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SYNTHESIS OF A COLLAGENASE INHIBITOR BY SMOOTH MUSCLE CELLS IN CULTURE

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SUMMARY

Serum-free medium that was incubated for 24 hours with confluent cultures of smooth muscle cells contains a potent inhibitor of mammalian collagenase but not of bacterial collagenase. Synthesis of this inhibitor by these cultures is blocked by cycloheximide. The inhibitor is sensitive to trypsin but is stable to heating at 60° for 10 minutes and to acid treatment. Reduction with mercaptoethanol followed by alkylation destroys the inhibitory activity. This inhibitor may play a physiological role in the slow turnover of collagen that is observed in the vasculature.

INTRODUCTION

It is well established that the degradation of collagen is catalyzed by the enzyme, collagenase (1) and that this enzyme is responsible for the turnover of collagen in tissues. Collagenase activity has been demonstrated in a variety of tissues (2-9) and regulation of its activity is essential for the normal remodeling of connective tissue.

Smooth muscle cells present in the medial layer of blood vessels have been shown to be active in collagen synthesis (10). Recent studies by Ross et al (11) have suggested that the smooth muscle cells in the vasculature may be responsible for the increased collagen content of the blood vessels in atherosclerosis. Although the synthesis of collagen by smooth muscle cells has been extensively studied, little is known about its degradation in this system. The present studies indicate that smooth muscle cells in culture synthesize a potent inhibitor of collagenase and support the view that

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this inhibitor may play a critical role in the control of collagen degradation in blood vessels.

MATERIALS AND METHODS

Human skin fibroblasts (CRL-1187, American Type Culture Collection) were grown in culture (75cm^2 dishes) in Minimal Essential Medium supplemented with 10 percent fetal calf serum, 2mM glutamine and 1 percent penicillin and streptomycin (GIBCO). At confluency, the medium was removed, the cell sheet was washed with phosphate buffered saline (GIBCO) and 10 ml of the above medium (minus serum) was added. After 3 days at 37°, the medium was harvested and concentrated by ultrafiltration. This material was dialyzed against 50mM cacodylate buffer, pH 7.0 containing 5mM $CaCl_2$.

Smooth muscle cells from porcine aorta were grown in culture by the techniques described by Ross et al (10). After growth of replicate dishes to dense cultures, the cell layer was washed three times with Hanks Balanced Salt solution. Serum-free medium was added and after 24 hours processed as above.

Collagen extracted from the skin of calf fetuses (12) was labeled with [^{14}C]-acetic anhydride (109mCi/mMole; Amersham) by the method of Gislow et al (12). The [^{14}C]-acetylated collagen was mixed with unlabeled collagen to a specific activity of approximately 1x10^5 cpm/mg.

Collagenase activity present in the medium of human fibroblasts was activated by limited proteolysis with 1.5μg/ml of trypsin (14). After 60 minutes at 37° tryptic activity was destroyed by the addition of excess trasylol (Sigma) and this material was used as the source of collagenase. Collagenase assays were performed by the method of Harper et al (15). A typical assay in 0.45 ml of 50mM cacodylate buffer, pH 7.4, contained 5mM $CaCl_2$, 0.5 mg of [^{14}C]-acetylated collagen (gel volume of 0.25 ml) and an aliquot of the enzyme preparation. After incubation at 37° for 16 hours, the material was centrifuged in a Beckman microfuge for 5 minutes at full speed. An aliquot of the supernatant, representing digestion products of the fibril, was assayed for radioactivity.

Concentration of proteins in the medium was determined by the method of Lowry et al (16) using bovine serum albumin as the standard.

RESULTS

The human skin fibroblast medium contained a latent form of collagenase that could be activated by preincubation with trypsin (Table I) or by APMA[2] (β-aminophenyl mercuric acetate), observations that are consistent with the known properties of human collagenase. Under the conditions of the assay, the medium from cultures of smooth muscle cells did not exhibit detectable collagenolytic activity. Preincubation of the medium of smooth muscle cells with trypsin, APMA or plasmin (not shown) did not have any effect.

[2] The following abbreviations are used: APMA, β-aminophenyl mercuric acetate.

