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α_2-Macroglobulin in Sulci from Healthy and Inflamed Human Gingivae

ILEANA CONDACCI, GIORGIO CIMASONI,* AND CYRUS AHMAD-ZADEH

School of Dentistry and Hygiene Institute, Medical Faculty, University of Geneva, Geneva, Switzerland

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The concentration of α_2-macroglobulin (α_2-M) in human gingival sulci has been investigated in two studies: first, in gingival washings during a 21-day period of experimental gingivitis in eight human volunteers and, second, in crevicular fluid collected with filter paper strips before and after initial periodontal therapy in 11 patients. The concentration of total α_2-M was found to increase in the washings of the volunteers throughout the period of experimental gingivitis. In the group of patients receiving periodontal therapy, the absolute amount of α_2-M in the fluid showed a significant decrease after therapy. The gingival index of inflammation and the crevicular fluid flow also decreased significantly. The specific content of the inhibitor (μg of α_2-M per mg of fluid per min), however, was found to increase in the fluid with decreasing inflammation. As detected by crossed immunoelectrophoresis, the fluid collected in these patients before therapy, in the presence of severe inflammation, invariably showed peaks of both free and complexed α_2-M. In contrast, the fluid collected from the same sites after healing of the inflammation contained no detectable free α_2-M.

Inflammation of the gingiva is accompanied by an increased migration of polymorphonuclear leukocytes toward the oral cavity through the gingival sulcus (1). The cells discharge their endogenous enzymes in the gingival environment either by phagocytosis or by cell death. Numerous investigators have provided evidence supporting the concept that lysosomal enzymes, particularly proteases active at physiological pH, may be involved in the tissue damage associated with periodontitis (6).

The activity of proteases in the tissues is probably modulated by the presence of inhibitors either produced locally or circulating in plasma (7, 18, 23). The main plasma protease inhibitors are α_2-macroglobulin (α_2-M) and α_1-antitrypsin, accounting for more than 90% of the total protease-inhibiting capacity of serum (18). Their presence in the gingival environment has received limited attention.

Brill and Brönnestam (4) were the first to report the existence of α_2-M in the gingival fluid collected with filter paper strips from healthy human gingivae. Ohlsson et al. (19) reported the presence of α_1-antitrypsin and α_2-M in material from human gingival sulci and, after studying α_1-antitrypsin in more detail, showed that all of this inhibitor was in a bound form, both in healthy and inflamed sulci. α_1-Antitrypsin and α_2-M were found in gingival fluid from inflamed gingivae by Schenkein and Genco (21), in concentrations representing about 75% of those found in serum. More recently, Uitto and Raeste (24) confirmed that α_2-M is present in human gingival fluid and found no correlation between the concentration of α_2-M and that of collagenase in a group of nine patients. Until now, however, no attempts were made to distinguish between the free and the bound form of α_2-M in the gingival environment. This question was studied in the present investigation.

MATERIALS AND METHODS

Determination of α_2-M in gingival washings. In a first study, α_2-M was determined in gingival washings throughout a period of experimental gingivitis in eight human subjects. The washings were performed as previously described (5) by using individual appliances. These consisted of hard acrylic plates covering the total maxillary area, with a channel along the buccal and palatal marginal region. Physiological saline (5 ml) was circulated in the channel by a peristaltic pump for 15 min. The eight volunteers were first given daily oral prophylaxis for 10 days, until their index of gingival inflammation (13) approached zero. According to the procedure first outlined by Löe et al. (14), gingival inflammation was induced by omitting the brushing of the upper teeth for 21 days, after which normal oral hygiene was resumed. The index of gingival inflammation (13) was recorded before, during, and at the end of the 21-day no-brushing period. The index was determined at four sites (mesial, distal, buccal, and palatal) of the upper teeth, from the right first molar to the left first molar, and the average was calculated for each patient. The clinical indices and the gingival washings were performed on the same days.

The washings were concentrated 40 times, and α_2-M was determined by radial immunodiffusion (15) on 20-μl samples. Immunodiffusion plates low concentration-Partigen-α_2-M and human protein standard serum were from Hoechst-Pharma (Behringwerke AG, Marburg, Federal Republic of Germany).

