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Fibroblast Heterogeneity in Collagenolytic Response to Cyclosporine

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Abstract To investigate the mechanism of cyclosporine (CS)-induced fibrotic gingival enlargement, the effect of CS on the collagenolytic activities of 14 different human gingival fibroblast strains derived from healthy individuals with non-inflammed gingiva was examined in vitro. There was marked heterogeneity among individuals in basal levels of collagenase activity, and there was also variation among the subpopulations derived from one strain. Fibroblasts from different individuals also varied markedly in their collagenolytic response to CS (0.1 to 0.75 μg/ml). In most strains, CS decreased collagenase activity, but in some, the drug caused no change or significantly increased activities. In most of the subpopulations CS significantly decreased collagenolytic activity.

Two of the fibroblast strains and the subpopulations described above were examined for the production of immunoreactive collagenase and tissue inhibitor of metalloproteinase (TIMP). The two strains made similar amounts of collagenase, but differed markedly in TIMP levels; CS affected their collagenase production differently but had similar effects on TIMP. Among the subpopulations there was variation in the production of collagenase, although none made detectable levels of TIMP; they also varied in the production of both proteins in response to CS. In two of the subpopulations and in both strains at some concentrations, the effect of CS on the relative levels of collagenase and TIMP could account for the decreased collagenase activity; i.e., the level of collagenase was unchanged or decreased, and TIMP production was unchanged or increased.

This study demonstrates the variation among individuals as well as intrastrain heterogeneity of human gingival fibroblasts with regard to collagenase activity and the production of collagenase and TIMP. The heterogeneity of the collagenolytic response of different gingival fibroblast strains and their subpopulations to CS treatment may partly explain the susceptibility of only some individuals to CS-induced gingival enlargement.

Key words: drug-induced gingival fibrosis, extracellular matrix, collagen, collagenase, tissue inhibitor of metalloproteinases, cell subpopulations

Collagen, the major structural protein in connective tissue, is synthesized by fibroblasts, and its production and degradation in physiological and pathological states requires precise regulation. In fibrotic conditions such as scleroderma, keloids, and gingival fibrosis, for example, the finely regulated balance between collagen synthesis and degradation may be disturbed, resulting in the accumulation of excess interstitial collagen. Gingival fibrosis can be a hereditary condition [1–3] or it may occur as a side effect of several drugs including phenytoin, sodium valproate, primidone, nifedipine, and cyclosporine (CS) [4–10]. Human gingival fibroblasts, like fibroblasts from other tissues, synthesize collagen, various matrix metalloproteinases (MMPs), including collagenase (MMP-1), as well as tissue inhibitor of metalloproteinase (TIMP) [11–16]. Increased collagen synthesis, reduced collagenase production, or increased TIMP production by fibroblasts (in response to drugs such as CS and phenytoin), leading to reduced collagenolytic activity, may all contribute to the dense collagen deposition characteristic of fibrous gingival enlargements.

Evidence from several laboratories suggests that CS directly affects gingival fibroblast functions in vitro, including proliferation, collagenolysis, and the synthesis of extracellular matrix products [17,18]. Indeed, several in vitro studies have shown that the effect of CS on non-

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lymphoid cells may be direct and unrelated to an interaction with lymphocytes [19 and references therein]. The observation that not all patients on CS therapy develop gingival enlargement further suggests that there is interindividual heterogeneity in direct fibroblast response to CS. Sander et al. [20] demonstrated interindividual differences in the sensitivity of lymphocytes to CS, and Hassell et al. [21] showed that fibroblast strains from different individuals varied in their synthesis of collagen and total protein in response to CS.

Interindividual variation in cellular response to agents such as CS may be related to the presence of agent-specific responsive subpopulations within the target cell population. The functional heterogeneity of fibroblast subpopulations from a variety of tissues including gingiva is well-established [22–24], and fibroblasts also exhibit heterogeneity in response to various stimuli, including mononuclear cell-derived mediators [25] and tumor-derived products [26]. In susceptible individuals, therefore, environmental stimuli, including drugs such as CS, may select a functionally distinct fibroblast subpopulation or clone with increased proliferative rates and abnormally high production of extracellular matrix molecules, or activate susceptible clones to this phenotype. This has been proposed to occur in a number of fibrotic conditions, including phenytoin-induced gingival enlargement [27–31]. The inherent interindividual heterogeneity of fibroblast strains and their heterogeneous responses to various stimuli, therefore, may contribute to the selective development of gingival enlargement in some patients treated with CS.

Excessive collagen synthesis and deposition is characteristic of fibroses [32,33], but decreased collagenolysis may also be involved, due to increased levels of TIMP, decreased production of collagenase, or synthesis of a defective collagenase [34–37]. The present in vitro study focused on the role of collagenolysis in the pathogenesis of CS-induced gingival fibrosis. Although it has been shown in our laboratory [38,39] and others [21] that CS apparently can indirectly contribute to the development of this condition via its effects on immune cells, the collagenolytic response of fibroblasts and fibroblast subpopulations from different individuals as target cells for the direct effects of CS has not been examined. Therefore, in order to investigate the concept of fibroblast subpopulation heterogeneity and interindividual differences in susceptibility to CS in the pathogenesis of gingival enlargement, we examined 14 fibroblast strains from different individuals and subpopulations from one of the strains for the direct effects of CS on their collagenolytic behavior.

MATERIALS AND METHODS

Fibroblast Strains

Fourteen human gingival fibroblast strains were derived from healthy individuals with clinically non-inflamed gingiva, each strain being derived from a gingival biopsy from a different donor. The tissue was minced using sterile techniques, and washed several times in Hanks' balanced salt solution (HBSS; Gibco, Grand Island, NY) supplemented with gentamicin (Gibco) at 100 μg/ml and 2.5 μg/ml Fungizone (Gibco). Minced tissue pieces were placed into 25 cm^2 flasks (Falcon; Oxnard, CA), allowed to adhere, and maintained in 3 ml of Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% newborn calf serum (NCS; Gibco) and gentamicin (100 μg/ml) (referred to as complete medium). The flasks were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells eventually were passaged by trypsinization with 0.25% trypsin (Enzart, Armour Pharmaceutical Co., Kankakee, IL) in EDTA-containing phosphate-buffered saline.

Derivation of Fibroblast Subpopulations

Fibroblast subpopulations were obtained by differential trypsinization [23]. Briefly, the fibroblast monolayer was washed twice with HBSS (Ca^{2+}-, Mg^{2+}-free) and then exposed to 0.05% trypsin in HBSS containing 0.3 mM EDTA for 3 min. Cells detached from the flask in this manner were aspirated and transferred to three other flasks. The cells remaining attached to the original flasks were incubated in complete medium for 24 h, at which time the trypsinization procedure was repeated and the cells were transferred to three new flasks. Human gingival fibroblast subpopulations obtained in this way have been shown to be functionally heterogenous [23].

