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SYNTHESIS OF COLLAGEN, COLLAGENASE AND COLLAGENASE INHIBITORS BY CLONED HUMAN GINGIVAL FIBROBLASTS AND THE EFFECT OF CONCANAVALIN A

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Individual clones of human gingival fibroblasts that differ in morphology and growth characteristics have been found to synthesize collagen (types I, III and V), collagenase and collagenase inhibitors, and to be capable of degrading native collagen mats. Although collagenase activity was normally low, synthesis of the enzyme could be stimulated ten-fold by Concanavalin A. These results demonstrate that individual fibroblasts have the ability to both synthesize and degrade collagen.

INTRODUCTION

Collagen remodelling, which occurs at a particularly rapid rate in gingiva (1) involves the continual synthesis and degradation of collagen by the connective tissue cells. Although the synthesis of collagen is a known function of fibroblasts, whether or not individual cells have the ability to both synthesize and degrade collagen has not been established. Gingival fibroblasts *in vivo* (2) and *in vitro* (3,4) have been reported to degrade collagen via a phagocytic pathway. Gingival fibroblasts in culture have also been shown to synthesize collagenase, albeit in latent form (5,6,7). Although these same cultures also synthesize collagen, it is known that cultures of fibroblasts contain a heterogeneous population of cells (8,9,10) and it cannot be assumed that the same cell is synthesizing both collagen and collagenase. To answer this question, therefore, we have studied the ability of clonally-derived cell populations to synthesize and degrade collagen. Con A was used to amplify the synthesis of collagenase.

Abbreviations used are Con A, Concanavalin A; PCMB, p-hydroxymercuribenzoate; DMEM, Dulbecco's modified Eagles medium; FCS, fetal calf serum; β-APN, β-amino proprionitrile; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; FN, fibronectin.

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MATERIALS AND METHODS

Cell Culture. Human gingival fibroblasts prepared from non-diseased tissues were obtained at the third subculture from the American Type Culture Collection (Gin-1). The cells were grown in DMEM supplemented with 15% heat-inactivated FCS and antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamycin sulphate and 300 ng/ml fungizone) (growth medium). For cloning, the cells were plated in microwells (Linbro, 0.28 cm^2) in 200 μl of a 1:1 mixture of growth medium and conditioned medium. To prepare the conditioned medium, fresh growth medium was added to confluent cell cultures for 24 h, passed through a 0.22 μm Millipore filter and in some cases stored at -20°C before use. The cells were plated by limiting dilution at 0.5-2.0 cells/well. After two weeks about 2-5% of the cells had formed large circular colonies of about 2-3 mm diameter. Each colony was subcultured into four wells (Linbro, 2.0 cm^2). About 70% of these clones grew to confluence and were subcultured every 1-2 weeks for at least five subcultures. For enzyme and inhibitor assays, confluent cell cultures were washed well and serum-free growth medium was added for 48 h. The medium was then collected, centrifuged at 10,000 rev/min for 5 min and either assayed fresh or stored at -20°C. Cell morphology was viewed in a Leitz-Diavert inverted phase contrast microscope and photographed with a Nikon M-355 camera using Pan-X film. (Con A), when used, was added to cultures at a concentration of 25 μg/ml.

Collagen Synthesis. Cloned cells were cultured in 300 ul of serum-free medium deficient in glycine and proline but supplemented with 2 μCi each of [^{14}C] glycine and [^{14}C] proline and 50 ug/ml each of β-APN and ascorbate. After incubation for 24 h, the medium was harvested, FCS was added to a concentration of 0.5% v/v, and the mixture was exhaustively dialyzed against 1% acetic acid. A sample was taken to measure the non-dialyzable radioactivity and the remaining solution divided into two equal samples. One sample was digested with 50 μg pepsin at 15°C for 4 h to convert procollagens to α-chains. After freeze-drying, both pepsin digested and non-digested samples were analyzed by SDS-PAGE, and identified as described previously (11).

Collagenase Assay. Collagenase activity was carried out using a [^{14}C]-methylated collagen substrate (12). 20 μl of the collagen substrate (6,000 dpm) in 0.05 M-Tris/HCl, 5 mM $CaCl_2$, pH 7.4 was added to 100 μl of culture fluid in microtest wells. Incubation was carried out for 24 or 48 h at 22°C. Latent collagenase was assayed following trypsin activation (10 μl of a 0.1 mg/ml solution of trypsin for 10 min at 22°C), or following the addition of PCMB to 1mM. Trypsin activation was terminated by adding 10 μl of soybean trypsin inhibitor (1.0 mg/ml). Collagenase activity was determined using a combination of SDS-PAGE and fluorography to separate and quantitate collagen digestion products (12).

