DEGRADATION OF CONNECTIVE TISSUE MATRICES BY MACROPHAGES

I. Proteolysis of Elastin, Glycoproteins, and Collagen by Proteinases Isolated from Macrophages*

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Turnover of the extracellular connective tissue matrix occurs during tissue remodeling, wound healing, and in a variety of pathological conditions including rheumatoid arthritis, chronic obstructive pulmonary disease, atherosclerosis, and tumor invasion. It has been suggested that the degradation of connective tissue proteins involves two distinct steps—an initial extracellular cleavage of insoluble proteins mediated by neutral proteinases, followed by endocytosis and completion of digestion within lysosomes (1). Mononuclear phagocytes are prominent cells in the host defense characteristic of chronic inflammatory responses. These phagocytes contain a rich complement of lysosomal hydrolases (2–5) and are active secretory cells, producing neutral proteinases including plasminogen (Plg)[1] activator (6–8), elastase (a metalloproteinase that degrades elastin) (8–10), collagenase (11, 12), a casein-degrading serine proteinase (13), and, under some circumstances, lysosomal hydrolases (5). Although macrophages have been shown to phagocytose collagen fibrils in connective tissues (14, 15), and elastin particles injected intraperitoneally (16, 17), it is still conjectural whether macrophages participate directly in the extracellular degradation of the matrix macromolecules. Plg activator can mediate extracellular protein degradation by macrophages in the presence of Plg (7, 18), but other enzymes such as collagenase are present in extracellular fluids in an inactive or latent form (8, 11). Macrophages also play an important role in modulating the capacity of other cell types to secrete neutral proteinases (19–21) and degrade matrix macromolecules (20). Accordingly, we have investigated the role of mouse macrophage proteinases in the extracellular and intracellular degradation of connective tissue matrices.

In the present report we have characterized the components of complex, insoluble extracellular matrices secreted by rat vascular smooth muscle cells, endothelial cells, and fibroblasts, and have analyzed the ability of purified macrophage neutral proteinases and mixtures of neutral proteinases secreted by resident and inflammatory macrophages to degrade them. We also investigated the degradation of the extracel-

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[1] *Abbreviations used in this paper:* CPA, clostridiopeptidase A; DME, Dulbecco's modified Eagle's medium; HGE, human granulocyte elastase; LH, lactalbumin hydrolysate; PE, porcine pancreatic elastase; Plg, plasminogen; SDS, sodium dodecyl sulfate; SMC, smooth muscle cells; TG, thioglycollate elicited.

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lular matrix by hydrolases in macrophage lysosomes, and the synergy between the neutral proteinases and lysosomal enzymes in degrading matrix macromolecules to amino acids and oligopeptides. In the accompanying reports we have extended these observations to the degradative capacity of live mouse macrophages growing in contact with extracellular matrices (22), and have determined by morphological and biochemical techniques the contributions of extracellular, pericellular, and intracellular events to degradation of matrix macromolecules (23).

Materials and Methods

Materials. Tissue culture dishes were obtained from Falcon Plastics, Oxnard, Calif. (35-mm dishes) or Costar, Data Packaging, Cambridge, Mass. (16-mm wells in 24-well plates, and 25-cm^2 flasks). Eagle's minimal essential medium, Dulbecco's modified Eagle's medium (DME), McCoy's 5A medium, penicillin-streptomycin solution, and lactalbumin hydrolysate (LH) were obtained from Grand Island Biological Co., Grand Island, N. Y. Fetal bovine serum was purchased from either Grand Island Biological Co., or Irvine Scientific, Irvine, Calif., and was heat inactivated before use. Tryptose phosphate broth, *Escherichia coli* 026:B6 lipopolysaccharide W, and brewer's thioglycollate broth were purchased from Difco Laboratories, Detroit, Mich. Ascorbic acid (Sigma Chemical Co., St. Louis, Mo.) was recrystallized from ethanol (24, 25). L-[3,4(n)-^{3}H]proline (25-50 Ci/mmol), L-[1-^{3}H]fucose (3 Ci/mmol), and L-[U-^{14}C]lysine (300 mCi/mmol) were purchased from either New England Nuclear, Boston, Mass., or Amersham Corp., Arlington Heights, Ill. ACS scintillation fluid was purchased from Amersham Corp., and Biofluor from New England Nuclear. Rabbit antiserum to rat fibronectin adsorbed with bovine fibronectin was a gift of Dr. Eva Engvall, La Jolla Cancer Research Foundation, La Jolla, Calif. Elastin from bovine ligamentum nuchae was purchased from Elastin Products Co., St. Louis, Mo. Sephadex G-25, G-10, and Sepharose 4B were purchased from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J. α_2-Macroglobulin was purified from human haptoglobin type 1-1 plasma by Cibacron blue chromatography (26), and α_1-proteinase inhibitor was purchased from Worthington Biochemical Corp., Freehold, N. J. Pepstatin and leupeptin were purchased from Vega-Fox Biochemicals Div., Newbery Energy Corp., Tucson, Ariz. Other materials were purchased from standard suppliers.

Macrophage and Other Tissue Proteinases. Macrophage elastase form B was purified from the conditioned medium of thioglycollate-elicited (TG) mouse macrophages by the method of Banda and Werb (9; unpublished data). The preparations used had specific activities of >5,000 U/mg. Purified human granulocyte elastase (HGE) was kindly provided by Dr. Alan J. Barrett, Strangeways Research Laboratory, Cambridge, England. Mammalian collagenase was obtained from the culture medium of rabbit fibroblasts induced with 20 ng/ml 12-*O*-tetradecanoyl-phorbol-13-acetate (27), then activated with trypsin (11, 21). Collagenase was then partially purified by affinity chromatography on a column of immunoglobulin from a sheep antiserum to rabbit collagenase (28) linked to Sepharose 4B. Plg was prepared from human or bovine plasma by affinity chromatography on lysine-Sepharose, and verified to be plasmin-free by the ^{125}I-fibrin plate assay (7, 21). Plasmin was prepared by activating bovine or human Plg with urokinase (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.) as described previously (21). Cathepsin D from bovine spleen was obtained from Sigma Chemical Co.

