each of the four fractions was concentrated to 10 % of the initial volume in a Diaflow apparatus using a YM-10 membrane and dialysed against the collagenase assay buffer (Tris/$CaCl_2$ buffer containing 0.2 M-NaCl/0.02 % NaN_3). The 0.2 M-NaCl and 0.8 M-NaCl fractions were pooled, concentrated and used to determine collagenase activity. The 0.4 M-NaCl fraction was concentrated and used to determine TIMP activity. The eluate and wash solutions of the Heparin–Sepharose column containing serine proteinases were pooled and chromatographed on an Ultrogel AcA 54 column in the same buffer containing 0.2 M-NaCl/0.1 % Triton X-100. The protein fractions containing enzyme activity with M_r 25000 ± 5000 were pooled, concentrated and used to determine serine proteinase activity.

Enzyme and inhibitor assays

Enzyme assays were performed at least in duplicate and with appropriate controls. Tryptic activation of latent collagenase and the collagenase assay were performed as described previously (Zeydel *et al.*, 1986). Cleavage of type I collagen by collagenase was determined by SDS/polyacrylamide-gel electrophoresis (PAGE) (Laemmli, 1970) as described (Takahashi & Simpser, 1981). For the TIMP assay, a known amount of active collagenase (Takahashi & Biempica, 1985) was first incubated with the sample containing TIMP, and then the remaining collagenase activity was measured. TIMP activity was expressed in units: 1 unit of TIMP inhibits 2 units of collagenase by 50 %. Other non-specific neutral proteinase activity was measured by using ^{14}C-labelled soluble casein as described (Zeydel *et al.*, 1986).

Three kinds of lysosomal enzyme activity were measured as follows. Serine proteinase activities were assayed with native and synthetic substrates. (1) A native substrate for elastase, reduced tritiated ligamentum nuchae elastin, was used (Takahashi *et al.*, 1973; Zeydel *et al.*, 1986). (2) A synthetic substrate for elastase, Suc-$(Ala)_3$-pNA, was also used at pH 7.5 (Bieth *et al.*, 1974). Enzyme activity was expressed in units: 1 unit of elastase is the amount that releases 1 μmol of *p*-nitroaniline/h at 37 °C; absorbance of the latter is measured at 410 nm (ϵ 8800 $M^{-1} \cdot cm^{-1}$). (3) A synthetic substrate for cathepsin G, Suc-$(Ala)_2$-Pro-Phe-pNA (pH 7.5), was also used at 37 °C (Barrett, 1981). Enzyme activity was expressed in units as described for the elastase assay with the synthetic substrate in (2). The degradation of acid-soluble type I collagen by the protein fraction containing serine proteinases of MI tissue was determined by SDS/PAGE; the reaction mixture, containing substrate and enzyme with and without pretreatment with inhibitor, was incubated in assay buffer containing 10 mM-EDTA and 0.02 % NaN_3 at 25 °C. Enzyme activity was terminated by the addition of di-isopropylphosphofluoridate (DIFP) to 5 mM, and the samples were subjected to SDS/PAGE. For inhibitor assays, the sample was preincubated with the inhibitor for 30 min at 25 °C and a portion was assayed. The inhibitors used were DIFP (5 mM) or PMSF (2 mM) or the specific chloromethane-peptide inhibitors (Powers *et al.*, 1977); these are MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl (0.2 mM) for elastase and Z-Gly-Leu-Phe-CH_2Cl (0.02 mM) for cathepsin G. For the latter assays, the concentration of methanol in the assay was 1 %; this did not significantly affect the assay. The experiments included two enzyme controls: one contained no inhibitor, and the other contained no inhibitor but 1 % methanol. The latter enzyme activity was used as the base value of 100 %.

Cathepsin D activity was measured by using methyl-[^{14}C]glycinated bovine haemoglobin at pH 3.0 (Takahashi *et al.*, 1980).

β-D-Glucosaminidase activity was measured by using 2-methylumbelliferylglucopyranoside at pH 3.0 (Takahashi *et al.*, 1985).

Immunoassays

The type I collagen content of the tissue was determined by e.l.i.s.a. (Rennard *et al.*, 1980), employing rabbit anti-(mouse skin type I collagen) antiserum using a method similar to that described by Zeydel *et al.* (1986). Western immunoblot staining (Towbin *et al.*, 1979) of type I collagen was also performed. The sample proteins were subjected to SDS/PAGE using LKB 2050-001 Midget Electrophoresis Unit, and protein bands of the gel were transferred to nitrocellulose using a LKB-Multiphor II Nova Blot system. The transferred protein bands were stained immunologically by sequential reaction, first with the rabbit antiserum and then with ^{125}I-labelled Protein A; radiolabelled protein bands were autoradiographed.