Assay of Collagenase Inhibitors. Inhibition of collagenase activity was measured using a stock collagenase solution prepared from the culture medium of porcine gingival tissue (7) and the assay system described above. substrate in 24 h). 20 μl of collagen substrate was added and the incubation carried out for 24 h at 22°C. The sensitivity of the inhibitor to heat was tested by incubating the medium at 100°C for 10 min prior to assay. Sensitivity to trypsin and PCMB was tested using the collagenase activating conditions described above. Digestions were analyzed by SDS-PAGE.

Preparation of Collagen Mats. A dispersion of native bovine tendon collagen (a gift from Ethicon Inc., Sommerville, N.J.) was used to prepare collagen mats (13). About 100 ug collagen per Linbro well (2.0 cm^2) was dried for 24 h at 22°C. The mats were then equilibrated with medium and either 10^4 or 10^5 cells/well were seeded onto the mats in growth medium for 24 h. The medium was replaced with serum-free growth medium for 1-3 days and the patterns of collagenolysis were examined by phase contrast microscopy after removing the cells with 0.5% Nonidet-P40 or 0.01% trypsin in citrate saline.

RESULTS

Morphology and Growth of Clones. Approximately 2-5% of cells plated produced clones of about 2 mm diameter after two weeks. Another 5-10% produced smaller, slowly or non-proliferating colonies which were not further utilized. Approximately 70% of the larger colonies could be subcultured at least five times and data from these clones are presented here. Some marked differences were found between the clones. Examples of clone morphologies are shown in Fig. 1. Most commonly, the clones consisted of small to medium-sized, densely packed and sometimes multilayering cells which were very elongated and spindle shaped, aligning in parallel orientations (e.g. Fig. 1a). Some clones comprised cells which were more polygonally-shaped, with more irregular orientation (not shown). A few clones consisted of very large cells of intermediate morphology which grew to lower cell densities (Fig. 1b). The latter cells grew at considerably reduced rates on repeated subculture and resembled typically senescent fibroblasts (14,15). Large variations in growth rates and saturation densities were generally observed amongst the clones. Saturation densities for example, varied over a ten-fold range (~1-10 x 10^4 cells/cm^2).

Degradation of Collagen Mats. All ten clones studied degraded substantial amounts of collagen during 1-3 days of culture on collagen mats and showed a similar pattern of lysis to that of mixed gingival fibroblasts (Fig. 1c, d). Active and latent collagenase levels were very low suggesting that the collagen was being degraded via a phagocytic pathway.

Collagen Synthesis. A total of 13 (7 in Table 1, plus 6 not shown) HGF clones were assayed for collagen, collagenase and collagenase inhibitor. Each clone was active in synthesizing and secreting protein into the medium (Table 1). Approximately 70-80% of the non-dialyzable proline radioactivity in the medium was incorporated into collagenous proteins which were identified on SDS-PAGE as comprising > 85% type I collagen. However, between clones differences were observed in the proportions of different collagen types synthesized. Thus, Type III collagen synthesized by different clones ranged from trace amounts to