Enzymes for Matrix Analysis. TPCK-trypsin (Worthington Biochemical Corp.) or trypsin type III (Sigma Chemical Co.) was dissolved at 1 mg/ml in 50 mM Tris-HCl buffer, pH 7.6, and then pretreated with 5 mg of bovine elastin/ml to adsorb contaminating elastase. Porcine pancreatic elastase (PE) was purchased from Sigma Chemical Co. (type IV) or Worthington Biochemical Corp. (type ESFF). Clostridiopeptidase A (CPA)[2] (bacterial collagenase type CLSPA) was purchased from Worthington Biochemical Corp.

[2] To avoid confusion with mammalian collagenase, bacterial collagenase is referred to as clostridiopeptidase A (CPA) throughout this paper.

Analytical Methods

AMINO ACID ANALYSIS. Samples for amino acid analysis were hydrolyzed under nitrogen in constant boiling HCl and analyzed on a Durrum D500 analyzer as described previously (24). Cross-linking amino acids (including desmosine and isodesmosine) were estimated by chromatography of hydrolyzed samples from [^{14}C]lysine-labeled matrices on Sephadex G-10, essentially as described for collagen cross-links (29).

SODIUM DODECYL SULFATE (SDS)-POLYACRYLAMIDE GEL ELECTROPHORESIS. Matrix proteins were analyzed by gel methods described previously (30) before or after pepsin treatment (31).

GEL FILTRATION OF SOLUBLE PEPTIDES. Peptides solubilized from radiolabeled matrices were separated on a 11.8 × 26-cm column of Sephadex G-25 equilibrated with 0.2 M Tris-HCl buffer, pH 7.6, containing 100 μg bovine serum albumin/ml and 0.1% NaN_3. 1.5-ml fractions were collected. For this column the void volume corresponded to fraction 14 and the totally included volume to fraction 27.

ENZYME ASSAYS. Macrophage elastase in conditioned culture medium was assayed as described previously using [^{3}H]$NaBH_4$-reduced elastin as substrate (8). Total elastase (active plus latent) was determined with ^{3}H-elastin substrate in the presence of 0.132 mg SDS/ml; active elastase was determined with ^{3}H-elastin alone. One unit of elastase solubilized 1 μg of elastin/h at 37°C. Plasminogen activator was measured by assaying aliquots of conditioned medium on ^{125}I-labeled fibrin plates as substrate in the presence of Plg. Plg activator was determined by assaying 25–100-μl aliquots of medium in 50 mM Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. One unit of Plg activator solubilized 1 μg of ^{125}I-fibrin/h at 37°C (7, 8). Collagenase was measured using ^{14}C-labeled collagen fibrils as substrate (21). One unit of collagenase solubilized 1 μg of collagen/min at 37°C. Protein content was determined by the method of Lowry et al. (32).

LYSOSOME PREPARATIONS. Subcellular fractions enriched in lysosomes were prepared from 5 × 10^7 TG or endotoxin-elicited macrophages or P388D1 macrophages by suspending pellets of cells that had been washed twice with phosphate-buffered saline in 0.25 M sucrose. The cells were disrupted in a Dounce homogenizer with a tight-fitting pestle (Kontes Co., Vineland, N. J.), then centrifuged at 500 *g* for 10 min to remove nuclei and debris. The supernate was then centrifuged at 10,000 *g* for 30 min to collect a lysosome-rich pellet. The lysosomes were then suspended and solubilized in 5 ml of 0.1% Triton X-100.

CULTURE OF MACROPHAGES. Unstimulated (resident) macrophages were obtained by lavage from the peritoneal cavity of Swiss mice (Charles River Breeding Laboratories, Wilmington, Mass.), and inflammatory macrophages by lavage 4 d after injection of one of the following eliciting substances: 1 ml of 3% brewer's thioglycollate broth (15, 31); 30 μg of endotoxin (*E. coli*, 026:B6 lipopolysaccharide); 0.5 ml of 5 mM $NaIO_4$ (metaperiodate) in 0.15 M NaCl; or 1 mg pyran copolymer (a gift of Hercules Chemical Co., Inc., New York). P388D1 macrophages were maintained in culture as described previously (33). To prepare conditioned media, 2.5 × 10^5 macrophages/cm^2 were plated in tissue culture dishes in DME-10% fetal bovine serum for 2 h, washed vigorously three times, and then incubated in DME supplemented with 0.2% LH (1 ml/5 × 10^5 macrophages) for 48–72 h.

Preparation and Analysis of Extracellular Matrices

PREPARATION OF LABELED MATRIX FROM SMOOTH MUSCLE CELLS (SMC). Labeled extracellular matrices were prepared essentially as previously described (18). Rat heart SMC from fourth to ninth passage stock cultures of the R22 strain (30) were seeded into 35-mm plastic dishes (10^5/dish) or 16-mm wells of 24-well plates (0.5 × 10^5/well). The cells were grown in minimal essential medium containing 10% fetal bovine serum, 2% tryptose phosphate broth, and penicillin-streptomycin. 50 μg/ml of ascorbic acid was added to cultures daily and [^{3}H]proline or [^{3}H]fucose was added at 5 d after seeding. The cells were cultured for another 7 d with two weekly medium changes and then lysed by the addition of 0.25 M NH_4OH for 30 min at room temperature (18). The insoluble extracellular matrix, which remained firmly anchored to culture dishes, was washed vigorously with distilled water followed by 70% ethanol and air dried. Matrices were sterilized with 70% ethanol for 5 min and then washed with phosphate-buffered saline before the addition of macrophages.

PREPARATION OF GLYCOPROTEIN-DEPLETED MATRIX. Unless indicated otherwise, glycoprotein-

depleted matrices were prepared by incubation of complete matrices with 20 μg/ml elastin-treated trypsin for 5 h at 37°C. Complete matrices were sham incubated with buffer only (0.1 M Tris-HCl, pH 7.6, containing 0.01 M $CaCl_2$) at the same time. The matrices were washed extensively with water, 70% ethanol, and phosphate-buffered saline before the addition of macrophages.

PREPARATION OF COLLAGEN MATRICES. Matrices from the rat fibroblastic subclone R22 ClF (30) were labeled and prepared as described for the R22 SMC matrix.