Assay of Collagenase Activity

Collagenase activity in serum-free culture supernatants of the fibroblasts in the absence or presence of CS (Sandoz, Ltd.; Basel, Switzerland) was measured using reconstituted [^{14}C]-proline and [^{3}H]-glycine labeled collagen fibril gels as substrate [40]. Fibroblasts (between pas-

sage 2 and passage 7) were seeded in wells of 24-well tissue culture plates (Linbro; McClean, VA) at 1×10^5 cells/well. The cells were cultured for 24 h in complete medium at 37°C in 5% CO_2 in air. At that time, medium was removed and replaced with serum-free AMEM-F10 medium (Gibco) contains 100 μg/ml gentamicin and CS (dissolved in ethanol containing 20% Tween 20 (Sigma Chemical Co.; St. Louis, MO) and diluted in DMEM [41]) or CS solvent at 0.1, 0.25, 0.5, 0.75, and 1 μg/ml. Medium was collected at 2-day intervals for 8 days or at day 6 after addition of CS and stored frozen at −20°C. At the time of the assay, the supernatants were thawed and the collagenase in the medium was activated by incubation with 25 μg trypsin (Sigma) for 10 min at 37°C. The trypsin was inactivated by adding a 5-fold excess of soybean trypsin inhibitor (SBTI; Sigma). The supernatants were incubated on the collagen gels for 18–24 h at 34°C in 0.05 M Tris-Cl buffer with 0.01 M $CaCl_2$ and 0.2 M NaCl, pH 7.5, and counted in a liquid scintillation spectrometer. Results are expressed as total units of collagenase per 10^6 cells or units of collagenase per mg protein. The method of Lowry et al. [42] was used for protein determination. One unit of collagenase activity degrades 1 μg collagen per minute at 34°C.

Assay of Immunoreactive Collagenase and TIMP

Immunoreactive collagenase and TIMP were measured in conditioned media using established enzyme-linked immunosorbent assays (ELISA). The ELISA for collagenase is that of Cooper et al. [43], modified by using 0.05 M Tris-HCl, pH 8.3, containing 0.005 M $CaCl_2$ as the coating buffer, and the same buffer at pH 7.5 as the wash buffer. The TIMP ELISA was performed as described [44,45]. These assays are insensitive to the presence of enzyme-inhibitor complexes [45].

Statistical Analysis

The data are expressed as mean ± standard deviation and were analyzed using a one-way analysis of variance (ANOVA).

RESULTS

Collagenase Activity

a. Basal activity. The basal level of collagenase activity varied markedly among the fourteen fibroblast mass strains and also among the different GN 32 subpopulations (Table I). The activities of the mass strains varied from 0.3 ± 0.02 to 8.8 ± 1.3 units/mg. The activity of each of eight strains (GN 23, 32, 41, 43, 48, 51, 53, and 55) was significantly different from ten or more of the others and the activities of the remaining six were significantly different from five to nine of the others, demonstrating significant variation among individuals.

The collagenase activity of the GN 32 parental strain (P) was significantly different from that of each of the subpopulations derived from it except E. The subpopulations themselves were

TABLE I. Heterogeneity in Collagenase Activity of Human Gingival Fibroblast Strains and Subpopulations†

Cell strain	Collagenase activity (U/mg protein) ± SD
GN 16**	2.7 ± 0
GN 23*	4.3 ± 0.3
GN 32*	8.8 ± 1.3
GN 41*	0.3 ± 0.02
GN 42**	2.8 ± 0.13
GN 43*	5.3 ± 0.3
GN 45**	2.6 ± 0.05
GN 48*	3.1 ± 0
GN 49**	3.5 ± 0.53
GN 51*	2.4 ± 0.15
GN 52**	2.7 ± 0.55
GN 53*	1.1 ± 0.29
GN 54**	2.2 ± 1.14
GN 55*	4.1 ± 0
Subpopulations	**Collagenase activity ($U/10^6$ cells) ± SD**
GN 32 P***	1.4 ± 0.16
GN 32 A****	2.1 ± 0.16
GN 32 B*****	2.7 ± 0.12
GN 32 D*****	4.0 ± 0.14
GN 32 E*****	1.6 ± 0.14
GN 32 F****	2.2 ± 0.17

†The collagenase activity in serum-free culture supernatants of the fibroblasts was measured using reconstituted [^{14}C]-proline and [^{3}H]-glycine labeled collagen fibril gels as substrate as described in Materials and Methods. The data represent the means and standard deviations of triplicate cultures of a representative experiment and were analyzed statistically using a one-way analysis of variance (ANOVA).
*Collagenase activity was significantly different from that of ten or more of the other strains.
**Collagenase activity was significantly different from that of five to nine of the other strains.
***Collagenase activity was significantly different from all subpopulations except E.
****Collagenase activity was significantly different from three of the other subpopulations.
*****Collagenase activity was significantly different from all four of the other subpopulations.

TABLE II. The Effect of CS on Collagenase Activity of Human Gingival Fibroblasts in Group 1†

Cell line	Collagenase activity[a] [CS conc. (μg/ml)]				
	0	0.1	0.25	0.5	0.75
GN 16	2.7 (0)	0.95 (0.02)***	0.37 (0)**	0.28 (0.07)**	0.27 (0.03)**
GN 32	8.8 (1.3)	1.0 (0.2)**	3.9 (0.2)**	3.7 (0.06)**	6.0 (0.38)****
GN 43	5.3 (0.3)	3.0 (0.58)****	2.0 (0.01)***	0.8 (0)**	0.1 (0)**
GN 51	2.4 (0.15)	N.D.[b],*	1.7 (0.1)	1.3 (0.41)****	1.4 (0)****
GN 23	4.3 (0.3)	3.0 (0.6)	2.9 (0.14)***	1.6 (0.43)***	4.2 (0)
GN 41	0.3 (0.02)	0.156 (0)****	0.2 (0.07)**	N.D.[b],*	0.23 (0.06)
GN 42	2.8 (0.13)	1.6 (0.01)****	1.8 (0)****	0.7 (0.1)***	2.6 (0.07)
GN 55	4.1 (0)	3.6 (0.57)	0.9 (0)****	2.3 (0.28)****	7.3 (1.6)