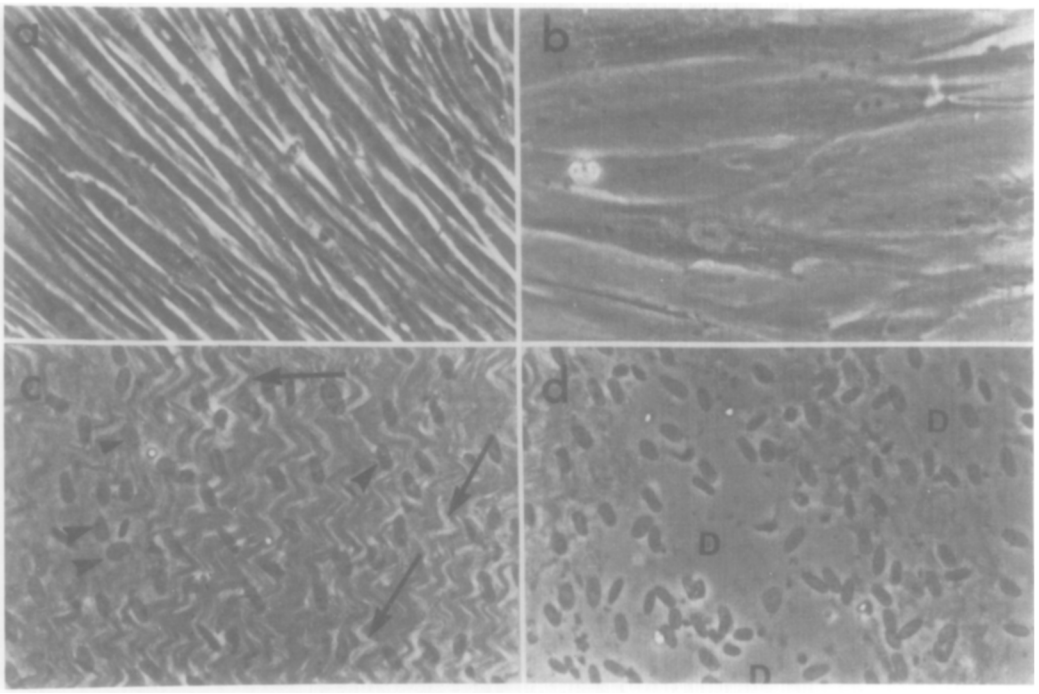

Fig. 1 Morphological phenotypes of HGF clones
Clones HG 22 and 25 (a,b) were grown to confluence in Linbro 2 cm^2 wells. These clones had been subcultured 2-3 times and were photographed when the biochemical assays were done. In photographs of collagen mats, only cell nuclei (arrowheads) and not cell bodies are visible due to NP40 treatment used to enhance visualization of lysis areas (c,d). c) Clone 21 grown on collagen mats showed small areas of collagen degradation after 24 h (nuclei, arrowheads; lysis, arrows). d) A similar clone (18), after 48 h, showed areas of extensive degradation (D).

15% of the total collagen; the average being 5.6% (Table 1). The α_1(V) chain was observed in all clones on longer exposure of fluorographs and represented between 1-3% of the total collagen. The procollagen profiles were closely similar between clones with little processing of procollagens evident. In the presence of Con A the total amount of non-dialysable radioactivity was changed only slightly relative to controls in cultures labelled in the first or second 24 h. However, as shown in Fig. 2, during the first 24 h increased processing of procollagen to pc-intermediates was evident. A small increase in radiolabelled collagen compared to controls was also observed. In the second 24 hr the radiolabelled collagen decreased substantially coinciding with the appearance of appreciable amounts of active collagenase. Con A also appeared to stimulate the synthesis of several non-collagenous proteins as indicated in the fluorographs.

Collagenase Activity. In each clone studied, a small amount of active collagenase was evident from the observation of 3/4 α-fragments (Fig. 3). However, in many cases there was insufficient activity to obtain accurate quantitation. The presence of latent collagenase was evident from the slight but definite increase in activity following trypsin activation (Table 1) or the addition of PCMB (not shown). Addition of Con A resulted in a marked increase in latent collagenase activity during the first 24 h of culture and also in active collagenase after fresh medium containing 25 μg/ml Con A was added for an additional 24 h (Table 2). This increase in collagenase was blocked in the presence of 25 μg/ml cycloheximide.

Collagenase Inhibitor Activity. All clones studied produced at least one and probably two inhibitors of collagenase activity. One inhibitor, the activity of which is shown in Table 1, was only slightly affected by trypsin (either 10 μg or 100 μg for 10 min at 22°C) or PCMB (not shown), but was destroyed by

TABLE 1 SYNTHESIS OF COLLAGEN, COLLAGENASE AND COLLAGENASE INHIBITOR BY CLONES

		^{14}C-PROTEIN		COLLAGENASE ACTIVITY		COLLAGENASE INHIBITION		
Expt.	Clone	CPM/10^3 Cells	% Type III Collagen	Active	Trypsin-Activatable (% α_1(I) Digestion)	Untreated	Trypsin-treated	Boiled
						(% original collagenase activity)		
3	HG 1	157	TR	0.7	4.1	7.4	35.8	82.0
	HG 2	108	TR	2.0	2.3	20.2	32.3	91.4
	HG 5	179	4.9	0.1	3.0	TR	12.8	74.8
	HG 6	132	5.2	1.2	3.8	TR	4.4	86.4
	HG 7	310	15.0	5.2	3.3	7.6	21.2	83.2
	HG 8	373	13.5	1.0	5.4	2.6	15.0	84.0
5	HG 8	138	9.4	TR	0.9	33.0	11.6	81.0
	HG 14	217	2.1	TR	4.2	12.4	64.8	90.2
1-6	Ave	105±49.0	5.6±4.6	1.3±1.7	3.4±1.4	4.6±4.9	11.4±7.8	38.2±6.4
	n	30	24		8		13	

Data are given for two experiments in which complete data were available, and the average values (± S.D.) for these and six other experiments. The clones in experiments 3 and 5 were at first and third subcultures, respectively. The procedures used to calculate the percent type III collagen, the collagenase activity and the collagenase inhibitory activity are described in Materials and Methods. The ^{14}C-protein represents the total non-dialysable protein in the medium in cultures labelled for 24 h with [^{14}C] glycine and [^{14}C] proline. TR-trace, bands were apparent but could not be accurately quantitated; n - total number of clones assayed.