PREPARATION OF ENDOTHELIAL MATRICES. Basement membranes produced by cultured bovine aortic endothelial cells were prepared by culturing cells of the Al_4Cl-1 strain in the presence of [^{3}H]proline with daily additions of 50 μg/ml ascorbic acid on the unlabeled extracellular matrix produced by R22 SMC (34). 2 wk after seeding, the producer endothelial cells were removed by NH_4OH lysis and the labeled endothelial cell matrix was processed as described for the R22 SMC matrix.

ANALYSIS OF MATRIX COMPOSITION. The composition of the matrix was determined by sequential enzyme digestion with elastin-adsorbed trypsin, PE, and clostridiopeptidase A (CPA). The matrix was incubated, in turn, with 10 μg/ml of each enzyme in 0.1 M Tris-HCl buffer containing 10 mM $CaCl_2$ for 3 h at 37°C as described previously (30).

STUDIES OF MATRIX DIGESTION. In all experiments enzymes, medium, or cells were incubated with matrix, in duplicate, and results were expressed as the mean of these values. Duplicates were usually within 5% of each other. For experiments in which macrophages were cultured on extracellular matrix, cells were plated onto the matrix at 2.5×10^5 macrophages/cm^2 in either DME-0.2% LH or McCoy's 5A medium. The cells were allowed to attach to the matrix for 2-3 h and then washed with medium to remove nonadherent cells. Fresh medium (1 ml with 16-mm wells, 2 ml with 35-mm dishes) was then added and the progress of digestion was followed by sampling 50- to 100-μl aliquots of medium at timed intervals into 5 ml of scintillation fluid and counting in a liquid scintillation spectrometer. At the end of the experiment, the medium or reaction mixture was removed from the matrix, the macrophages were lysed by the addition of 0.25 M NH_4OH, and composition of the residual matrix was determined.

For study of the degradation by enzymes secreted by macrophages, conditioned medium (pH 7.4) was placed on the matrix and the radioactive components were monitored as described for live macrophages. Purified enzymes were incubated on the matrix in 0.1 M Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$. Lysosomal enzymes were incubated with matrix in 0.1 M acetate or Tris-acetate buffer at pH values indicated in the Results.

CALCULATION OF MATRIX DIGESTION. To compare the rate of degradation of matrix by living macrophages and medium conditioned by macrophages, a linear rate of enzyme secretion was assumed: therefore, the exposure of matrix to enzymes produced by cells growing on matrix for t hours was assumed equivalent to exposure of matrix to conditioned medium for $t/2$ hours.

For experiments in which the degradation of matrix was monitored by the release of radioactive components from ^{3}H-labeled matrices, the relative digestion of the matrix components was expressed as a percentage of the total radioactivity released. The composition of the glycoprotein, elastin, and collagenous components of the matrix was determined as a percentage of the trypsin-, PE-, or CPA-sensitive material solubilized by sequential enzyme digestion. In some experiments the composition was then corrected for the relative abundance of the amino acid used for labeling (e.g., collagen contains more proline than either glycoproteins or elastin [Table I]).

Results

Properties of the Biosynthetically Prepared Insoluble Extracellular Matrices. The internal elastic lamina produced by vascular SMC in vivo consists of microfibrillar and related glycoproteins, elastin, and several types of collagen (35-40). In previous work we demonstrated that R22 SMC secrete and assemble an insoluble extracellular matrix consisting of components solubilized by trypsin, PE, and CPA (24, 30). Transmission electron microscopy showed that the SMC matrix contains morphologically identifiable collagen fibers, amorphous elastin, microfibrillar glycoprotein, and other mate-

rials (23). No cell-associated proteins such as actin were detected on the insoluble matrix by morphological and immunological methods (not shown). In the present study we first characterized the R22 SMC matrix components in greater detail by means of sequential enzyme digestion to better analyze the role of macrophage proteinases in the degradation of matrix macromolecules. The collagen of the insoluble extracellular matrix, analyzed by gel electrophoresis after pepsin solubilization, was largely type I, as shown by the presence of bands co-migrating with α_1(I) and α_2 subunits, with small amounts of other collagen subunits (Fig. 1 a). Although SMC secreted fibronectin (20 μg/ml), this glycoprotein was not detected in the insoluble matrix by immunologic methods (not shown), and it is normally not present in the internal elastic lamina of blood vessels (39). However, complete matrices contained several glycoproteins, including a major protein of 250,000 dalton that was extracted with SDS and dithiothreitol. These proteins were no longer in evidence after trypsin digestion (10 μg/ml for 30 min) (Fig. 1 b). Longer treatments with trypsin removed the morphologically identifiable microfibrils (23) and most of the fucose-rich proteins from the matrix. The R22 SMC secreted 72,000-dalton tropoelastin and assembled it into insoluble elastin.