Other methods

The protein content of tissue samples were determined by the Lowry method (Lowry *et al.*, 1951), after solubilizing the homogenates with 1 M-NaOH for 24 h at 25 °C (Takahashi *et al.*, 1978). For soluble samples, the Bradford (1976) method was used. Total collagen content of the tissue was determined by measuring Hyp content (Takahashi & Lee, 1987). The collagen content was expressed as mg of collagen/100 mg of tissue protein, based on the assumption that the Hyp content of collagen is 10 % (1 μmol of Hyp = 1 mg of collagen; 10 μmol of Leu equivalents = 1 mg of protein). Total amino acid content of the sample (including Hyp) was determined using an automatic amino acid analyser or by the ninhydrin reaction using L-Leu as a standard (Takahashi & Shefer, 1987). The data were analysed using a variance test to determine significance between groups. Values are given as means ± S.D. from *n* determinations.

RESULTS AND DISCUSSION

Collagen content

The specific collagen content of the RV of rats was about 2-fold greater than that of the LV. However, the mass of LV was about 5-fold greater, and therefore the total collagen content was over 2-fold greater in the LV than in the RV (results not shown). As shown in Fig. 1, the total collagen content per 100 mg of protein of the LV increases with age ($P < 0.02$). Evan's Blue dye did not interfere with the determination of Hyp in the tissue [based on two groups ($n = 5$) of normal rats: one perfused with Evan's Blue and the other with PBS]. The yield of freeze-dried tissue of MI ($n = 10$) was 180 ± 20 mg per g of tissue, and of non-infarcted tissue control ($n = 10$), it was 200 ± 20 mg. Protein content was 134 ± 12 mg and 144 ± 10 mg per g of tissue respectively. There were no significant differences in the contents of both protein and Hyp in the samples of LV tissue studied (20–200 mg). The collagen contents of non-infarcted tissue controls were similar to those of normal LV (Fig. 1). A 50% decrease in collagen content occurred in the LV of rats in which MI was produced (3 and 8 months of age) by ligating the LAD for 3 h, as compared with that of the non-infarcted tissue controls (Fig. 1). This decrease was highly significant ($P < 0.02$). The decrease appeared to correlate with the duration of ligation time, i.e. 1 h ($n = 4$), 2 h ($n = 4$) or 3 h ($n = 10$). The collagen contents in these groups of MI tissues were respectively 75 ± 8%, 65 ± 7% and 50 ± 10% of those of the non-infarcted tissue controls. Significant differences occurred between 1 h and 3 h values ($P < 0.03$), although a similar degree of significance was not apparent between either of these groups and the 2 h values. A marked decrease in collagen content also occurred in the 6 M-GdnHCl and residual fractions as compared with corresponding fractions of the non-infarcted tissue controls or normal LVs (Fig. 2).

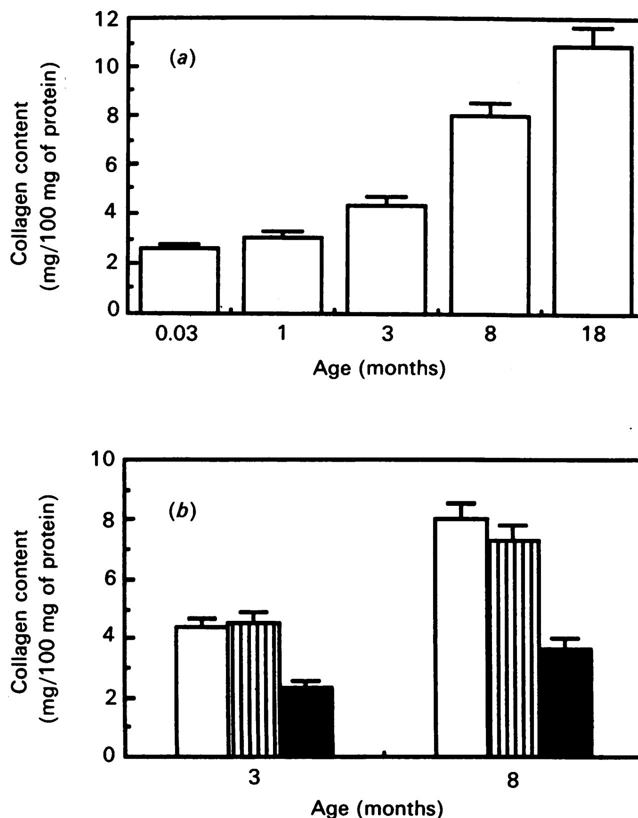

Fig. 1. Collagen content of rat LV: (a) normal Wistar and Fischer rats and (b) comparisons of MI, non-infarcted and normal LV tissue