†Collagenase activity in serum-free culture supernatants of the fibroblasts in the absence or presence of the indicated concentrations of CS was measured using reconstituted [^{14}C]-proline and [^{3}H]-glycine labeled collagen fibril gels as substrate as described in Materials and Methods. The data are expressed as the means and standard deviations of triplicate cultures of a representative experiment and were analyzed statistically using a one-way analysis of variance (ANOVA).
[a]Collagenase activity in units/mg protein (±SD).
[b]None detected.
*$P < 0.001$.
**$P < 0.005$.
***$P < 0.01$.
****$P < 0.05$.

heterogenous, with the enzyme activity of each of two subpopulations (A and F) being significantly different from that of three of the other four subpopulations, while the activity of each of the remaining three (B, D, E) was significantly different from every other subpopulation (Table I).

b. Effect of CS. The mass strains were also heterogenous in their collagenolytic response to CS. Three patterns of response to CS as a function of its concentration were observed (Tables II, III). Group 1 was defined as those strains in which significantly decreased collagenase activity was evident at lower CS concentrations (0.1 and/or 0.25 μg/ml). Over one-half of the fibroblast strains were included in this category, viz., GN 16, 23, 32, 41, 42, 43, 51, and 55 (Table II). The collagenase activities of all eight of the

TABLE III. The Effect of CS on Collagenase Activity of Human Gingival Fibroblasts in Groups 2 and 3†

Cell strain	Collagenase activity[a] [CS conc. (μg/ml)]				
	0	0.1	0.25	0.5	0.75
Group 2					
GN 45	2.6 (0.05)	3.1 (1.1)	2.7 (0.14)	2.8 (1.1)	1.6 (0)***
GN 48	3.1 (0)	2.7 (0.09)	3.9 (0.16)	1.6 (0.27)***	1.7 (0.2)**
GN 49	3.5 (0.53)	3.7 (1.1)	4.4 (0.5)	0.8 (0)**	N.D.[b],*
Group 3					
GN 52	2.7 (0.55)	2.6 (0.26)	2.8 (0.09)	3.2 (0.56)	3.7 (0.29)***
GN 53	1.1 (0.29)	1.5 (0.15)	2.9 (0.28)***	2.6 (0.1)***	2.1 (0.17)***
GN 54	2.2 (1.1)	2.5 (1.1)	2.8 (0.67)	3.5 (0.34)	2.1 (0.86)

†Collagenase activity in serum-free culture supernatants of the fibroblasts in the absence or presence of the indicated concentrations of CS was measured using reconstituted [^{14}C]-proline and [^{3}H]-glycine labeled collagen fibril gels as substrate as described in Materials and Methods. The data are expressed as the means and standard deviations of triplicate cultures of a representative experiment and were analyzed statistically using a one-way analysis of variance (ANOVA).
[a]Collagenase activity in units/mg protein (±SD).
[b]None detected.
*$P < 0.005$.
**$P < 0.01$.
***$P < 0.05$.

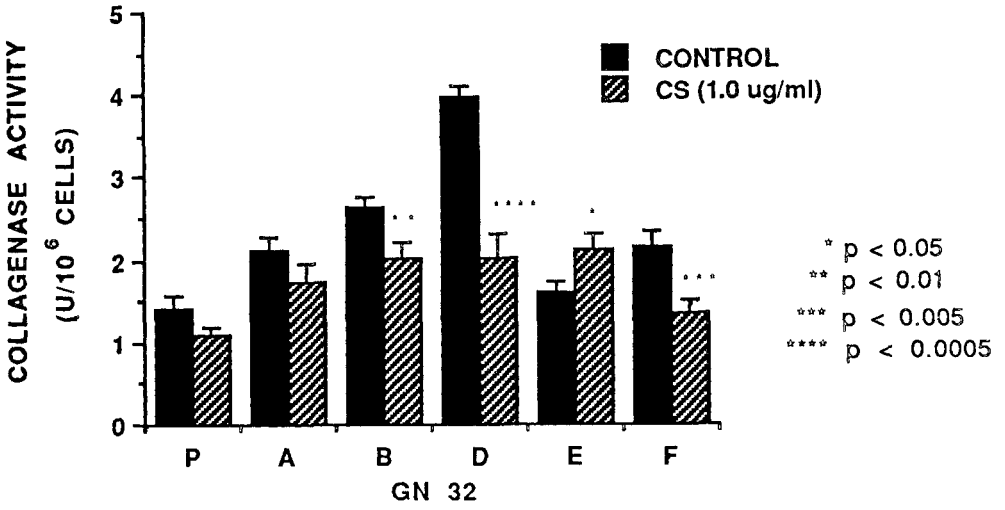

Fig. 1. Effect of CS on the collagenase activities of a human gingival fibroblast strain and subpopulations derived from it. The fibroblast mass strain (P) and its subpopulations (A–F) were cultured in the absence or presence of CS (1 μg/ml) in serum-free AMEM-F10 medium. Culture supernatants were collected at 2 day intervals for 8 days, and assayed for collagenase activity using radiolabeled type I collagen gels as substrate. The data represent the total units of collagenase/10^6 cells and are expressed as the means and standard deviations of triplicate cultures of a representative experiment. Statistical analysis was by one-way analysis of variance (ANOVA).

strains in Group 1 were also significantly inhibited by CS at 0.5 μg/ml, and four of them (GN 16, 32, 43, or 51) were significantly inhibited by CS at 0.75 μg/ml. In the remaining four strains, however, at 0.75 μg/ml CS there was no significant change relative to control levels (GN 23, 41, 42, 55). In Group 2 (GN 45, 48, and 49), CS significantly decreased the collagenase activity only at the highest concentrations tested (0.5 and/or 0.75 μg/ml) (Table III) and in group 3 (GN 52, 53, and 54), CS had no effect or significantly increased collagenase activity. (Table III).

In the GN 32 family, CS caused no significant change in the collagenase activity of the parental strain P and subpopulation A (Fig. 1). However, the enzyme activities of three of the subpopulations (B, D, F) were significantly decreased by CS treatment, whereas CS significantly increased the collagenase activity of subpopulation E.

Synthesis of Immunoreactive Collagenase and TIMP

a. Basal production. Two representative strains (GN 16 and 23) in which CS decreased collagenase activity and the GN 32 strain and its subpopulations were assayed by ELISA for immunoreactive collagenase and TIMP production. The basal levels of collagenase produced by GN 16 and GN 23 were not different from one another, but the two strains were heterogenous in their production of TIMP, with GN 16 producing approximately three times as much TIMP as GN 23 (Table IV). This is consistent with the data that GN 16 has a lower basal collagenase activity than GN 23 (Table I).