Amino acid analysis validated the procedure of sequential enzyme digestion. The

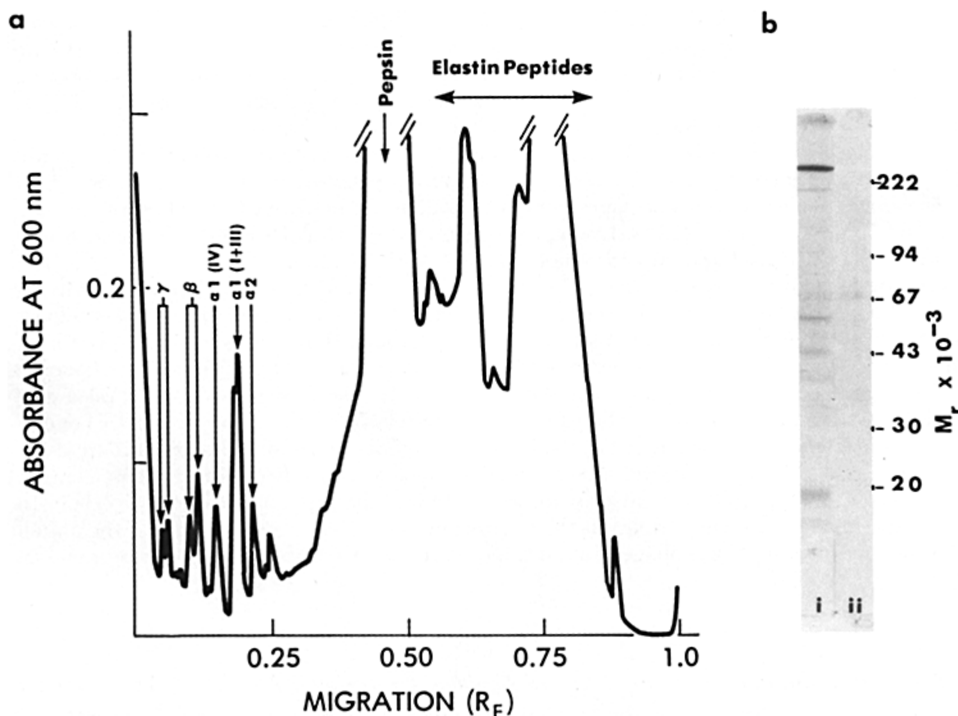

FIG. 1. Collagen and glycoprotein content of R22 SMC insoluble matrix. (a) Densitometric scan of SDS-polyacrylamide gel of pepsin-solubilized R22 matrix. Markers show the migration of pepsinized standards α_1(I)-, α_2-, β-, and γ-subunits of rat tail collagen type I, α_1(III) from rabbit fibroblasts, and α_1(IV) from bovine anterior lens capsule, and bovine ligament elastin. (b) Gels showing SDS-gel sample buffer extractable proteins from a freshly prepared matrix from R22 cells grown for 6 d. (i) Complete matrix extracted; (ii) matrix extracted after trypsin pretreatment. Migration of molecular weight standards is shown on the right.

trypsin-solubilized peptides were remarkably similar to microfibrillar glycoproteins (Table I). It is not yet clear whether the hydroxyproline in the trypsin-soluble material represents a small amount of denatured collagen, trypsin-sensitive type III collagen, uncross-linked tropoelastin (40), or an intrinsic component of microfibrils, as suggested by others (38). Subsequent digestion of the extracellular matrix by PE liberated peptides with amino acid composition indistinguishable from that of rat aortic elastin (Table I). The PE-sensitive component also contained complex amino acid cross-links derived from lysine. The remaining portion of the extracellular matrix, digested only by CPA, was enriched in glycine, proline, and hydroxyproline and was similar to rat type I collagen (Table I).

Two further observations are relevant to the validation of the sequential digestion procedure for analysis of components remaining in the extracellular matrix. When CPA was used before trypsin and PE, the rate of liberation of collagen peptides was reduced considerably, suggesting that the presence of glycoprotein and elastin com-

TABLE I

Amino Acid Composition of R22 SMC Matrix Components Solubilized by Trypsin, PE, and CPA

Amino acid	Residues/1,000 residues*					
	R22 trypsin solubilized	Microfibril	R22 PE solubilized	Rat aorta elastin	R22 CPA solubilized	Rat collagen
Gly	115	110	321	335	332	327
Ala	81	65	189	203	116	106
Val	71	56	90	86	45	22
Pro	76	64	106	111	115	117
Hyp	8	ND‡	21	13	78	100
Ile	41	48	26	28	18	10
Leu	78	69	65	69	45	25
Tyr	25	36	24	40	6	3
Phe	26	38	18	25	18	13
Thr	74	56	33	14	28	20
Ser	65	62	25	16	36	41
Asp	86	113	24	11	44	47
Glu	103	114	30	21	60	74
Met	9	16	0	1	3	6
His	15	15	2	4	5	5
Arg	47	45	14	10	33	49
Lys	53	45	11	11	16	29
Hyl	1	ND	1	0	7	6
Cys/2	26	48	0	0	0	0
X	0	0	~1	~2	0	0
Fucose	87	ND	<2	ND	<1	ND

* R22 matrix consisted of 40% trypsin-sensitive peptides, 41% PE-sensitive peptides, and 13% CPA-sensitive peptides by weight. Bovine ligament microfibrillar protein composition was taken from Rucker and Tinker (41) and should be compared with enzyme-digested microfibrillar protein from Ross and Bornstein (37). Newborn rat aorta elastin composition was from Looker and Berry (35) with values for Hyp, Hyl, and cross-linking amino acids (X) added from Bentley and Hanson (42). Thr and Ser values were not corrected for loss. Amino acid analysis of type I collagen was from Piez and Gross (43). Cross-linking amino acids (desmosine and others) were determined by [^{14}C]lysine incorporation followed by gel filtration of acid hydrolysates (29). Carbohydrate was determined as the percent of [^{3}H]fucose incorporated into each component.

‡ ND, not determined.

ponents may protect the collagen from rapid digestion (W. Laug and P. A. Jones. Manuscript in preparation.). When trypsin-treated matrices were partially digested by PE, the residual elastin was susceptible to some solubilization (5–15%) during a second treatment with trypsin. These data are in keeping with previous studies on the synergism between PE and trypsin in liberation of peptides from elastin (44) and must be borne in mind during the use of sequential digestion to determine remaining matrix components after digestion with macrophage enzymes.

Typical R22 SMC matrices contained 25 μg of insoluble matrix (range, 10–50 μg) per cm^2. A typical matrix contained 10 μg glycoprotein, 10 μg elastin, and 5 μg collagen/cm^2 as determined by protein analysis (24) and sequential enzyme digestion of [^{3}H]proline-, [^{3}H]valine-, and [^{3}H]fucose-labeled matrices corrected for enrichment of the particular amino acids (Table I).

The molecules secreted by endothelial cells are composed of glycoproteins, including laminin and fibronectin (39, 45), a mixture of collagens consisting of type I (20%), type III (60%), type IV (5%), and type AB (15%) (31, 46, 47 [R. Stern, University of California, San Francisco, Calif. Personal communication]), but no elastin. The vascular fibroblastic clone R22 ClF secreted collagens and fibronectin (24, 34, 48), as determined by sequential enzyme digestion.