Collagen contents of LVs are plotted against age of animals, with the exception of one-day-old newborn rats, where the whole heart was analysed. Collagen content is expressed as mg of collagen/100 mg of tissue protein. (*a*) Fischer rats of ages one day ($n = 5$), one month ($n = 3$) and 18 months ($n = 3$); and Wistar rats of ages 3 months ($n = 10$) and 8 months ($n = 3$). (*b*) Solid columns, MIs produced in Wistar rats (3 months; $n = 10$ and 8 months; $n = 3$) by ligating the LAD for 3 h; striped columns, non-infarcted tissue controls; open columns, normal LVs. Bars show S.D. Assay conditions were as described in the text.

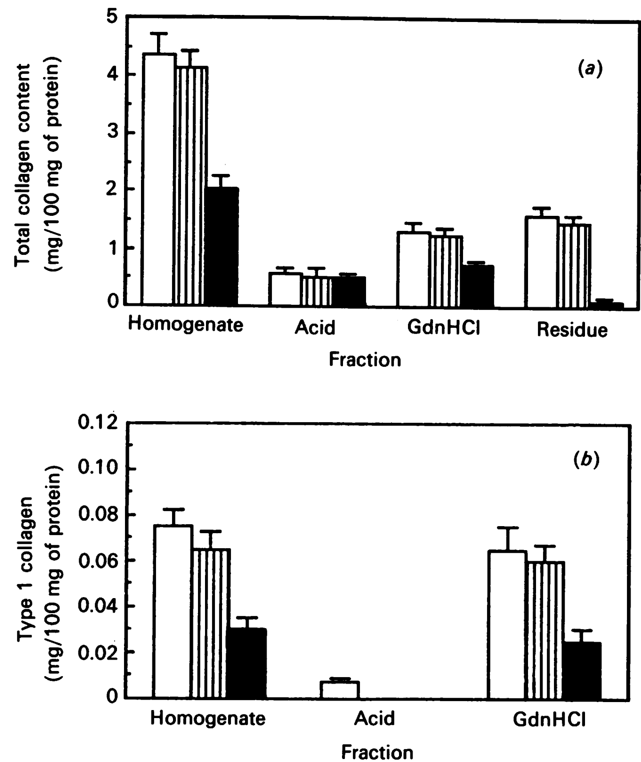

Fig. 2. Collagen extraction from LVs of rats: (a) extractable and residual collagen and (b) type I collagen

MIs were produced by ligating the LAD of Wistar rats for 3 h. Collagen content is expressed as mg of collagen/100 mg of tissue protein. Type I collagen content was determined by indirect inhibition e.l.i.s.a. using rabbit antiserum raised against mouse skin type I collagen. Open columns, normal LVs; striped columns, non-infarcted tissue controls; solid columns, MI tissue. Data points represent the means of two separate experiments with analyses in duplicate. Bars show S.D. Other conditions were the same as in Fig. 1.

Immunoassays

To characterize the type of collagen that had decreased in MI tissue of rats as described above, the collagen was studied further by use of e.l.i.s.a. with an antiserum that had been raised against mouse skin type I collagen. (Mouse skin type I collagen was used since we did not have a rat type I collagen of comparable purity.) In the