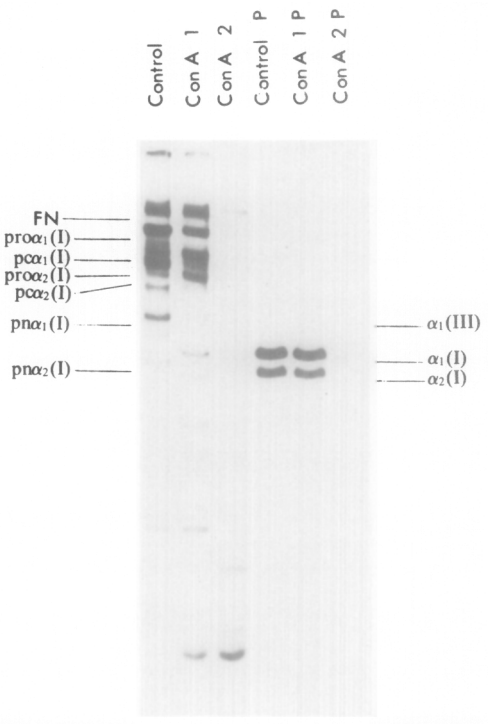

Fig. 2 Fluorographs of radiolabelled proteins synthesized by HGF clones. Cloned cells were incubated in serum-free media in the presence of [^{14}C] proline and [^{14}C] glycine for 24 h. After dialysis aliquots with (P) or without prior digestion with pepsin were analyzed on SDS-PAGE and radiolabelled proteins localized by fluorography. The effect of the presence of 25 μg/ml Con A in the culture media for 24 h (Con A1) or 48 h (Con A2) was also determined. The migratory positions of the procollagens and collagen chains were determined from standards (11) and their collagenous nature demonstrated by susceptibility to bacterial collagenase (not shown).

boiling. In the presence of 25 μg/ml cycloheximide, inhibitor activity was reduced to approximately 20% of the original activity. In the presence of Con A the inhibitory activity was reduced in some cultures but was unaffected in others. Evidence for a second inhibitor was derived from the trypsin or PCMB activation of collagenase which was particularly noticeable following Con A treatment (Fig. 3, Table 2). Although this could be interpreted as activation of a proenzyme, the activation conditions were identical to those used to destroy a trypsin-and organomercurial-sensitive inhibitor of collagenase in porcine gingival fibroblast cultures (16).

DISCUSSION

This study has demonstrated that clones of human gingival fibroblasts, showing a wide range of interclonal variation in size, morphology