α_2-M in the gingival exudate. In a second study, the concentration of total α_2-M and the free and bound fractions of α_2-M were determined in micro amounts of gingival exudate (crevicular fluid) collected at selected sites in 11 patients with very inflamed gingivae and periodontitis. The patients subsequently underwent initial periodontal therapy which resulted in a marked decrease in gingival inflammation. After completion of therapy a second sample was obtained from the same selected sites. The fluid was collected on two upper anterior teeth by inserting a paper strip at the depth of 1 mm in the gingival sulcus (between the tooth and the gingiva) of each of the two selected teeth after drying of the area. The time of collection, usually 3 to 5 min, and the weight of each strip before and after collection were recorded. The gingival index of inflammation (13) was scored before collecting the fluid at the same sites where the paper strips were inserted. One strip (2 by 5 mm) from the first upper anterior tooth was utilized for the determination of total α_2-M by the technique of Laurell (12). It was first placed in a microtube, and 40 μl of physiological saline was added. The tubes were kept at 4°C for 24 h and shaken intermittently. They were then centrifuged, and 10 μl of supernatant was used for the electroimmunoassay (12).

The second strip (5 by 10 mm), with the fluid collected from the second upper anterior tooth, was used for the determination of the free and complexed fractions of α_2-M by a modification of the isoelectric focusing and crossed immunoelectrophoresis technique of Ohlsson and Skude (20). The strip was soaked with 15 μl of physiological saline and laid directly on the polyacrylamide gel containing the ampholytes with the pH range 3.5 to 9.5 (polyacrylamide gel plates L.K.B., 1804-101; L.K.B. Products, Bromma, Sweden). Immediately after completion of the electrophoretic run, a 5-mm slice of the gel, containing the separated sample, was cut with a surgical knife and transferred to a 1% agarose plate for the crossed immunoelectrophoresis. The agarose plate was divided into two parts, the superior part containing about 7 μl of antiserum against α_2-M (rabbit anti-human α_2-M ORCD-04-05; Hoechst-Pharma, Behringwerke AG) per ml of gel, the remaining part being free of antiserum. The slice of polyacrylamide was inverted so that the protein-bearing surface of the gel faced downward onto the antiserum-free agarose gel surface at about 2 mm from the limit of the antiserum-containing portion of the gel. This second electrophoresis was performed with 8 to 10 V/cm at 16°C for 4 h. A 0.1 M barbital buffer (pH 8.6) was used in the electrode vessels and in the agarose gel. After completion of the electrophoresis run, the glass plate with the agarose gel was washed in 0.1 M NaCl overnight, pressed, dried, and stained with Coomasie brilliant blue R 250.

The determination of both the total α_2-M and the free and bound fractions was repeated with fluid collected at the same sites in the same patients after about 1 month of routine periodontal therapy (oral hygiene instructions, scaling, and root planing). The gingiva was then much less inflamed as indicated by a significant decrease of the index of inflammation and of the gingival fluid flow. In two of the patients fluid could be obtained also during the healing phase.

Samples of human serum, alone or with added elastase from human polymorphonuclear leukocytes, were also submitted to crossed immunoelectrophoresis. For this purpose strips of filter paper were soaked with 20 μl of fresh human serum diluted 10-fold with distilled water. Elastase (20 μg), partially purified in our laboratory from human polymorphonuclear leukocytes (9), was added to 200 μl of 0.2 M Tris-hydrochloride buffer (pH 8). After incubation for 1 h at 37°C, 20 μl of this mixture, absorbed on a filter paper strip, was used for the crossed immunoelectrophoresis.

Finally, commercial serum (Hoechst-Pharma, Behringwerke AG) was also analyzed for the presence of free and bound α_2-M. The run was again performed with filter paper strips soaked with 20 μl of commercial serum. The isoelectric focusing and immunoelectrophoresis apparatus was an L.K.B. 2117 Multiphor.

RESULTS

Total α_2-M in the gingival washings. The average index of gingival inflammation of the eight volunteers increased during the no-brushing period and reached a peak at day 20. This was followed by a rapid decline when the hygiene procedures were resumed (Fig. 1). The concentration of α_2-M, as determined by radial immunodiffusion, tended to increase in the gingival washings collected during this period of experimental gingivitis (Fig. 1). A great variability was observed among individuals, as shown in the figure by the high values of standard errors

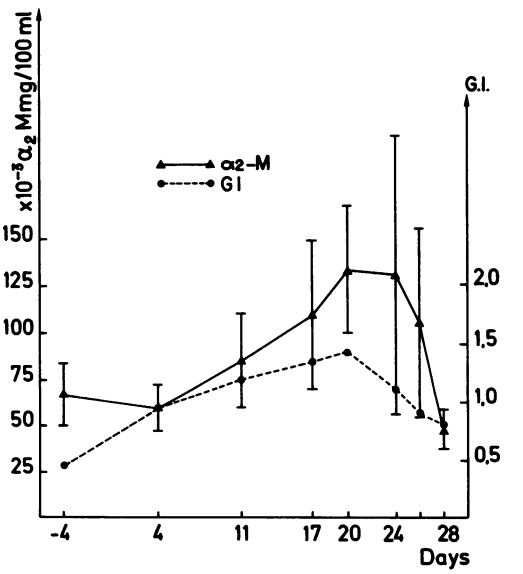

FIG. 1. Concentration of α_2-M in the washings during experimental gingivitis (average of eight volunteers ± standard error of the mean). The average index of gingival inflammation (GI) is also presented.