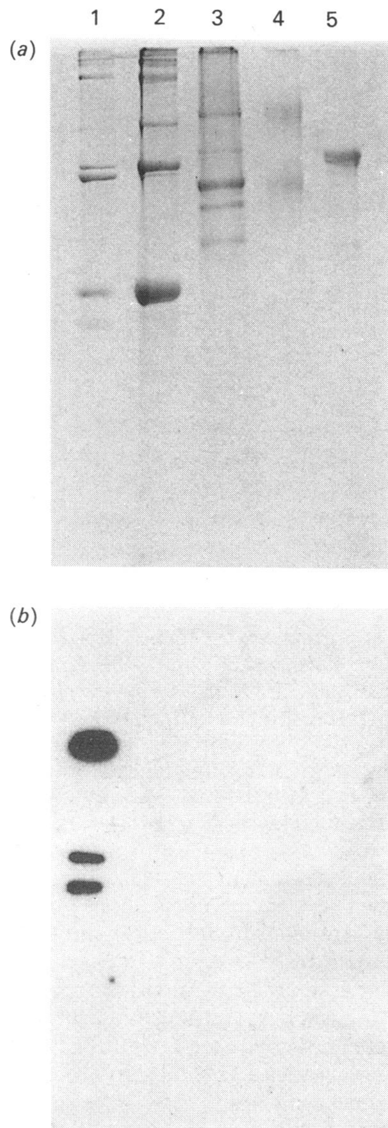

Fig. 3. Cross-reactivity of rabbit anti-(mouse skin type I collagen) antiserum with types III and IV collagens, and other proteins: (*a*) Coomassie Brilliant Blue staining and (*b*) radioautography

Protein samples (1 μg per slot) were reduced with 0.1 M-2-mercaptoethanol for 2 h at 25 °C and subjected to SDS/PAGE (6.25 % gel) in two gels. Protein bands were (*a*) stained by Coomassie Brilliant Blue and (*b*) immunoblotted. Lane 1, mouse type I collagen (0.4 μg); lane 2, mouse liver type III collagen (4 μg); lane 3, EHS type IV collagen (4 μg); lane 4, EHS laminin (4 μg); lane 5, rat fibronectin (4 μg). Antiserum was at 1:50. Assay conditions were as described in the text.

first instance, then, we had to establish the cross-reactivity of the antiserum with rat collagen. For that purpose we used type I collagen purified from rat tail tendon. In direct e.l.i.s.a. reaction, with the antiserum used in 1:50 and 1:1500 dilutions (and 1 μg of antigen coated on the well), rat tail type I collagen gave 80 % and 40 % respectively of the reaction obtained with mouse skin type I collagen. In indirect e.l.i.s.a., the rat type I collagen gave 30 % of the reaction given by mouse type I collagen (antiserum was used in 1:1500 dilution and the wells were coated with 0.2 μg of mouse type I collagen). In the latter assay, GdnHCl was added to a concentration of 0.6 M to determine the effect of this reagent on the immunological reaction. The 0.6 M-GdnHCl in fact reduced the reactivity from an assigned 100 % in the absence of the reagent to 85 %. The antiserum against the Mouse type I collagen was then tested for the kinds of epitopes involved in the antigen. mouse type I collagen was heated at 60 °C for 20 min to destroy the helical structure of the protein. In direct e.l.i.s.a., the antigen denatured in this manner gave 30 % of the reactivity given by undenatured collagen containing a triple helical structure. Mouse type I collagen treated with 6 M-GdnHCl gave 50 % of the reactivity given by undenatured collagen. Thus the antiserum appeared to recognize epitopes associated with the collagen triple-helical structure to an extent of about 50–70 %, and to a lesser degree it recognized epitopes in the primary amino acid sequence. The cross-reactivities of this antiserum to type III collagen and other related proteins were then tested by direct e.l.i.s.a. Antiserum (1:100) was treated with mouse skin type I collagen (from 1 to 1000 ng) alone or in the presence of a 10- to 50-fold excess of the following proteins: type III collagen (mouse liver), type IV collagen [Engelbreth–Holm–Swarm (EHS) mouse], fibronectrin (rat) or laminin (EHS). None of these four proteins affected the binding of antibody with mouse type I collagen; the immunoblot staining of these proteins was also negative (Fig. 3). These results suggest that the antiserum does not cross-react with either native or denatured type III collagen.

By using these methods and by correcting for the lower cross-reactivity of the antiserum with rat collagens, it appears that the major type of collagen in the GdnHCl extract of the LV of normal rats is type I collagen; this type becomes decreased in the MI tissue. Type I collagen was detected in the acid extract of the LV of normal rats (Fig. 2*b*). No type I collagen was detected in the acid extract of MI tissue, suggesting that the collagen had become degraded to a size that did not react immunologically with the antiserum. The immunoblot staining of heart extracts (using GdnHCl) showed patterns typical of type I collagen from mouse skin and rat tail tendon (Fig. 4). However, no firm conclusions can yet be made regarding the amount of type I collagen present in the LV without further evidence that the antiserum is totally free of antibodies directed against antigenic determinants present in the other types of collagens; however, by indirect e.l.i.s.a., this does seem to be the case. The purpose of determining total and type I collagen content in several fractions is to establish whether the physical changes that occur *in vivo* due to degradation of the extracellular matrix collagen become reflected in the extracted or non-extracted fractions. The use of Gdn has some advantages in that it distinguishes a particular fraction relative to others. The method is simple and maintains intact the primary structure of the collagen chains, but the reagent destroys native collagen helical structure.