Mixing experiments shown in Table I indicate that the addition of smooth muscle cell medium to the fibroblast collagenase resulted in a marked inhibition of enzymatic activity suggesting the presence of an inhibitor in the spent medium of smooth muscle cell cultures. Preincubation of the smooth muscle cell medium with trypsin resulted in partial inactivation of the inhibitor (Table I). Although higher concentrations of trypsin (up to 60μg/ml) did not completely inactivate the inhibitor, it is likely that the conditions used were not optimal for complete inactivation of the inhibitor. Preincubation of the smooth muscle cell medium with APMA caused only a slight inactivation of the inhibitor.

The inhibitory effect of the smooth muscle cell medium on fibroblast collagenolytic activity was dependent on the protein concentration of the medium (Figure 1). A 50 percent inhibition in fibroblast collagenolytic activity was observed at between 3 to 4μg of crude protein in the medium.

Properties of the inhibitor elaborated by smooth muscle cells.

When the smooth muscle cell medium was acidified to pH 2.0 and neutralized before the assay, the inhibitory effect on fibroblast collagenolytic activity was retained (Table II). α2 macroglobulin in serum is known to inhibit collagenase activity and as shown in Table II, the addition of 50μg of protein in fetal calf serum caused a marked inhibition of fibroblast collagenase activity. However, unlike the smooth muscle cell medium, the inhibition of collagenase activity by serum was lost if the latter was subjected to acid treatment. α macroglobulin is completely inactivated by acidification to pH 2.0 (17) and the acid stability of the collagenase inhibitor indicates that it is not α2 macroglobulin. Additional evidence that the inhibitor in the medium of smooth muscle cells is not α2 macroglobulin is obtained from data on the molecular size. α2 macroglobulin has a molecular weight of approximately 700,000 but the molecular weight of the collagenase inhibitor is approximately 30 to 60,000.

TABLE I

Inhibition of fibroblast collagenase by medium of smooth muscle cells

	System	Preincubation	cpm in supernatant
1.	Fibroblast medium (3.5µg protein)	None	3802
2.		+1.5µg/ml trypsin	26075
3.		+0.2mM APMA	26268
4.	Smooth muscle cell medium (11.3µg protein)	None	0
5.		+1.5µg/ml trypsin	0
6.		+15.0µg/ml trypsin	0
7.		+0.2mM APMA	0
	Mixing experiment		
	System 2 plus 4		2018
	System 2 plus 6		15000
	System 2 plus 7		4047

Preincubation conditions of the assay are described in the text.

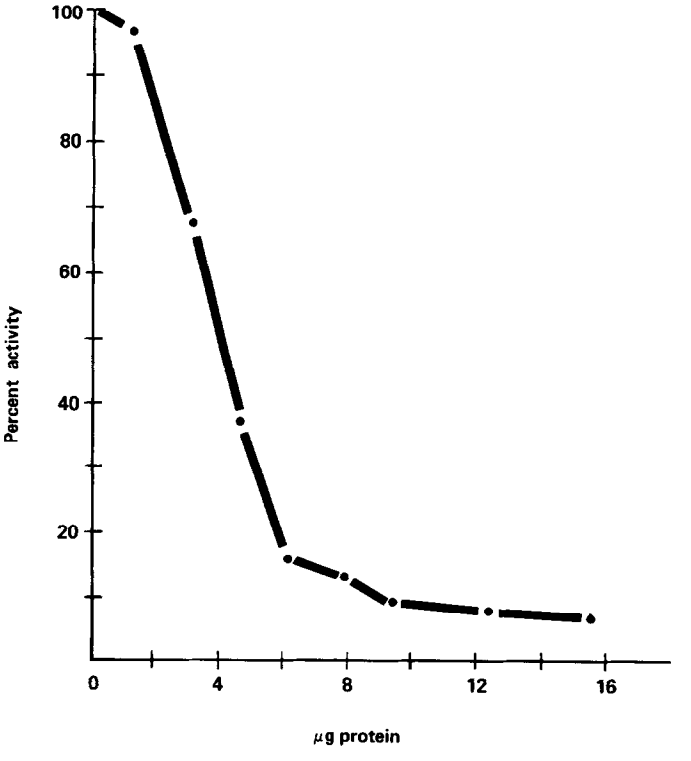

Figure 1

Effect of protein concentration in the smooth muscle cell medium on collagenase activity.