throughout the experiment. However, the difference between the values of α_2-M concentrations found at day −4, in the absence of inflammation, and those of day 20, when the inflammation reached the highest level, was statistically significant ($0.01 < P < 0.05$). This was confirmed by the highly significant difference found between day 20 and day 28 ($0.001 < P < 0.01$).

Total α_2-M in the crevicular exudate. As explained above, crevicular exudate was collected before and after periodontal therapy in a second group of 11 patients. Each time two collections were performed: the first sample was used for determination of the total α_2-M, and the second was used for the determination of the free/bound ratios of α_2-M. Table 1 shows the values of the clinical measurements and the results of the analysis of total α_2-M by the Laurell technique. As expected, the high degree of gingival inflammation at the site of collection decreased very significantly after oral hygiene instructions and subgingival curettage. The same pattern can be observed for the weight and especially the flow of crevicular fluid: this last parameter decreased from an average of 0.7 mg/min to the mean value of 0.07 mg/min after periodontal therapy (Table 1).

The absolute amount of total α_2-M recovered from the strips was lower after therapy in eight patients and increased slightly in three, the mean decrease being statistically significant (Table 1).

However, when taking into account the weight of exudate, it can be seen (Table 1) that the actual concentration of α_2-M, expressed as micrograms of α_2-M per milligram of fluid, was significantly higher in all but two instances after periodontal therapy, i.e., when the degree of inflammation was minimal. This was even more evident when considering the flow rate (micrograms of α_2-M per milligram of fluid per minute) of fluid (Table 1). A great variability was observed among individuals. It is worth mentioning that this way of expressing the data shows that the specific content of the inhibitor in the gingival sulcus is actually increasing with decreasing inflammation.

Free and bound α_2-M in the crevicular exudate. Figure 2 shows the results of three assays with the technique of crossed immunoelectrophoresis with freshly collected human serum (Fig. 2a), the same human serum plus elastase (Fig. 2b), and commercial serum (Fig. 2c). As expected, fresh human serum (Fig. 2a) showed a major peak of free α_2-M at pH 5.0 and a very small peak of bound α_2-M at a pH of about 5.6. When the enzyme elastase was added to whole human serum, a much greater peak of bound α_2-M could be demonstrated (Fig. 2b). Finally, in samples of commercial serum, all of the α_2-M was found in the bound form (Fig. 2c).

The patterns of crossed immunoelectrophoresis of free and bound α_2-M in the crevicular exudate from 8 among the 11 patients before and after periodontal therapy are shown in Fig. 3. The average gingival inflammation for these patients, measured at this second site of collection, went from 2.4 to 0.8, whereas the gingival fluid flow decreased from an average of 1.306 mg/min before therapy to 0.177 mg/min after therapy. In two other patients the exudate was collected before periodontal therapy (average gingival inflammation, 2.25; fluid flow, 1.171 mg/min), during the healing phase (gingival inflammation, 1.0; fluid flow, 0.201 mg/min), and after

TABLE 1. Scores of gingival index of inflammation (GI), weight and flow of crevicular fluid, and values of total α_2-M before and after periodontal therapy in 11 patients[a]

Patient no.	GI		Wt of fluid (mg)		Flow of fluid (mg/min)		μg of α_2-M		μg of α_2-M/mg of fluid		μg of α_2-M/mg/min	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	3.0	1.0	3.17	2.16	1.580	0.410	6.40	3.80	2.02	1.76	4.05	9.27
2	2.0	0.0	2.90	0.70	0.480	0.035	3.20	1.60	1.10	2.28	6.67	45.72
3	2.0	1.0	1.83	0.17	0.170	0.007	2.10	0.40	1.15	2.35	12.35	57.14
4	2.0	1.0	1.19	0.35	0.240	0.018	1.70	0.80	1.43	2.28	7.08	44.44
5	2.0	1.0	2.31	0.55	0.920	0.032	0.50	0.76	0.22	1.38	0.54	23.75
6	2.0	0.0	0.39	0.38	0.054	0.017	0.12	0.20	0.31	0.52	2.22	11.76
7	3.0	1.0	1.34	0.09	1.340	0.005	1.28	0.18	0.96	2.00	0.96	36.00
8	3.0	1.0	1.18	1.00	2.020	0.083	1.40	1.96	1.19	1.96	0.69	23.62
9	3.0	1.0	1.22	0.67	0.542	0.067	1.76	0.72	1.44	1.08	3.25	10.74
10	2	0.0	0.80	0.33	0.145	0.036	0.50	0.22	0.63	0.67	3.45	6.11
11	3.0	1.0	0.83	0.45	0.160	0.041	0.60	0.38	0.72	0.84	3.75	9.26
Avg	2.45	0.72	1.56	0.62	0.70	0.07	1.78	1.00	1.02	1.56	4.09	25.25
SD	0.52	0.46	0.89	0.57	0.67	0.12	1.77	1.09	0.53	0.69	3.50	17.87