Enzyme activities

The Evan's Blue dye present in the non-infarcted tissue control did not affect enzyme activities in the fractions studied. Collagenase precipitated in the 25–60 % ammonium sulphate fraction could only be reproducibly determined in fractions obtained from the heparin–

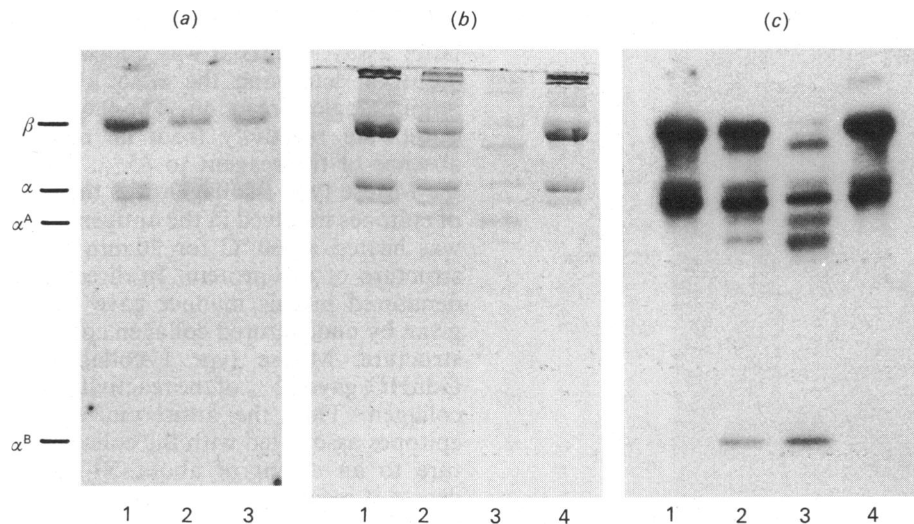

Fig. 4. Immunoblot staining of type I collagen (*a*) and of cleavage products produced by the action of myocardial collagenase: comparisons with Coomassie Brilliant Blue staining (*b*) and radioautography (*c*)

(*a*) The acid-soluble rat tail tendon type I collagen and GdnHCl fraction of the LV of normal rats (80 ng per slot) were subjected to SDS/PAGE (7.5% gel) and immunoblot staining using rabbit anti-(mouse type I collagen) antiserum. Other conditions were the same as in Fig. 3. Lane 1, antigen (80 ng); lane 2, rat tail tendon type I collagen; lane 3, GdnHCl fraction of normal LV. (*b*), (*c*) The reaction mixture of mouse skin type I collagen (12.5 μg) and the protein fraction (1.25 μg) containing collagenase of MI tissue, obtained from a heparin–Sepharose CI 6B column, was subjected to SDS/PAGE (7.5% gel) in two gels and stained as in Fig. 3. The gel shows the reaction mixture of substrate and collagenase at 25 °C for 0 h (lane 1), 3 h (lane 2) and 16 h (lane 3), and substrate plus collagenase pretreated with *o*-phenanthroline (10 mM) at 16 h (lane 4). Antiserum was at 1:100 dilution. Other conditions were the same as in Fig. 3. The preparation of collagen and collagenase fractions was as described in the text.

Sepharose column (Fig. 5). This column eliminated over 95% of other proteins in the eluate, and most of the serine proteinase and non-specific neutral proteinase activities were not adsorbed. Most of the collagenase was eluted with 0.2 M-NaCl and some with 0.8 M-NaCl; most of the TIMP was eluted with 0.4 M-NaCl. The TIMP activity was 4-fold greater in non-infarcted tissue controls (3.2 units/100 mg of protein) than in the MI tissue (0.8 units/100 mg of protein). Interestingly, the collagenase derived from the MI tissue contained this enzyme in an active form, whereas in the non-infarcted tissue controls, the enzyme was present only in an inactive form. To obtain maximal collagenase activity, all samples were activated with trypsin before assay. Activity of collagenase in the MI tissue was about 3-fold greater than in the non-infarcted tissue controls (Fig. 5). Collagenase activity was completely inhibited by EDTA (10 mM), *o*-phenanthroline (10 mM), and rat serum (1:50 dilution); the collagenase activity was insensitive to DIFP (5 mM), PMSF (2 mM), NEM (10 mM) and *p*-chloromercuribenzoate (0.2 mM). Collagenase preparations obtained from the MI-tissue cleaved acid-soluble type I collagen and produced characteristic fragments of $\frac{3}{4}$ and $\frac{1}{4}$ of the molecule (Fig. 4). These properties are similar to those of collagenases isolated from various other tissues. The activity of non-specific neutral proteinases in the MI tissue was also about 3-fold greater than in the non-infarcted tissue controls (Fig. 5).