Digestion of Extracellular Matrix Proteins by Macrophage Proteinases. Mouse macrophages secrete Plg activator, elastase, and collagenase when stimulated appropriately (6, 10, 12). Although macrophage Plg activator has little proteolytic activity of its own, it is able to generate an active proteinase, plasmin, after interaction with Plg. Macrophage collagenase cleaves collagens of types I, II, and III (11). Macrophage elastase hydrolyzes elastin as well as fibronectin, laminin, fibrinogen, and proteoglycans (2, 9, 49). Accordingly, we examined the roles of plasmin, collagenase, and elastase in degrading extracellular matrices. We used purified plasmin and highly purified macrophage elastase, but because highly purified macrophage collagenases are not available, rabbit fibroblast collagenase was chosen as a representative mammalian collagenase.

Plasmin was less effective than trypsin in removing the insoluble glycoprotein components of the extracellular matrix even though it has tryptic specificity (Fig. 2).

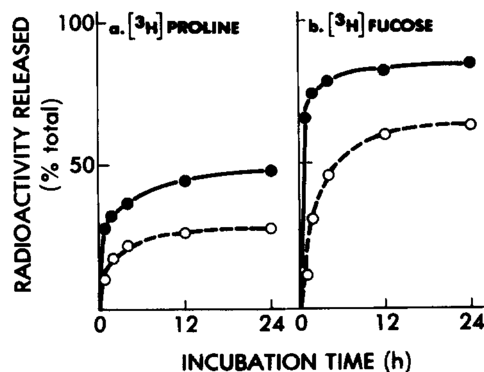

Fig. 2. Comparison of the solubilization of R22 SMC matrix by plasmin and trypsin. (a) Matrix labeled with [^{3}H]proline to study solubilization of protein; (b) matrix labeled wtih [^{3}H]fucose to allow selective examination of the solubilization of glycoprotein components. (●) Trypsin (10 μg/ml); (○) plasmin (10 μg/ml).

Only 50–70% of the glycoprotein component was removed by exhaustive plasmin digestion in these experiments with [^{3}H]proline- and [^{3}H]fucose-labeled matrices. It is not yet clear if this difference between plasmin and trypsin in digestion represents heterogeneity within the glycoprotein components of this matrix, or if plasmin makes more limited cleavage in the glycoproteins, leaving fragments of glycoprotein still attached to the elastin and collagen components. Plasmin did not degrade elastin or collagen.

Purified macrophage elastase hydrolyzed both complete matrix and matrix depleted of glycoproteins. The rate of degradation was greatly increased after removal of glycoproteins (Table II). Macrophage elastase solubilized the glycoprotein components and elastin, but not collagen.

Purified mammalian collagenase from rabbit fibroblasts digested only the collagen components of the R22 SMC extracellular matrix. The rate of degradation of collagen was greater in the glycoprotein-depleted matrix (Table II). It has been observed that collagen protects glycosaminoglycans from degradation (50, 51). Glycoprotein components may also protect collagen from digestion by collagenase. Thus the protection of fibrillar components of connective tissue matrix by glycoproteins may be a general mechanism (22) by which connective tissue architecture is maintained in the face of some limited proteolytic digestion.

Comparison of Macrophage Elastase with Other Elastinolytic Enzymes. The glycoprotein-, elastin-, and collagen-degrading ability of PE and HGE was compared with that of macrophage elastase. The macrophage elastase and cultured macrophages degraded complete matrices more slowly than glycoprotein-depleted matrices. In contrast, the complete and glycoprotein-depleted matrices were hydrolyzed to the same extent by PE and HGE (Table III). HGE also regularly degraded a small proportion (10%) of the collagen of the R22 SMC matrix (not shown).

Degradation of Endothelial and Fibroblastic Matrices by Macrophage Enzymes. The degradation of extracellular matrices produced by endothelial and fibroblastic cells was

TABLE II

Degradation of Complete and Glycoprotein-depleted R22 SMC Matrices by Purified Macrophage Elastase and Mammalian Collagenase

Matrix	Enzyme	Amount added	Incubation time	Matrix protein solubilized			Acid-soluble digestion products*
				Glycoprotein	Elastin	Collagen	
		U	*h*	%	%	%	*% total*
Complete	Elastase	6.0	16	35	12	0	31
		18.0	16	63	72	0	51
	Collagenase	1.2	24	6	2	31	<5
Glycoprotein depleted‡	Elastase	6.0	16	NP§	16	2	17
		18.0	16	NP	85	3	34
	Collagenase	1.2	24	NP	4	64	<5

* Solubilized peptides were treated with 5% (wt/vol) trichloroacetic acid and percentage of acid-soluble and insoluble peptides was determined. The acid-soluble peptides were <5% soluble when precipitated a second time with 40% (wt/vol) trichloroacetic acid.

‡ Glycoproteins were removed from the [^{3}H]proline-labeled matrix by trypsin pretreatment as described in Methods.

§ NP, not present.

TABLE III
Degradation of Complete and Glycoprotein-depleted R22 SMC Matrices by Elastinolytic Enzymes and Macrophages

Enzyme treatment	Incubation time	Radioactivity solubilized	
		Complete matrix	Glycoprotein-depleted matrix
	h	%	%
PE	8	11.6	11.7
HGE	8	13.6	9.5
Macrophage elastase	24	8.3	17.2
Live macrophages	48	4.0	18.2

The percentage of radioactivity solubilized by purified elastases or by live macrophages from complete or glycoprotein-depleted matrices labeled with [^{3}H]proline was determined. Incubation was for 8 h with purified enzymes and 48 h with TG macrophages.

TABLE IV
Degradation of Endothelial and Fibroblastic Matrices by Plasmin, Elastases, and Mammalian Collagenase

Matrix*	Enzyme	Radioactivity solubilized	Glycoprotein degraded	Collagen degraded
		% total	%	%
Endothelial (Al_4Cl-1)	Macrophage elastase	43	61	9
	Plasmin	22	28	10
	Collagenase	19	1	58
	HGE	55	72	21
Fibroblastic (R22-ClF)	Macrophage elastase	2	37	0
	Plasmin	5	88	2
	Collagenase	28	0	28
	HGE	15	95	9

5.7 U of purified macrophage elastase (form B), 10 μg of bovine plasmin, 1.2 U of rabbit fibroblast collagenase, or 1.1 μg of HGE was used in each assay with the [^{3}H]proline-labeled matrices and incubation was for 24 h.