Table IV also shows that among GN 32 and its subpopulations, collagenase production varied from non-detectable levels to 156 ng/ml. [The amount of collagenase made by GN 32 E was 45

TABLE IV. Heterogeneity in Collagenase and TIMP Synthesis by Human Gingival Fibroblast Cell Lines and Subpopulations†

Cell stain	Collagenase (ng/ml) ± S.D.	TIMP (ng/ml) ± S.D.
GN 16	4073 ± 499	2910 ± 132
GN 23	4004 ± 17	955 ± 96
Subpopulation	**Collagenase (ng/ml) ± S.D.**	**TIMP (ng/ml) ± S.D.**
GN 32 P	90 ± 8	73 ± 28
GN 32 A	126 ± 37	N.D.[a]
GN 32 B	77 ± 8	N.D.[a]
GN 32 D	156 ± 9	N.D.[a]
GN 32 E	N.D.[a]	N.D.[a]
GN 32 F	62 ± 8	N.D.[a]

†Immunoreactive collagenase and TIMP were measured in fibroblast conditioned media by enzyme-linked immunosorbent assay (ELISA) as described in Materials and Methods.
[a]None detected.

ng/ml, just below the reliable lower limit of the assay (50 ng/ml), and therefore was indicated as not detectable. However, this amount apparently was sufficient to result in measurable collagenase activity (Fig. 1)]. Three of the five subpopulations were significantly different from the parental strain in collagenase production: D ($P < 0.001$), E ($P < 0.005$), and F ($P < 0.005$). The subpopulations themselves were heterogenous: two (D, E) were each significantly different from three of the other four subpopulations, and three (A, B, and F) were each significantly different from two of the other four subpopulations in their production of immunohdreactive collagenase.

TIMP production among GN 32 and its subpopulations varied from non-detectable levels (all subpopulations) to 73 ng/ml (P), and all subpopulations were significantly different from the parental strain ($P < 0.05$). In contrast to collagenase production, there was no detectable heterogeneity in TIMP production among the subpopulations (Table IV).

b. Effect of CS. Collagenase production by GN 16 was significantly decreased by CS at all concentrations tested (Fig. 2a). In contrast, CS increased GN 23 collagenase production by 11% at 0.25 μg/ml, and decreased it by 9%, 27%, and 31% at 0.1, 0.5, and 0.75 μg/ml, respectively. It appears, therefore, that there is a general trend for CS to decrease collagenase production by GN 23, but when analyzed using ANOVA, these changes were found not to be statistically significant (Fig. 2b). TIMP production by the two strains, on the other hand, was affected similarly by all CS concentrations tested except 0.5 μg/ml. In both strains, CS at 0.1 μg/ml had no effect on TIMP production, 0.25 μg/ml significantly increased it, and at 0.75 μg/ml CS significantly reduced the level of TIMP (Fig. 2a,b). (At 0.5 μg/ml CS had no effect on TIMP production by GN 16 and significantly increased it in GN 23.)

In the GN 32 strain and its subpopulations, CS had no significant effect on collagenase production by the mass strain and subpopulations A and D (Fig. 3a). In the remaining three subpopulations, CS caused significant increases in collagenase production. TIMP production by the mass strain was significantly decreased by CS to a non-detectable level, and the drug increased TIMP production by subpopulations D, E, and F from non-detectable levels to up to 205 ng/ml (E). CS had no effect on TIMP production by subpopulations A and B (Fig. 3b).

Correlation of Collagenase Activity With Synthesis of Collagenase and TIMP

Because collagen breakdown at a given site in vivo depends in part on the balance between local concentrations of collagenase and TIMP, the effects of CS on collagenase activity were compared with its effects on collagenase and TIMP production. CS significantly decreased the collagenase activity of GN 16 at all concentrations tested (Table II). However, only at 0.1, 0.25, and 0.5 μg/ml could the effect of CS on collagenase and TIMP production apparently account for the decrease in activity: at these concentrations collagenase production was decreased and TIMP production was either unchanged or enhanced (Fig. 4). In the GN 23 strain, CS at 0.25 and 0.5 μg/ml significantly decreased collagenase activity, caused an increase (11%) or decrease (27%) in collagenase production, while markedly increasing TIMP production by 62% and 145%, respectively (Fig. 5). At 0.1 μg/ml, CS had a slight (9%) but statistically insignificant decrease in collagenase production, no effect on TIMP production, and no significant effect on collagenase activity. At 0.75 μg/ml, collagenase activity was not different from the control level; while the amount of collagenase was reduced by 31%, TIMP was reduced by 97%. This dramatic decrease in the amount of TIMP could account for the return of collagenase activity to the control level (Fig. 2b; Table II). CS also significantly inhibited the collagenase activities of four of the GN 32 subpopulations (B, D, E, and F) but only in two (D and F) could its effects on collagenase and TIMP production account for the decrease in activity (Fig. 6).

DISCUSSION

The excessive collagen deposition associated with connective tissue disorders [27,46], including hereditary and drug-induced gingival enlargements [1–4,6,8,9], may be explained by several mechanisms. There may be accelerated production of collagen by fibroblasts, [32,33] or alternatively, reduced intracellular or extracellular collagen degradation [36,37,47,48]. Preferential expansion of a metabolically active fibroblast population could also result in collagen accumulation. One or more of these mechanisms may be involved in the development of