Lysosomal enzyme activities were determined by direct assay in the supernatants obtained after centrifugation of the homogenates at 27000 ***g*** for 30 min; over 95% of the Evan's Blue-stained material was in the precipitate. Elastase and cathepsin G activities in the MI tissue were each 2-fold greater than those of the non-infarcted tissue controls (Table 1); they were inhibited almost completely by DIFP (5 mM) and PMSF (2 mM), suggesting that they are typical serine proteinases. The other typical lysosomal enzyme activities, such as cathepsin D and β-D-glucosaminidase, were not increased significantly over the values of control tissues (Table 1). Synthetic substrates for these enzymes, although less specific, were used because they are soluble in buffers and their assay is simple. The selective inhibition of both serine proteinases in the protein fraction obtained from the Ultrogel AcA 54 column (see the Materials and methods section) was achieved by employing specific peptide inhibitors (Powers *et al.*, 1977). The elastase activity of the fraction with both native and synthetic substrates was inhibited almost completely by 0.2 mM-MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, but only slightly (15 ± 5%) by 0.2 mM-Z-Gly-Leu-Phe-CH_2Cl (Table 2). In contrast, 0.02 mM-Z-Gly-Leu-Phe-CH_2Cl almost completely inhibited the cathepsin G activity of the fraction, while 0.02 mM-MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl caused little or no inhibition. Results indicated that both are specific inhibitors for elastase and cathepsin G, as reported previously (Powers *et al.*, 1977). The gel electrophoretic patterns of the acid-soluble type I collagen, treated with the serine proteinase fraction obtained from the MI tissue in the presence of 10 mM-EDTA, showed depolymerization of dimers (β) and trimers (γ) to modified collagen molecules; there was a concomitant increase in the amount of free (modified) α chains, and degradation of the latter into small peptides. These changes did not occur when the collagen was incubated with the same fraction pretreated with 0.2 mM-MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, but occurred to some extent with the same fraction pretreated with 0.02 mM-Z-Gly-Leu-Phe-Val-CH_2Cl (Fig. 6). This indicates that the

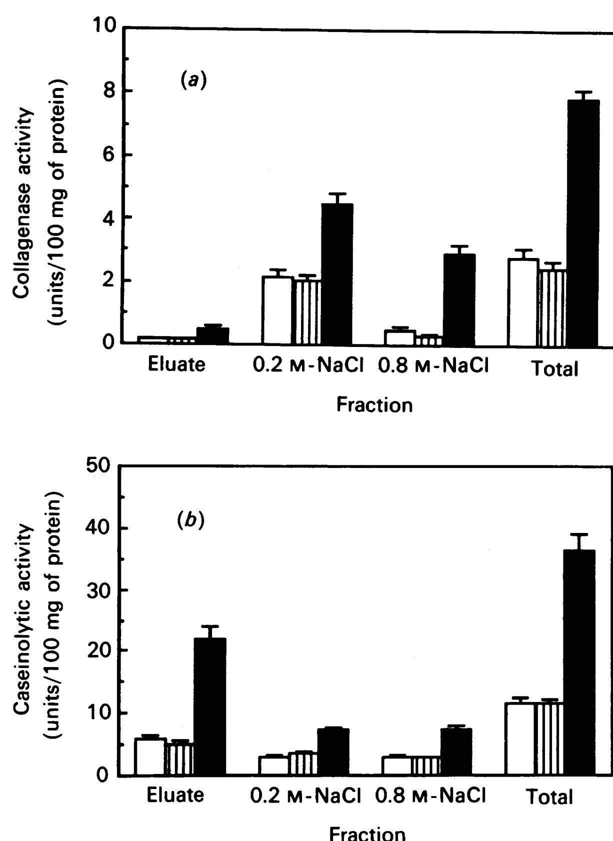

Fig. 5. Neutral proteinase activity in LVs of rats: (*a*) collagenase activity and (*b*) non-specific neutral proteinase activity