* The distribution of [^{3}H]proline label, as a percentage of the total radioactivity incorporated determined by sequential trypsin, PE, and CPA treatments, was, respectively: endothelial matrix, 66, 1, 33; fibroblastic matrix, 6, 2, 92. Because of the low amount of PE-sensitive material, results for trypsin and PE were combined.

examined in comparison with the elastin-rich R22 SMC matrices used in other experiments. Macrophage elastase and plasmin degraded the glycoproteins of these matrices readily (Table IV). HGE rapidly degraded the glycoproteins and also some of the collagen, in keeping with its previously reported collagenolytic activity (52). Collagenase degraded only the collagen components of these matrices.

Effect of Plasma Inhibitors on Degradation of Matrix by Macrophage Enzymes. Macrophages are normally found in tissues surrounded by plasma filtrates. Therefore, the effect of plasma inhibitors in regulating degradation of matrix was examined. Although plasmin activity was inhibited by both α_2-macroglobulin and α_1-proteinase

inhibitor, macrophage elastase was sensitive only to α_2-macroglobulin (Table V).

Evidence for a Latent Form of Macrophage Elastase in Macrophage-conditioned Medium. The capacity of conditioned medium from TG macrophages to degrade SMC matrix was compared with that of purified macrophage elastase. Although the conditioned medium had >20 U of elastase activity/ml, as assayed with ^{3}H-elastin in the presence of SDS, the degradation of matrix by either cultured cells or conditioned medium was less than that seen with one unit of purified macrophage elastase after a 48-h incubation (Table VI). Inclusion of plasminogen accelerated the rate of elastin degradation by medium on complete matrices only (data not shown). The data in Table VI suggest that 90–95% of elastase activity secreted by macrophages is present in a latent form that is activated or revealed in the standard elastin assay with SDS. The assay of elastase in the absence of SDS appears to reflect more accurately the degradative potential of the secreted macrophage proteinase. Plasmin did not activate

TABLE V
Effect of Plasma Inhibitors on Degradation of R22 SMC Matrix by Macrophage Enzymes

Enzyme	Inhibitor added	Inhibitor concentration	Radioactivity solubilized
		μg/ml	*% total*
Macrophage elastase	None	0	10.2
	α_2-Macroglobulin	25	1.0
	α_1-Proteinase inhibitor	50	12.7
Plasmin	None	0	31.0
	α_2-Macroglobulin	25	2.1
	α_1-Proteinase inhibitor	50	8.2

3 U of macrophage elastase or 10 μg bovine plasmin was incubated with [^{3}H]proline-labeled matrix for 24 h.

TABLE VI
Comparison of the R22 SMC Matrix Degradation by Macrophage Elastase, Cultured Macrophages, and Medium Conditioned by Macrophages

Source of elastase*	Elastase activity‡		Radioactivity solubilized
	Active	Total	
	U	*U*	*% total*
Macrophage elastase, purified	1.0	1.2	12.3
Macrophage elastase, partially purified	0.9	1.3	15.7
Macrophage-conditioned medium	1.5	10.7	8.4
Live macrophages	3.1	20.2	8.1

* Purified macrophage elastase (>5,020 U/mg) or elastase (13 U/mg) partially purified by dialysis against 10 mM $NaHCO_3$ was incubated with matrix for 48 h in DME-LH. Medium was collected from TG macrophages (5×10^5 cells/well, 1 ml DME-LH) after 48 h and 0.5 ml was incubated with matrix for 48 h as described in the Methods. Live TG macrophages (5×10^5/well) were incubated on matrix for 48 h, and the enzyme activity present in the medium was determined at this time.

‡ Total elastase activity of the enzymes or medium was determined in the ^{3}H-elastin assay with SDS. Active elastase activity was determined with ^{3}H-elastin without SDS. Units shown are those present in the matrix degradation mixtures.

the latent macrophage elastase (data not shown), in contrast to its activation of collagenase (11). Similar results were seen with medium and enzymes on trypsin-pretreated matrices (compare Table VI with Tables II, III, and VII).

Effect of Various Eliciting Stimuli on the Degradative Potential of Macrophage-conditioned Medium. The data obtained with purified enzymes indicated that macrophage elastase and Plg activator played distinct roles in the proteolysis of the SMC matrix. Accordingly, the degradation of glycoproteins, elastin, and collagen by macrophages that secrete differing amounts of Plg activator and elastase was examined. Macrophages were elicited by a variety of inflammatory stimuli to modulate the rates of secretion of these proteinases (Table VII). In general, the rate of matrix degradation in the absence or presence of Plg was proportional to the amount of elastase and Plg activator present in the conditioned medium. Pyran copolymer-elicited macrophages secreted abundant Plg activator but little elastase, and consequently medium from these cells degraded only the matrix glycoproteins. P388D1 and TG macrophages secreted comparable amounts of enzyme, but the medium from P388D1 macrophages degraded more matrix. It is possible that the elastase in the medium of P388D1 macrophages was present in an active form. The medium from resident and endotoxin-elicited macrophages degraded the matrix poorly. Degradation of collagen could be detected only for P388D1 and TG macrophages, in keeping with previous observations on the small amounts of collagenase secreted by some stimulated macrophages (11).

pH and Inhibitory Profile of Digestion of Matrix by Macrophage Lysosomal Proteinases. Lysosomal extracts from P388D1 macrophages were used to study the capacity of lysosomal hydrolases to degrade R22 SMC matrix, directly and in the

TABLE VII

Comparison of R22 SMC Matrix Degradation by and Secreted Proteinases in Conditioned Medium from Macrophages Elicited by Various Inflammatory Stimuli

Eliciting agent	Secreted enzymes		Matrix degradation		Matrix protein degraded		
	Plg activator	Elastase	Plg added	Radioactivity solubilized	Glycoprotein	Elastin	Collagen
	U/ml	*U/ml*	*μg/ml*	*% total*	%	%	%
Resident	2.5	2.5	0	5.1	14	1	0
	—	—	10	7.3	21	10	0
Endotoxin	14	0.2	0	3.2	5	2	0
	—	—	10	9.4	32	11	0
Periodate	27	11.2	0	9.8	32	9	0
	—	—	10	28.7	66	53	0
Thioglycollate	48	16.8	0	13.2	35	12	2
	—	—	10	27.4	57	48	7
Pyran copolymer	94	1.9	0	3.5	9	0	0
	—	—	10	16.0	57	4	0
P388D1	58	26.3	0	31.3	62	35	1
	—	—	10	66.2	78	79	18