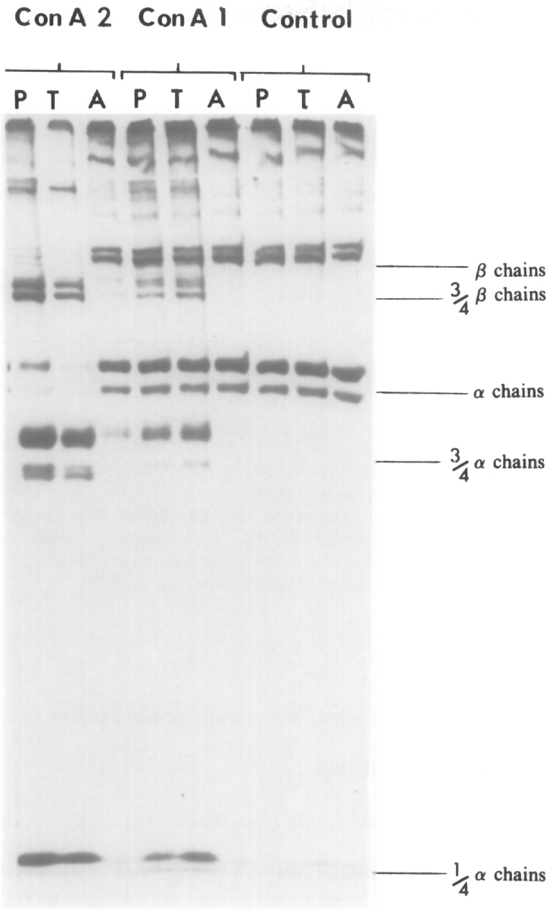

Fig. 3 Fluorographs demonstrating collagenase activity in culture media of HGF clones.
100 µl culture medium from 24 h cultures was incubated with 20 ul of radiolabelled collagen substrate for 24 h at 22°C, pH 7.4 (A). Activation of medium with trypsin (T) and PCMB (P) was also carried out to detect latent collagenase. Collagenase activity was analyzed in the media of cells grown in the absence (control) and presence of 25 µg/ml Con A for 24 h (Con A1) and 48 h (Con A2). Radiolabelled digestion products were separated on SDS-PAGE and vizualized by fluorography.

and growth rates, are similar in their ability to synthesize collagen, collagenase and collagenase inhibitors. It is conceivable, therefore, that individual fibroblasts, or their close progeny, can function in both the synthesis and degradation of collagen during the remodelling of the connective tissue matrix. The mechanism of collagen degradation, however, remains to be elucidated. Although collagenase production by fibroblasts can be demonstrated, it is not known whether the enzyme functions in the

TABLE 2
EFFECT OF CONCANAVALIN A ON COLLAGENASE AND COLLAGENASE INHIBITOR PRODUCTION

Expt.	Condition	COLLAGENASE ACTIVITY			COLLAGENASE INHIBITION			
		Active	Trypsin-Activatable	n	Untreated	Trypsin-treated	Boiled	n
		($\%\ \alpha_1$ (I) digestion)			(% original collagenase activity)			
1-5	Control	1.3	3.4	8	9.2	22.8	76.4	13
	+ Con A	3.2	34.8	7	30.0	55.2	70.2	8
6	Control Day 1	6.6	10.4	2	37.4	61.8	68.6	2
	+ Con A Day 1	3.6	46.3	2	50.2	77.4	72.0	2
	+ Con A Day 2	26.9	73.3	2	62.2	89.2	82.4	2

Collagenase and collagenase inhibitor activity was measured in the media of cells cutlured in the presence of 25 µg/ml Con A for 24 h. In experiment 6 the medium was collected after 24 h (Day 1), then cultured with Con A for an additional 24 h (Day 2). Results are expressed as an average of the total clones (n) analyzed in each experiment.

extracellular degradation of collagen or in a preliminary step necessary for the phagocytosis of collagen fibrils.

The nature of collagens synthesized by the cloned fibroblasts compares on average with mixed cultures of gingival cells and also with *in vivo* results. For example, type III collagen comprises 5-30% of the total collagen in normal human gingival fibroblasts cultured *in vitro* (17). Similarly, *in vivo* studies on rat gingiva (18) have shown that type III collagen comprises 8-9% of the total collagen and that small amounts of type V collagen are also produced. The appearance of type V collagen in the clones demonstrates that fibroblasts produce this collagen in addition to type I and III. Differences in the proportion of type I and III collagens between the clones demonstrate variations in the detailed phenotypic expression of these cells. Similarly, differences have been observed in cloned fibroblasts from periodontal ligament (19) and in cloned bone cells from rat calvaria (20). Whether or not these differences correlate with other biochemical properties or specific morphological details has not yet been elucidated.

The collagenase synthesized by the cloned cells appears to be identical with the collagenases described for gingival fibroblasts (5,6,16)

and for gingival explants (7,21) in both its activity on collagen and its activation by trypsin and PCMB. The trypsin resistant inhibitor is probably the same as that characterized by Simpson & Mailman (22) and is similar to the inhibitor produced by periodontal ligament fibroblasts (16) with respect t its resistance to trypsin and PCMB. The evidence of a second inhibitor is based upon our ability to activate latent enzyme which is particularly evident with trypsin and PCMB in Con A-stimulated cultures. The activation conditions are consistent with the destruction of an inhibitor molecule from an enzyme inhibitor complex (23,24) rather than activation of procollagenase.

Under the influence of Con A the level of inhibitor activity, in contrast to the collagenase, was either unchanged or reduced, demonstrating that collagenase and inhibitor synthesis are not linked. The mechanism of action of Con A is not known, but appears to be mediated through the binding of Con A to the cell surface since the effect of Con A can be inhibited by α-methyl mannoside (Wang, Hurum & Sodek, in preparation). The action of Con A is not simply a cytotoxic effect as protein synthesis generally is not impaired and in some cases is increased. The reduced amount of labelled collagen in the medium may be related to the increased collagenolytic activity. However, a selective inhibition of collagen synthesis or secretion cannot be ruled out.

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