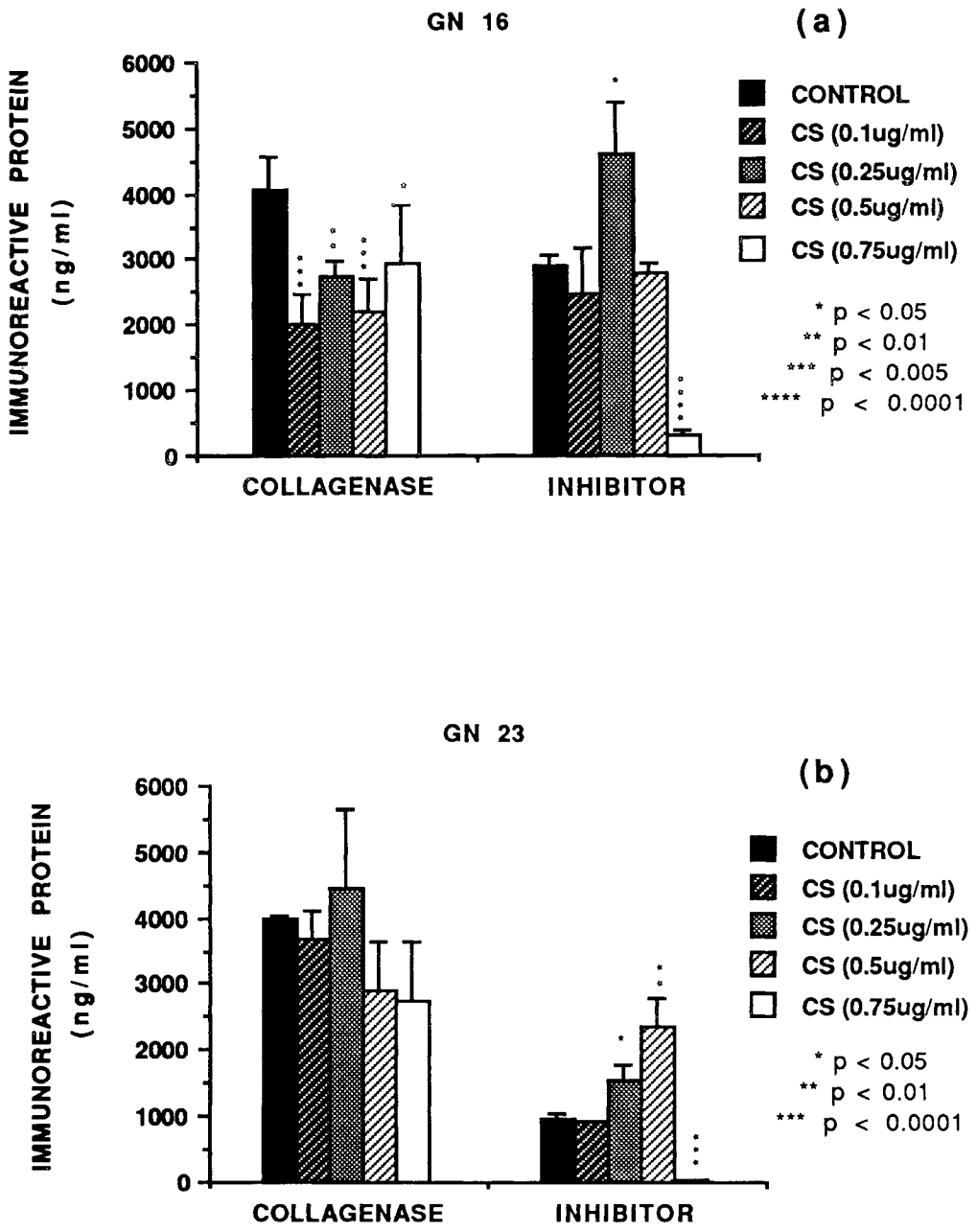

Fig. 2. Effect of CS on the production of immunoreactive collagenase and TIMP by human gingival fibroblasts. Two normal gingival fibroblast strains [GN 16 (a), GN 23 (b)] were cultured for 6 days in the absence or presence of CS (0.1 to 0.75 μg/ml) in serum-free AMEM-F10 medium. Immunoreactive collagenase and TIMP were assayed by ELISA. Results are expressed as ng protein/ml culture supernatant. The data represent the means and standard deviations of triplicate cultures of a representative experiment. Statistical analysis was by one-way analysis of variance (ANOVA).

fibrotic gingival enlargement. The present investigation focused on the direct effects of cyclosporine (CS) on the collagenase activity of several fibroblast strains and subpopulations.

The data have demonstrated variations in the direct response to CS of gingival fibroblasts derived from different individuals. In most strains, decreases in collagenolytic activities were evident at low CS concentrations or at relatively high concentrations only. One strain did not respond to CS, and there were two in which CS increased activity. Zebrowski et al. [49] reported