TABLE II

Properties of the collagenase inhibitor elaborated by smooth muscle cell cultures

Conditions	cpm in supernatant
Fibroblast collagenase (2.8µg protein)	18517
plus smooth muscle cell medium (3.1µg protein)	9380
plus acid treated smooth muscle cell medium	11054
plus heat treated smooth muscle cell medium	9870
plus reduced-alkylated smooth muscle cell medium	18371
plus fetal calf serum (50µg protein)	3294
plus acid treated fetal calf serum	17371

Details of the experiments are described in the text.

Furthermore, under conditions where α2 macroglobulin in fetal calf serum reacts with human α2 macroglobulin antibody, no interaction was obtained when the proteins in the spent medium of smooth muscle cells were analyzed. These results indicate that the collagenase inhibitor in the spent medium of smooth muscle cells is not α2 macroglobulin either synthesized *in vivo* or derived from the fetal calf serum used during the growth of the cultures. Table II also indicates that the inhibitor present in the smooth muscle cell medium was stable to heat treatment at 60° for 10 minutes.

When the smooth muscle cell culture medium was reduced with 2-mercaptoethanol (0.1M) and alkylated with excess iodoacetate (0.2M) the inhibitory effect on fibroblast collagenase was lost (Table II). These observations indicate that an intact sulfhydryl group is essential for inhibitory activity. The inhibitor elaborated by smooth muscle cell cultures did not have any effect on bacterial collagenase activity (data not shown) indicating that it may be specific to collagenases of mammalian origin.

To determine whether the inhibitor of fibroblast collagenase was syn-

TABLE III

Effect of cycloheximide and trypsin (pretreatment) on the synthesis of the collagenase inhibitor by smooth muscle cell cultures

Conditions	Units per dish#
Control	74
plus 2μM cycloheximide	11
trypsin treated control*	76

1 unit of collagenase inhibitor is the amount of dialyzed medium required for 50 percent inhibition of fibroblast collagenase (2.8μg protein). This presumptive unit is determined by titration with fibroblast collagenase.

* Cell sheet was pretreated with 0.05% trypsin in 0.02% EDTA for 1 min. The cell sheet was then rinsed with phosphate buffered saline and serum-free medium was added.

thesized, de novo, by smooth muscle cells, replicate dense cultures were exposed to serum-free medium containing 2μM cycloheximide. After 16 hours, the media from control and cycloheximide treated cells were harvested, concentrated by ultrafiltration and dialyzed against 50mM cacodylate buffer, pH 7.0, containing 5mM $CaCl_2$. The media were analyzed for their inhibitory activity. Table III indicates that in the presence of cycloheximide, the amount (units) of inhibitor in the medium was greatly reduced as compared to control media. In separate experiments, the cell sheet of dense smooth muscle cell cultures was pretreated briefly with 0.05 percent trypsin in 0.02 percent EDTA to remove serum proteins bound non-specifically to the cell surface. The trypsin was removed and the cell sheet was rinsed with phosphate buffered saline. The dishes were supplemented with serum-free medium. After 16 hours, the medium was harvested and assayed for the inhibitor (Table III). The amount of inhibitor in the medium of trypsin treated cultures was similar to that of control. All of the above obser-

vations support the view that the inhibitor is synthesized by smooth muscle cells and is not derived from traces of residual serum proteins that may have been present in the culture dishes.

DISCUSSION

Smooth muscle cells grown in culture retain the phenotypic expression of collagen synthesis that is observed *in vivo*, and this culture system has been used as a model for the study of biochemical parameters of normal and diseased blood vessels (18). Using this model system, the present studies have indicated that smooth muscle cell cultures are active in the synthesis of a potent inhibitor of fibroblast collagenase activity. The inhibitor has been partially characterized and its properties indicate that it is not α_2 macroglobulin, a known inhibitor of collagenase.

The turnover of collagen in blood vessels is most probably dependent on the ratio of the concentration of collagenase and the inhibitor. Although the present studies were primarily related to the collagenase inhibitor elaborated by smooth muscle cell cultures, it must not be construed that smooth muscle cells are inactive in the synthesis of collagenase. It is possible that the amount of collagenolytic activity in the medium of smooth muscle cells is small so that under conditions in which trypsin has partially destroyed the inhibitor, enzyme activity was masked. The collagenase inhibitor elaborated by smooth muscle cell cultures may account for the slow turnover of collagen that is observed in the vasculature.

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