[a] Before-versus-after P values were as follows: GI, $P < 0.001$; weight of fluid, $P < 0.001$; flow of fluid, $0.001 < P < 0.01$; α_2-M, $0.01 < P < 0.02$; α_2-M per milligram, $0.001 < P < 0.01$; α_2-M per milligram per minute, $P < 0.001$.

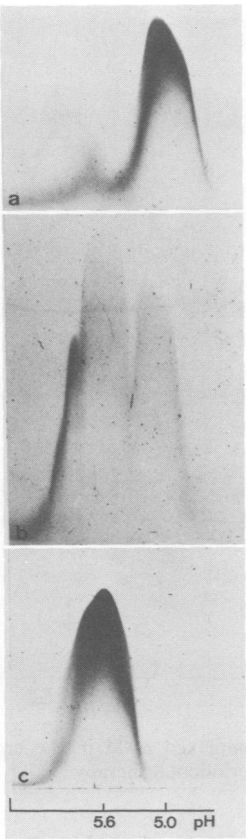

FIG. 2. Patterns of crossed immunoelectrophoresis of α_2-M in (a) fresh human serum, (b) fresh serum plus elastase, and (c) commercial serum. Peaks of bound and free α_2-M are visible at pH 5.6 and 5.0, respectively.

complete healing (gingival inflammation, 0.5; fluid flow, 0.110 mg/min); the results obtained by crossed immunoelectrophoresis in these samples of fluid are presented in Fig. 4.

The fluid collected in the presence of severe inflammation invariably showed peaks of both free as well as complexed α_2-M (Fig. 3 and 4). In contrast, the fluid collected from the same sites after healing of the inflammation contained no detectable free α_2-M (Fig. 3 and 4). A gradual disappearance of the peak of free α_2-M could be observed during the healing process in two of the patients (Fig. 4). The bound form of α_2-M in crevicular fluid appeared as multiple peaks centered at pH 5.6.

DISCUSSION

In the present investigation it was found that the total amount of α_2-M in the fluid of gingival sulci correlates with the degree of gingival inflammation. This was observed in the course of experimental gingivitis induced by discontinuation of dental hygiene and during the healing phase of periodontitis after therapy. These observations confirm data obtained by others either by immunoelectrophoresis (19) or radial immunodiffusion (24). The increase in α_2-M appears to be the result of the well-known increase in the production of crevicular fluid as a consequence of gingival inflammation (5). However, the actual concentration of α_2-M, i.e., the amount of inhibitor per amount of fluid produced, was higher in healthy sulci. This finding could be due to a difference in local synthesis of α_2-M in healthy and inflamed gingivae: it is known that not only is α_2-M synthesized in the liver, but also it can be produced locally by different types of cells, for instance, fibroblasts and macrophages (16, 25). Alternatively, in an inflamed tissue, some of the α_2-M could have been reabsorbed by the circulation after being bound to enzymes.

The technique for the determination of free and bound forms of α_2-M was first tested with fresh human serum and with commercial serum. We have confirmed that the largest part of the α_2-M is found in the free form in human serum, migrating at pH 5, with the presence of only traces of the complexed form, at a pH slightly lower than that described by Ohlsson and Skude (20). When active elastase was added to fresh human serum, a much greater peak of bound α_2-M could be detected (Fig. 2). Barrett et al. (2) have shown that the electrophoretic mobility of bound α_2-M is similar to that of the inactivated form of the α_2-M. This inactivated or bound form of α_2-M found, for instance, in commercial serum has been defined as the "fast" form of the α_2-M by these investigators. It is worth noting that the technique used in the present study confirmed their findings that all of the α_2-M contained in a preparation of commercial serum migrates at the higher pH (Fig. 2) and represents the fast form of the α_2-M. Inactivation of α_2-M in commercial serum could be due to the process of partial purification and stabilization.