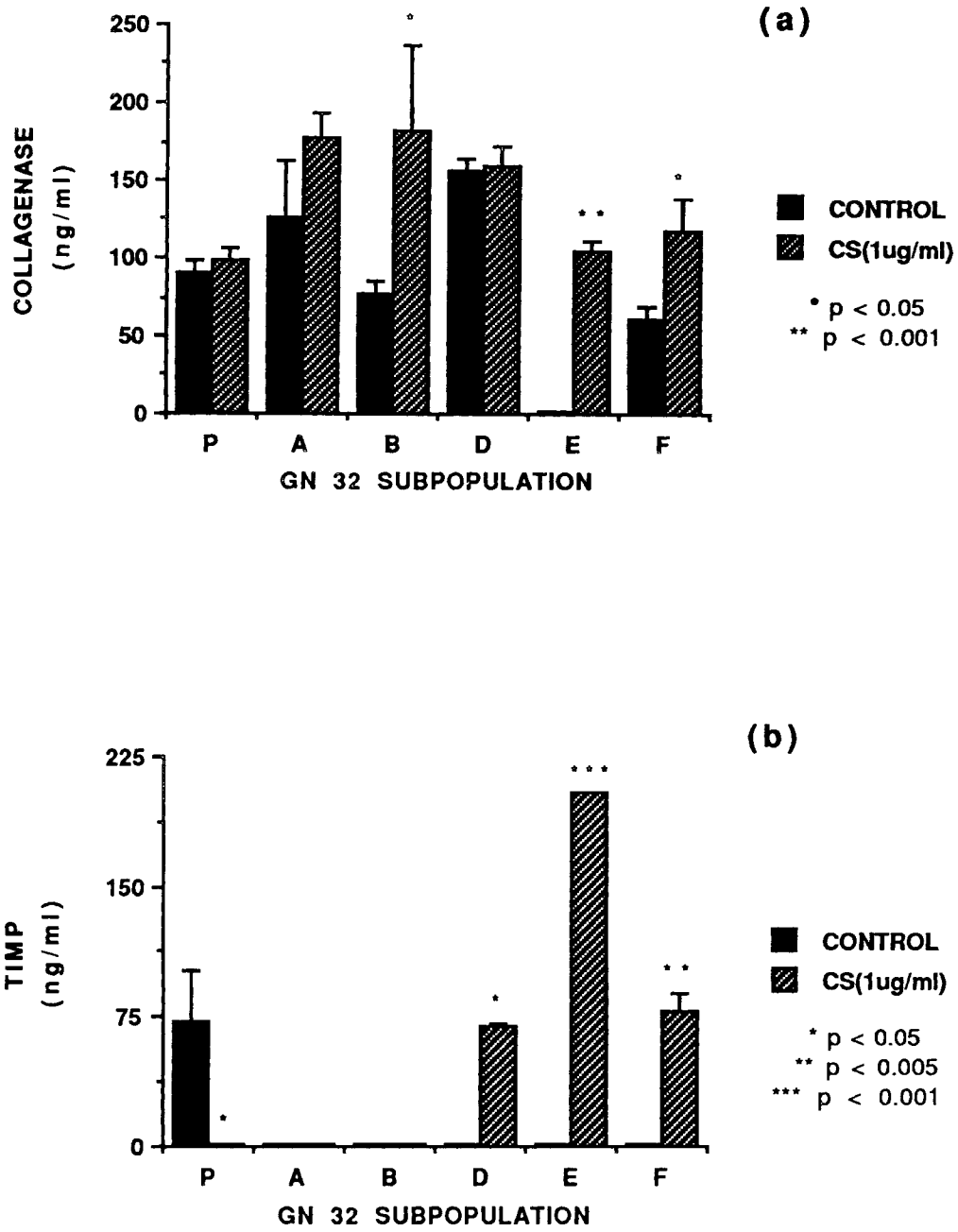

Fig. 3. Effect of CS on the production of immunoreactive collagenase and TIMP by a human gingival fibroblast strain and subpopulations derived from it. The fibroblast mass strain (P) and its subpopulations (A–F) were cultured in the absence or presence of CS (1 μg/ml) in serum-free AMEM-F10 medium. Culture supernatants were collected at 2 day intervals for 8 days. Immunoreactive collagenase (a) and TIMP (b) were measured by ELISA. Results are expressed as total ng protein/ml pooled culture supernatant. The data represent the means and standard deviations of triplicate cultures of a representative experiment. Statistical analysis was by one-way analysis of variance (ANOVA).

that CS at 0.25 and 0.5 μg/ml had no effect on the collagenase activity of a normal human gingival fibroblast strain and suggested that collagenase activity may not play a significant role in the drug-induced gingival enlargement. Our data suggest that the strain used in that study may respond collagenolytically to other CS concentrations and that fibroblast strains from other individuals may respond quite differently.

The present data show that the effect of CS on fibroblast collagenase activity apparently depends on the individual from whom the strain

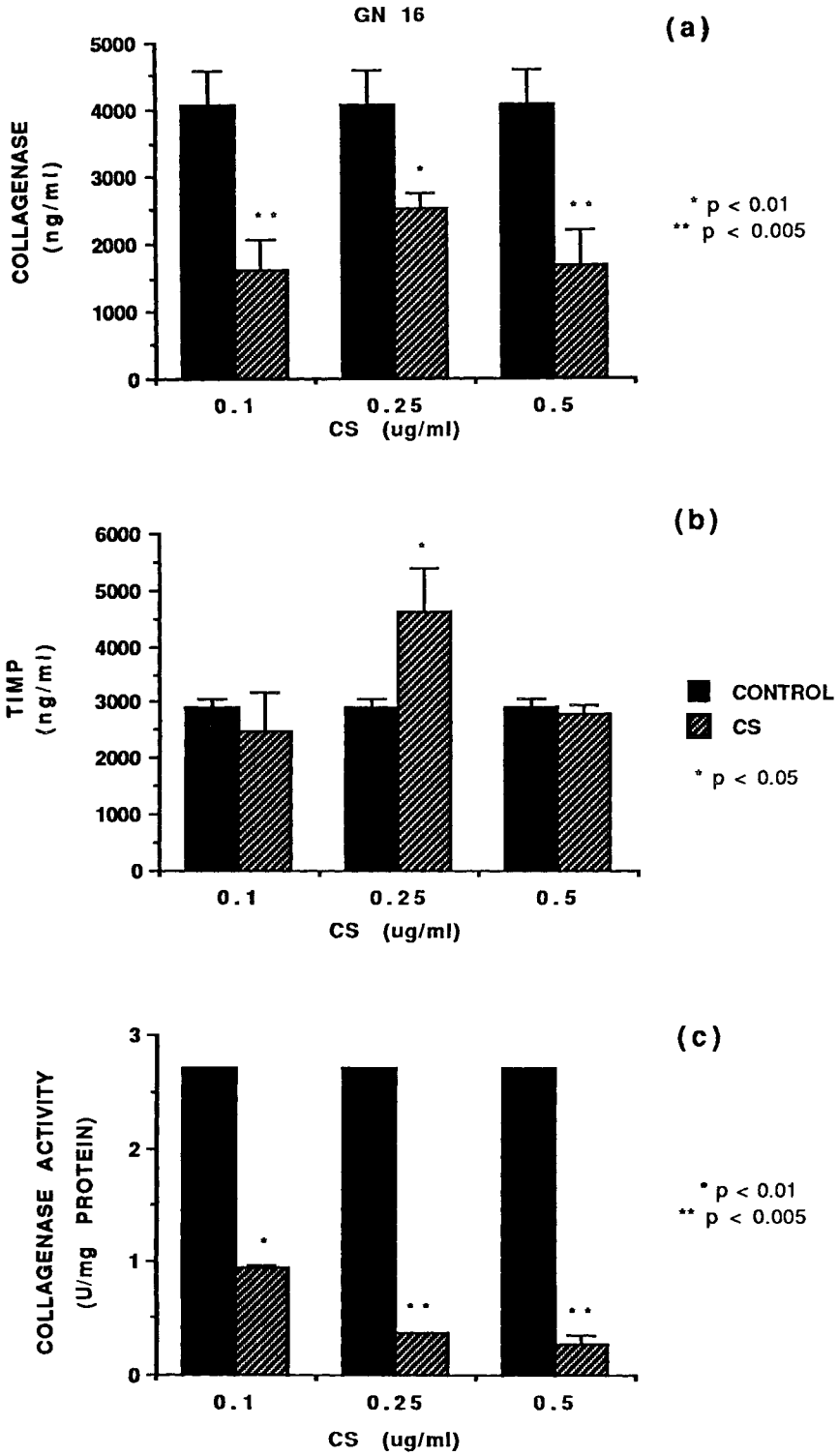

Fig. 4. Effect of CS on the collagenase activity, collagenase production, and TIMP production by the GN 16 human gingival fibroblast strain. Fibroblasts were cultured for 6 days in the absence or presence of CS (0.1, 0.25, or 0.5 μg/ml) in serum-free AMEM-F10 medium. Collagenase activity (c; expressed as units/mg protein) was measured using radiolabeled type I collagen gels as substrate, and collagenase (a) and TIMP (b) levels (expressed as ng protein/ml culture supernatant) were measured by ELISA. The data represent the means and standard deviations of triplicate cultures of a representative experiment and statistical analysis was by one-way analysis of variance (ANOVA).