Conditioned medium was collected from 2.5×10^6 macrophages elicited in vivo by the agent indicated after a 48-h incubation in 5 ml of DME-LH. Aliquots were assayed for Plg activator and total elastase (i.e., with elastin in the presence of SDS). For R22 SMC matrix degradation 1 ml of conditioned medium was placed in each well with or without Plg for 24 h, and released [^{3}H]proline-labeled radioactivity was measured. The components degraded were determined by sequential enzyme digestion.

presence of activators of thiol proteinases (53). The rate of matrix degradation was accelerated threefold when thiol enzymes were activated (Table VIII). The optimum pH was between 5.0 and 5.5 under both conditions, with little degradation at pH 3.6 and 6.4. The degradation of matrix in the presence of activator was completely abolished by 1 μl/ml pepstatin and 10 μg/ml leupeptin, inhibitors of cathepsin D and cathepsin B and related thiol enzymes. At pH 5.0 the glycoprotein, elastin, and collagen components of the matrix were degraded. Lysosomal extracts from TG and endotoxin-elicited macrophages also degraded the matrix to soluble peptides. Purified cathepsin D at pH 5.0 degraded the glycoprotein components of R22 SMC matrix, but little elastin or collagen (not shown). After 24 h, 67 and 92% of the radioactivity solubilized by TG macrophage extracts, without and with activator, respectively, were soluble in 5% trichloroacetic acid (data not shown). The fact that most of the solubilized radioactivity was acid-soluble suggested that lysosomal peptidases contribute to total solubilization of the matrix. Interestingly, the lysosomal enzymes of the TG and endotoxin macrophages were equally effective in degrading matrix (Table VIII), whereas, at neutral pH, the conditioned medium of TG macrophages was much more active in matrix digestion than that of endotoxin macrophages (Table VII). Although lysosomal enzymes may be secreted by macrophages (5), conditioned medium of TG macrophages did not degrade the SMC matrix at pH 5.0 (not shown).

Synergism of Macrophage-secreted and Lysosomal Hydrolases in R22 Matrix Degradation. Macrophage neutral proteinases and conditioned medium degraded matrix to peptides of varying size, mostly >5,000 dalton. The peptides produced by digestion of matrix with medium conditioned by TG macrophages were excluded from Sephadex G-25 chromatography, but after subsequent digestion by a lysosomal extract, the radioactivity was associated with the size of amino acids and oligopeptides (Fig. 3).

Discussion

In this paper we have demonstrated that purified macrophage proteinases, and mixtures of enzymes present in mouse macrophage-conditioned medium or in lyso-

TABLE VIII

Degradation of R22 SMC Extracellular Matrix by Lysosomal Extracts from Macrophages

Macrophage lysosomal extract	Assay pH	Radioactivity solubilized	
		Without activator	With activator
		%	%
P388D1	3.6	1.3	4.3
	4.4	3.3	12.5
	5.0	5.2	19.3
	5.4	6.9	19.3
	6.4	1.4	1.9
Thioglycollate	5.0	14.5	22.4
Endotoxin	5.0	13.5	20.4

Lysosomal preparations from 5 × 10^6 P388D1 cells or 2 × 10^6 TG- or endotoxin-elicited macrophages were placed in each well containing complete [^{3}H]proline-labeled R22 SMC matrix. Activator (1 mM dithiothreitol plus 1 mM EDTA) was added as indicated to selected wells.

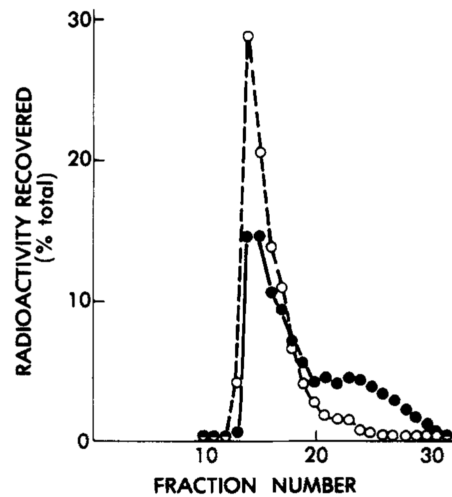

FIG. 3. Analysis of the size of peptides solubilized from R22 SMC matrix by macrophage enzymes. Conditioned medium from TG macrophages was incubated for 72 h on [^{3}H]proline-labeled R22 matrices. The solubilized peptides (○) were separated by Sephadex G-25 chromatography as described in Materials and Methods. An aliquot of medium containing solubilized peptides was incubated for an additional 42 h with macrophage lysosomal homogenate (0.5 mg) at pH 3.0, and then gel filtered (●).

somal preparations, degrade the connective tissue proteins secreted by smooth muscle cells, endothelial cells, and fibroblasts. The matrix substrates produced by these cells were particularly suited for our studies because their architecture and chemistry resemble those of connective tissues in vivo. We determined the role of each proteinase in solubilizing the insoluble extracellular matrix by using sequential digestion with trypsin, PE, and CPA to determine the disappearance of glycoproteins, elastin, and collagens.

At neutral pH, plasmin, a serine proteinase, and macrophage elastase, a metalloproteinase (9), both degraded the glycoproteins of SMC, fibroblastic, and endothelial matrices. Alone, plasmin solubilized 50–70% of the glycoproteins susceptible to trypsin, but in combination with macrophage elastase virtually all the glycoproteins were solubilized. Plasmin, generated by macrophage Plg activator, is a significant factor in matrix degradation by cultured macrophages (18, 23).

At acid pH the protein portions of the glycoproteins were degraded by carboxyl and thiol proteinases of macrophage lysosomes. Other lysosomal hydrolases such as peptidases and glycosidases probably participate in completion of digestion of the glycoproteins to amino acids and monosaccharides, as has been shown for glycosaminoglycan degradation by neutrophil enzymes (54).