In all samples of gingival fluid obtained from highly inflamed gingivae, we have consistently found both the free and bound forms of α_2-M. This finding was unexpected since in inflamed gingival sulci one regularly finds active (i.e., free) proteases like mammalian collagenase and neutrophil elastase (8, 10, 19, 24). Free α_2-M has also been reported to occur in synovial (17, 22) or pleural (3) effusions, but in such exudates free proteinases are usually not detected (17). Although there is no doubt about the reliability of the detection of both α_2-M forms in all our cases, we have no concrete explanation of this phenomenon.

A comment should be made on the presence

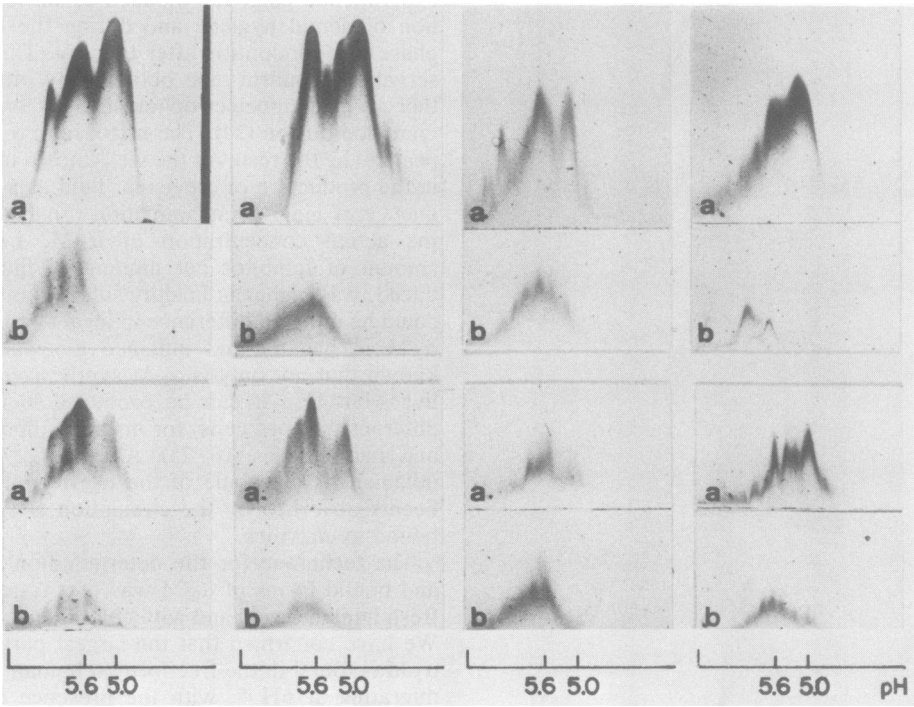

FIG. 3. Patterns of crossed immunoelectrophoresis of free and complexed α_2-M in the crevicular fluid collected from the same sites in eight patients before (a) and after (b) periodontal therapy.

of multiple peaks centered at pH 5.6 in crevicular fluid. The fluid might contain degradation products of α_2-M or complexes of α_2-M with nonenzymatic proteins (nonspecific complexes).

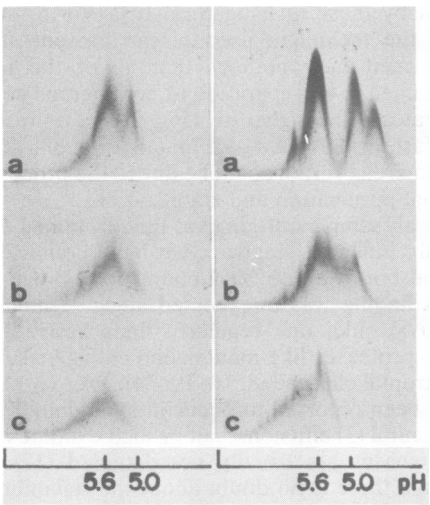

FIG. 4. Free and bound α_2-M in the crevicular fluid from two patients before periodontal therapy (a), during the healing phase (b), and after complete healing (c).

All of these forms of α_2-M are antigenically detectable, but might have slightly different mobility (around pH 5.6).

It may also appear difficult to explain that in the fluids we collected from healing or noninflamed gingivae virtually all of the α_2-M was found in bound form. This finding could be explained by the fact that neutrophil migration into the crevicular space is independent of the passage of fluid and also occurs in completely healthy gingivae (11). The presence of neutrophils results in the release of some neutral proteinase which could be captured by the inhibitors.

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