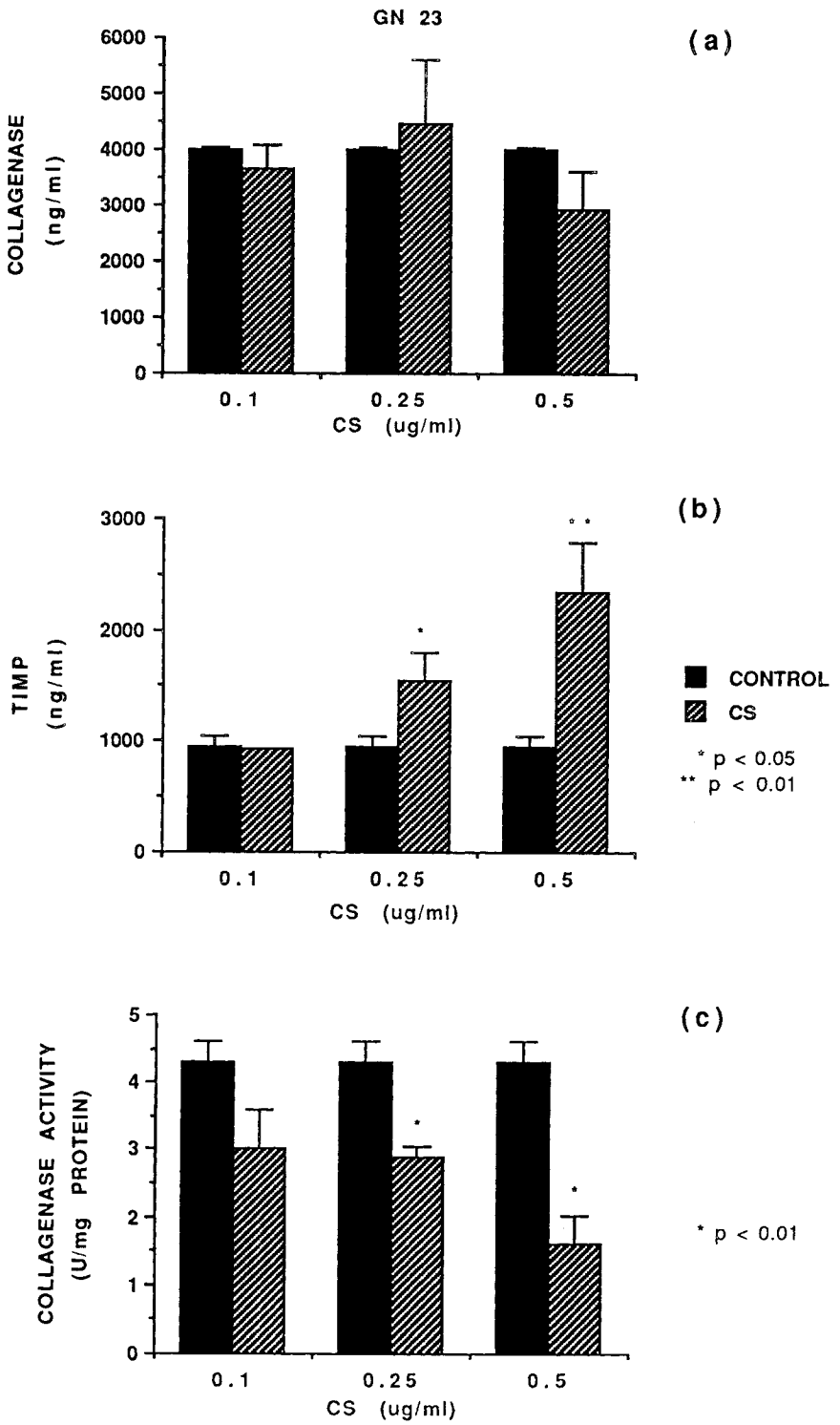

Fig. 5. Effect of CS on the collagenase activity, collagenase production, and TIMP production by the GN 23 human gingival fibroblast strain. Fibroblasts were cultured for 6 days in the absence or presence of CS (0.1, 0.25, or 0.5 μg/ml) in serum-free AMEM-F10 medium. Collagenase activity (**c**; expressed as units/mg protein) was measured using radiolabeled type I collagen gels as substrate, and collagenase (**a**) and TIMP (**b**) levels (expressed as ng protein/ml culture supernatant) were measured by ELISA. The data represent the means and standard deviations of triplicate cultures of a representative experiment and statistical analysis was by one-way analysis of variance (ANOVA).