Purified macrophage elastase degraded the SMC elastin readily, although to larger fragments than either HGE or PE, an observation in keeping with the more restricted specificity of the macrophage enzyme, which cleaves preferentially on the NH_2-terminal side of leucine, isoleucine, and phenylalanine residues of peptide bonds (M. J. Banda and Z. Werb. Unpublished observations). One other major difference among these elastases was the reduction in the rate of elastin degradation in the presence of matrix glycoproteins by macrophage elastase, macrophage-conditioned medium, and live macrophages, but not by PE or HGE, even though all three elastases degraded

the glycoproteins. Both the macrophage elastase and HGE degraded the glycoproteins of endothelial and fibroblastic matrices; in addition, HGE had limited collagenolytic potential on these matrices, as described previously for purified collagen (52). Previous studies have shown that proteinases from human neutrophil granules (55) and macrophages (49) degrade fibronectin.

Macrophage-conditioned medium had only limited collagenase activity, but purified mammalian collagenase was able to degrade some of the collagen of SMC, endothelial, and fibroblastic matrix. Because basement membrane collagens of types IV and AB are resistant to mammalian collagenases (56), it is possible that HGE and related enzymes and lysosomal thiol proteinases (52, 53) have important functions in the degradation of these collagens. Indeed, macrophage lysosomes contained thiol-dependent enzymes that degraded SMC matrix collagen at pH 5.0.

One major finding resulted from comparison of the matrix-degrading capacity of macrophage-conditioned medium and purified macrophage elastase with their activity on purified elastin. The data indicated that at least 90% of the potential elastinolytic activity present in conditioned medium, as assayed with elastin and SDS, was latent, probably because of a dissociable inhibitor that could be removed by exhaustive dialysis of the medium. The elastinolytic activity assayed on elastin without SDS more accurately reflected the matrix-degrading capability of the conditioned medium as compared to purified enzyme. Therefore, assays of macrophage elastase in the presence of SDS (8, 12) may reveal total potential catalytic capacity, but assays in the absence of SDS may demonstrate functional enzyme. The elastinolytic potential of live macrophages paralleled that of the culture supernates, suggesting that inhibitors may tightly control the catalytic activity of macrophage elastase. Thus, macrophage elastase may be regulated by inhibitor mechanisms previously recognized for collagenase (11, 21, 57, 58) and Plg activator (59).

We were able to alter secretion of Plg activator and elastase by eliciting macrophages by intraperitoneal injection of a variety of inflammatory agents. The differences in the secretion profiles of these cells were reflected by the degradation of the appropriate matrix components by medium conditioned by the macrophage. Resident macrophages secreted only small amounts of these enzymes and their medium degraded little matrix either in the absence or presence of Plg. Pyran copolymer-elicited and endotoxin-elicited cells secreted Plg activator but little elastase; consequently, their conditioned media degraded only the glycoproteins of SMC matrix. P388D1 macrophages and macrophages elicited with sodium metaperiodate or thioglycollate broth secreted both enzymes, and their media degraded both the glycoprotein and elastin components. The conditioned medium from P388D1 and TG macrophages also mediated detectable degradation of the SMC collagens. In contrast, the lysosomal preparations from P388D1, endotoxin-elicited, and TG macrophages degraded SMC matrix equally well. Thus, these inflammatory macrophages differ more in their secreted enzymes than in their lysosomal enzymes. The neutral proteinases degraded the matrix to peptides of >5,000 dalton, but in cooperation with lysosomal hydrolases, matrix was degraded to amino acids and oligopeptides.

The rate-limiting step in matrix degradation may be catalyzed by either neutral or lysosomal proteinases. If the former is the case, then the degradative capacity of macrophages in vivo should be reflected by their secreted enzymes. If phagocytosis and lysosomal digestion determine the rate of connective tissue degradation, then

phagocytic rate should reflect the degradative capacity of macrophages. In addition, inhibitors of secreted and cellular enzymes are likely to have important regulatory functions. In the accompanying papers (22, 23) we explore the connective tissue-degrading capacity of macrophages in culture and show that extracellular, pericellular, and intracellular events all contribute to macrophage-mediated turnover of connective tissue proteins.

Summary

We have investigated the ability of neutral and lysosomal enzymes of mouse macrophages to degrade the insoluble extracellular matrices secreted by smooth muscle cells, endothelial cells, and fibroblasts. Matrices produced by smooth muscle cells contained glycoproteins, elastin, and collagens, but matrices of endothelial cells and fibroblasts contained no elastin. Sequential enzyme digestion of residual matrix revealed that plasmin, a product of macrophage plasminogen activation, degraded 50–70% of the glycoprotein in the matrices but did not degrade the elastin or the collagens. Purified macrophage elastase degraded glycoprotein and elastin components but had no effect on the collagens. The rate of elastin degradation by macrophage elastase was decreased in the presence of the glycoproteins. In contrast, human granulocyte elastase effectively degraded the matrix glycoproteins, elastin, and, to a lesser extent, collagens. Mammalian collagenase degraded only collagens.

Conditioned medium from resident and inflammatory macrophages, containing mixtures of the secreted proteinases, degraded the glycoprotein and elastin components of the matrices. However, conditioned medium was less effective in degrading matrix than comparable amounts of purified macrophage elastase because >90% of the elastase in the medium was in a latent form. Inclusion of plasminogen in the assays accelerated degradation. In the presence of plasminogen, glycoproteins were degraded readily by medium from P388D1, pyran copolymer-, thioglycollate-, and periodate-elicited macrophages and, to a lesser extent, by medium from endotoxin-elicited and resident macrophages; medium from P388D1, thioglycollate-, and periodate-elicited macrophages was most effective in elastin degradation, and resident, endotoxin-elicited and pyran copolymer-elicited macrophages degraded almost no elastin.

The macrophage cathepsins D and B degraded all the matrix components at an optimum pH of 5.5 and acted with the secreted neutral proteinases to degrade the connective tissue macromolecules to amino acids and oligopeptides. These data indicate that macrophages at inflammatory sites contain and secrete proteolytic enzymes that could degrade the extracellular matrix.

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