Collagenase and non-specific neutral proteinase activities of normal LV tissue, MI tissue produced by ligating LAD for 3 h and non-infarcted tissue from 3-month-old rats were determined by measuring enzyme activities in the fractions obtained from a heparin–Sepharose CI 6B column. Enzyme activity is expressed in units: one unit of enzyme degrades 1 μg of substrate (native collagen or soluble casein)/min at 35 °C. Enzyme activities per 100 mg of tissue protein are plotted for each fraction; the total is the sum of the activities of each fraction. Open columns, normal LVs; striped columns, non-infarcted tissue controls; solid columns, MI tissue. Data points represent the means$\pm$S.D. (bars) of two separate experiments with analyses in duplicate. The preparation of the collagenase fraction was carried out as described in the text.

elastase activity of the fraction is capable of cleaving the terminal peptides of the collagen molecule involving the intramolecular cross-links; this does not occur when pancreatic elastase is used. It appears that the enzymes in the preparation do not attack the helical regions of the collagen molecule, but may have activities that give rise to proteolytically modified α chains that are released (Fig. 6).

We and others (Eghbali *et al.*, 1988) have found that over 80% of the LV collagen of rats is insoluble with usual extractants, and therefore appears to be extensively cross-linked (Gallop *et al.*, 1972). In the case of infarcted myocardium, collagen content decreases in the non-extractable fraction as well as in that extracted by 6 M-GdnHCl, so that degradation of well-established fibres must occur. Even though anaerobiosis would interfere with action of prolyl hydroxylase, and therefore synthesis of collagen (Prockop & Juva, 1965), this cannot be the main cause of diminished collagen content, since the observed decrease occurs within hours, too rapidly for biosynthesis to be significant. Thus increased degradation is probably responsible for the observed decreased content of collagen. Consistent with this idea, the infarcted areas of myocardium, when compared with non-infarcted tissue controls, exhibited increased activities of collagenase, non-specific neutral proteinase and serine proteinases, notably elastase and cathepsin G. The last two enzymes have been implicated in the turnover of the extracellular matrix collagens of cartilage (Starkey *et al.*, 1977), acting in a manner similar to that of the action of pepsin on collagens *in vitro*. Pepsin is known to solubilize type I and II collagens of tissues by cleaving the peptide bond near to the cross-linking regions of collagen molecules (Rubin *et al.*, 1965; Miller, 1971); these regions occur in the *N*- and *C*-termini of constituent α chains. In the present study, the observed degradation of myocardial collagen in the MI tissue could be due in part to the action of presumed lysosomal elastase (a general proteinase). We should note, however, that the tissue preparation used was the supernatant of a total homogenate, so that we cannot definitely assign the effective proteinase to an intracellular origin.

In general, the activities of collagenase, elastase and cathepsin G cannot be reproducibly quantified in the tissue extracts, unless serum, which contains proteinase inhibitors, is removed from the tissue. Serum inhibitors may be present to a much lesser degree in the tissue made

Table 1. Elastase, cathepsin G, cathepsin D and β-D-glucosaminidase activities in rat LV

The lysosomal enzyme activities in the supernatants of the homogenates of LVs from 3-month-old Wistar rats [normal controls (n = 10); rats with MIs produced by ligating the LAD for 3 h (n = 10); and non-infarcted tissue controls (n = 10)] were measured using native and synthetic substrates as indicated in parentheses below. Enzyme activity is expressed in units: one unit of enzyme releases radioactivity or chromogen to the soluble phase, in an amount corresponding to 1 μg (elastin or haemoglobin) or 1 μmol of synthetic substrate/h at 37 °C. Assay conditions are as in the text.

	Enzyme activities (units/h per 100 mg of LV protein)				
Tissue	Elastase (elastin)*	$10^{-3}\times$ Elastase (synthetic)*	$10^{-3}\times$ Cathepsin G (synthetic)*	Cathepsin D (haemoglobin)*	β-D-Glucosaminidase (synthetic)*
Normal tissue	0.35+0.03	7.70+1.0	5.91+0.7	8.8+1.0	2000+220
Non-infarcted tissue	0.29+0.03	7.70+1.0	6.06+0.6	8.2+1.0	1980+200
M.I.-tissue	0.72+0.06	16.97+1.5	13.39+1.0	10.2+1.8	2660+310

* See text for specific substrates.