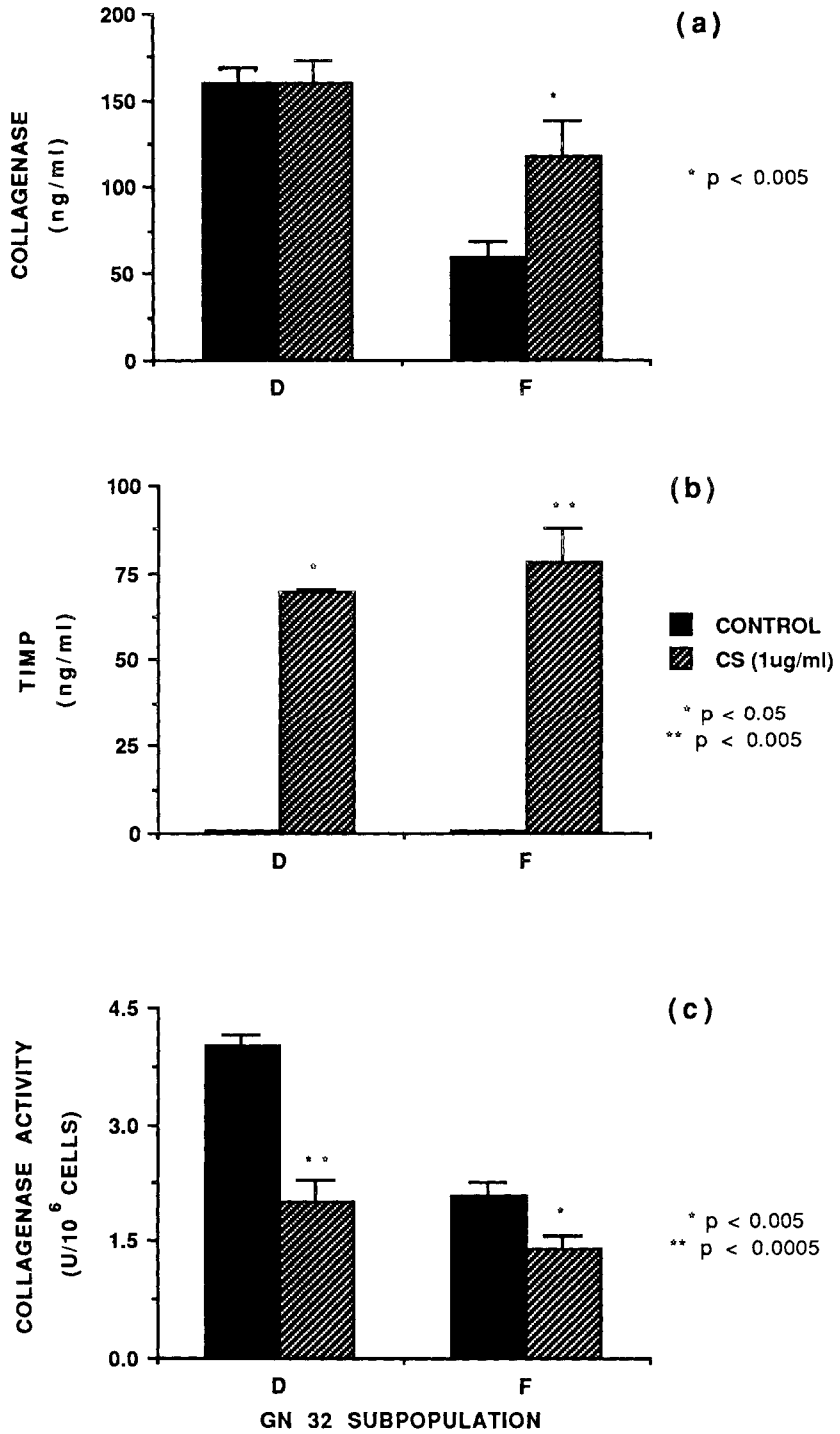

Fig. 6. Effect of CS on the collagenase activities, collagenase production, and TIMP production of human gingival fibroblast subpopulations. Subpopulations were derived from a human gingival fibroblast strain (GN 32) as described in Materials and Methods. The cells were cultured in the absence or presence of CS (1 μg/ml), and supernatants were collected at 2 day intervals for 8 days. Collagenase activity (**c**; expressed as total units/10^6 cells) was measured using radiolabeled type I collagen gels as substrate, and collagenase (**a**) and TIMP (**b**) levels were measured by ELISA. The data represent the means and standard deviations of triplicate cultures of a representative experiment and statistical analysis was by one-way analysis of variance (ANOVA).

was derived, the presence of responsive cell subpopulations, and the dose of the drug. Hassell et al. [21] reported similar observations of the effect of CS on normal gingival fibroblast protein and collagen production. There are differences among individuals in CS sensitivity of peripheral blood lymphocytes [20,50], and differential effects of CS on lymphocyte and monocyte subpopulations have also been noted [51,52]. A recent study also suggests that the effects of different CS concentrations on different immune cell populations could be clinically significant in immunosuppression [53].

That the fibroblasts from different individuals were heterogenous in their collagenolytic response to CS may partly explain the observation that not all patients taking CS develop gingival overgrowth [10]. Variability in the occurrence of gingival fibrosis has also been described in patients on phenytoin therapy [54]. Fibroblasts derived from the fibrotic tissue (responders) were significantly different in synthetic activity from comparable cells derived from normal individuals not taking phenytoin, as well as from patients taking the drug, but not exhibiting gingival enlargement (non-responders) [54].

The effect of CS on fibroblasts may be local; site-specific changes in fibroblast proliferation and synthetic activity in response to drugs have been reported [54,55] and attributed to cell selection in phenytoin gingival enlargement. Work in our laboratory [56] and others [17,18,20] has suggested that CS is not mitogenic for gingival fibroblasts or human spleen cells. Indeed, CS can exert a cytostatic activity on non-lymphoid cell lines [18,19 and references therein]. Therefore, CS does not appear to directly enhance the growth of sensitive fibroblast populations, but rather affects other metabolic functions such as collagen synthesis and breakdown.

Collagenase activity and thus collagen breakdown at a given site depend on the balance between local concentrations of collagenase and its inhibitors [57,58]. The different effects of CS on collagenase production by two different strains examined in this study is similar to the observation by MacNaul et al. [59] of variation among individuals in the induction of stromelysin mRNA by TNF in human synovial fibroblasts. In contrast, CS at most concentrations affected TIMP production by both of these strains in a similar manner. Strain- and dose-specific differences in the magnitude and direction of collagen and total protein synthetic response of human gingival fibroblasts to CS have previously been reported [21]. Our findings support these observations and extend them to collagenolysis and its regulation by TIMP.

In the GN 16 strain, decreased collagenase production, coupled with unchanged or elevated TIMP levels, may account for the profound (65–90%) inhibition of collagenase activity caused by CS. This is consistent with the work of Ito et al. [60], who showed that TNF-enhanced collagenolysis by human chorionic fibroblasts was due to increased collagenase production and decreased TIMP synthesis. On the other hand, the different pattern of effect of CS on collagenase and TIMP levels, and consequently collagenase activity in the GN 23 strain demonstrates that apparently, differences among individuals in the effect of CS on collagenase and TIMP production can account for differences in the magnitude and direction of collagenolytic response to the drug.

The observation that the effect of CS on collagenase activity could be attributed to its effects on the relative levels of TIMP and collagenase in only two of the subpopulations supports the concept of participation of select fibroblast subpopulations in the pathogenesis of drug-induced gingival fibrosis [23,29,56,61]. Heterogeneity of fibroblast populations and the presence of subpopulations of fibroblasts responsive to tumor cell-derived cytokines has also been reported from our laboratory [26].

Because the effect of CS on collagenase and TIMP production could not account for its effect on collagenase activity in all cases, CS may cause the synthesis of a defective or inactive collagenase, suggested for fibroblasts from phenytoin-enlarged gingiva [35]. Alternatively, the mechanism of CS action on fibroblast collagenolysis may involve factors other than collagenase and TIMP. For example, the effect of CS on other extracellular matrix proteases like stromelysin and plasmin, which may play roles in the activation of procollagenase in vivo [62], is unknown, as is the effect of CS on fibroblast synthesis of cytokines such as IL-1β, TNF, and TGF β, which can affect collagenolysis and contribute to fibrosis.

The mechanism of CS-induced gingival fibrosis in vivo probably involves both direct effects of CS on fibroblasts and indirect effects via CS-altered production of immune cell products, as shown in our laboratory [38,39] and others [21]. Infiltration of immune cells has been demonstrated in fibrotic tissue [63] and CS-enlarged

gingiva [7,8,64–66]. CS-induced alterations in the number, type, and/or functions of immune cells in gingiva may result in the disruption of normal fibroblast regulation, contributing to gingival enlargement. This study has demonstrated that the direct effect of CS on fibroblast collagenase activity and the production of collagenase and TIMP depends on the cell strain, the dose of the drug, and the presence of responsive fibroblast subpopulations. The heterogeneity of gingival fibroblast collagenolysis, both inherent and in response to direct exposure to CS, may in part explain why only some patients on CS therapy develop gingival enlargement. However, the role of indirect drug action via immune cell regulation of fibroblasts cannot be excluded in considering the mechanisms of connective tissue accumulation in CS-enlarged gingiva [39].

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