Table 2. Inhibition of serine proteinase activity of MI tissue by chloromethane substrates

The selective inhibition by peptide inhibitors of elastase and cathepsin G of the protein fraction (10 μg/0.1 ml) of MI tissue obtained from a Ultrogel AcA 54 column was determined by measuring the remaining enzyme activity using native and synthetic substrates as indicated in parentheses. Preparation of the serine proteinase fraction and inhibitor assays are as in the text.

Enzyme activity measured and substrate used	Inhibitor	Concentration (mM)	Activity remaining (%)
Elastase	None	0	100
(Elastin)	MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl	0.2	0
	Z-Gly-Leu-Phe-CH$_2$Cl	0.2	87
Elastase	None	0	100
[Suc-(Ala)$_3$-pNA]	MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl	0.2	0
	Z-Gly-Leu-Phe-CH$_2$Cl	0.2	85
Cathepsin G	None	0	100
	MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl	0.02	2
[Suc-(Ala)$_2$-Pro-Phe-pNA]	Z-Gly-Leu-Phe-CH$_2$Cl	0.02	95

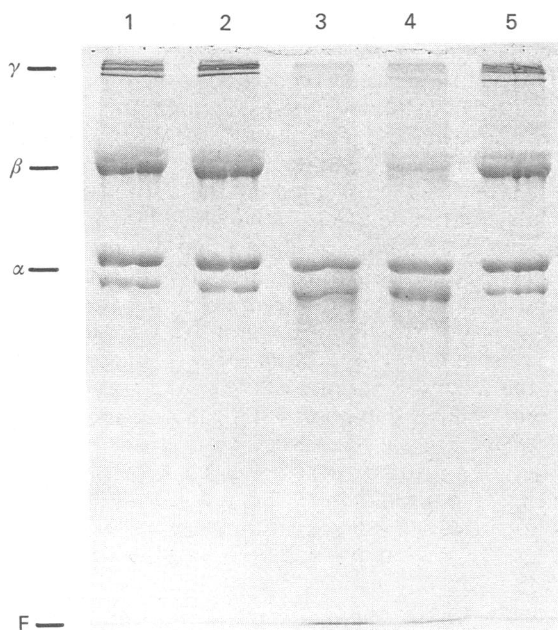

Fig. 6. Degradation products of type I collagen produced by the action of myocardial serine proteinases

The reaction mixture of acid-soluble rat tail tendon type I collagen (12.5 μg) and the protein fraction (2.5 μg) containing serine proteinase of MI tissue obtained from a Ultrogel AcA 54 column was subjected to SDS/PAGE (7.5% gel) and stained with Coomassie Brilliant Blue. Lane 1, reaction mixture of substrate and proteinase at 25 °C for 16 h: substrate alone; lane 2, substrate plus pancreatic elastase (2.5 μg); lane 3, substrate plus protein fraction; lane 4, substrate plus protein fraction pretreated with 0.02 mM-Z-Gly-Leu-Phe-CH$_2$Cl; lane 5, substrate plus protein fraction pretreated with 0.2 mM-MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl. Preparation of the serine proteinase fraction and inhibitor assays were carried out as described in the text. F, buffer front.

ischaemic by ligation of LAD, since blood flow is reduced. On the other hand, perfusion with Evan's Blue or PBS would be expected to remove most of these inhibitors from the tissues. The cellular locations of collagenase, elastase and cathepsin G in myocardium, or the effects of ischaemia on activation of procollagenase or procollagenase activator, or indeed the cause of the increased activities of collagenase, elastase and cathepsin G in the MI, are presently not known. However, the time period required for the appearance of extracellular procollagenase, after its intracellular synthesis, has been reported to be 3–6 h (Valle & Bauer, 1980; Brinckerhoff *et al.*, 1982), and the time period required for the appearance of inflammatory cells into infarcted tissue has also been reported to be 3–6 h (Mallory *et al.*, 1939; Fishbein *et al.*, 1978*b*). These findings suggest that the increased collagenase activity in the MI is due to the activation of free or bound forms of procollagenase resident in the myocardium. The increased activities of collagenase, non-specific neutral proteinase and serine proteinase may be responsible for the rapid degradation of extracellular matrix collagen in the MI.

Anatomically, the rat heart differs from that of the dog or human, and the extent and the course of MI following coronary occlusion differs among species. However, the ultimate effects of ischaemia on the degradation of extracellular matrix collagen of the heart may be similar in both experimental animals and humans.

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