

Concentrations of fibronectin in the sera and crevicular fluid in various stages of periodontal disease*

Dennis E. Lopatin^{1,2},
Elena R. Caffesse², Fred. L. Bye³
and Raul G. Caffesse^{2,3}

Departments of ¹Oral Biology and
²Periodontics and the ³Dental Research
Institute, School of Dentistry, The University
of Michigan, Ann Arbor, Michigan 48109-0402,
USA

Lopatin DE, Caffesse ER, Bye FL and Caffesse RG: Concentrations of fibronectin in the sera and crevicular fluid in various stages of periodontal disease. *J Clin Periodontol* 1989; 16: 359-364.

Abstract. While fibronectin (FN) has previously been demonstrated to be present in gingival crevicular fluid (GCF), its quality and quantity has not been reported. Since this information is relevant for ongoing studies on the use of FN for gingival reattachment, we performed these measurements and compared plasma levels in healthy subjects, patients with gingivitis and periodontitis, and in patients undergoing maintenance therapy. Plasma and GCF samples were obtained from 4 sites in each subject using a Periotron to permit quantification of samples. FN concentrations were determined in a microELISA using hyperimmune anti-FN antibody. Purified FN served as a reference for quantification. The functional activity of each sample was assessed by examining the natural affinity of FN for gelatin. Subjects with gingivitis and those in maintenance had significantly depressed levels of plasma fibronectin. While little fibronectin could be detected in the GCF of healthy sites regardless of patient category, examination of the most diseased sites in each group revealed that the concentration of FN in the GCF was highest in health and reduced when there was gingival inflammation. In no case was GCF FN found to be biologically active.

Key words: fibronectin; gingival crevicular fluid; plasma; ELISA

Accepted for publication 26 July 1988

An important component of the connective tissues is fibronectin (Ruoslahti et al. 1981, Yamada et al. 1984, Blumenstock et al. 1978, Mosher 1984). Fibronectin was first described by Morrison et al. in 1948 (Morrison et al. 1948). It is an α -2-globulin having a molecular weight of approximately 450,000 daltons, and is found in plasma at a concentration of 300 to 500 μ g/ml. One of its most basic functions is to promote the attachment of cells to the substratum (Marchase et al. 1976, Yamada et al. 1976, Yamada et al. 1985). Because many cells preferentially adhere to fibronectin, it serves as a promoter of cell migration and orientation (Ruoslahti et al. 1981, Yamada et al. 1984, Blumenstock et al. 1978, Mosher 1984). This

activity has led to attempts to use it to foster the reattachment of gingival tissue to the root surface in treatment of periodontal disease (Terranova & Martin 1982, Nasjleti et al. 1987, Nasjleti et al. 1986, Caffesse et al. 1985, Ryan et al. 1987).

Since fibronectin used at normal plasma concentrations has been shown to be effective in obtaining new tissue attachment in animal studies, we were interested in knowing what concentrations actually existed in the gingival crevicular fluid bathing the root surfaces (Smith et al. 1987). In addition, since plasma fibronectin levels are known to be affected by a variety of disease conditions (Mosher & Williams 1978, Brodin et al. 1984, Dejjgaard et al. 1984, Gerdes et al. 1983, Grossman et al. 1983), we wanted to determine the effects of periodontal disease status on the concentration of the protein in both the plasma and gingival crevicular fluid (GCF).

Material and Methods

Experimental subjects and clinical measurements

The research subject population was comprised of 4 groups of patients which included: (1) control or minimal disease ($n=8$; having minimal levels of gingivitis (GI=0,1; (Silness & Loe 1964)); (2) gingivitis ($n=5$; having significant amounts of inflammation (GI=2,3) with no loss of attachment); (3) adult periodontitis ($n=9$; having moderate to advanced periodontal lesions with attachment loss >4 mm and pocket depth >6 mm); (4) maintenance ($n=5$; having been treated for periodontitis and being under a 3-month prophylaxis recall). In order to provide a general perspective on the overall oral health of the subjects, the level of clinical periodontal attachment in relation to the cemento-enamel junction and pocket depth according to the criteria of the PDI (Ramfjord 1967) were recorded at

*This work was supported by United States Public Health Service Grant DE 02731 from the National Institute for Dental Research.

the distobuccal, buccal, mesiobuccal, distolingual, lingual, and mesiolingual aspects of the six PDI teeth (tooth nos. 3, 9, 12, 19, 25, 28). In addition, the same measurements were recorded for sites selected for GCF sampling in our patients: 2 sites having the least disease and 2 sites having the most disease for each patient, as determined by gingivitis scores and attachment levels.

Blood and gingival crevicular fluid collection

Blood samples collected in EDTA for fibronectin analysis were obtained from each subject. Prior to performing the clinical examination with instrumentation, 4 sites (2 minimally diseased and 2 most diseased) were selected, based on visual examination and radiographs. This was done so as not to mechanically disturb the site prior to sampling. Static gingival crevicular fluid samples were obtained from these sites by inserting Periopapers™ into the sulcus for a period of 30 s. The amount of fluid collected was estimated using a Periotron™ calibrated with human serum (0.1 μ l to 1.0 μ l). The Periopapers™ were then placed in vials containing 1 ml of phosphate-buffered saline (PBS, 0.05M PO₄, 0.15M NaCl, pH 7.4) containing Tween 20® detergent (PBS-T) and then incubated at room temperature for 24 h to allow for elution of the fibronectin. At the same time, measured serum samples were applied to other Periopapers™ and eluted in a similar fashion in order to provide controls to

permit estimation of elution efficiency of the fibronectin.

Determination of fibronectin concentrations

2 ELISA protocols were used to quantify the levels of fibronectin in plasma and GCF. The total concentration of fibronectin was determined by sensitizing 96-well polystyrene microtiter plates with rabbit anti-human fibronectin (IgG fraction, 5 μ g/ml) diluted in 0.1M sodium carbonate coating buffer (pH 9.6). The plates were filled (0.1 ml/well) with the antibody solution, incubated for 3–4 h at 37°C, and then stored at 4°C until used in the assay. Before use, the wells were washed 5 times with PBS-T. Human plasma samples or GCF periopaper extracts were diluted in PBS-T and added to the wells (0.1 ml/well). Graded concentrations of human fibronectin, purified by affinity chromatography (Vuento & Vaheri 1979) were added to selected antibody-sensitized wells to serve as standards. After incubation for 3 h at room temperature, the plates were washed 5 times with PBS-T, and alkaline phosphatase (calf intestine, type VII; Sigma Chemical Co., St. Louis, MO) conjugated to goat or sheep anti-rabbit immunoglobulin (Bio-Rad Laboratories, Richmond, CA) and added to each plate (0.1 ml/well). After overnight incubation at room temperature, the plates were washed 5 times with PBS-T. Alkaline phosphatase substrate (Sigma 104™, Sigma Chemical Co.) was then added to each well. After

30 min at room temperature, the absorbance at 405 nm was measured in a spectrophotometer (Multiskan; Flow Laboratories, Inc., McLean, VA). Concentrations of fibronectin in the plasma and GCF samples were calculated from a standard curve generated by the simultaneous titration of the purified human fibronectin standards. The total biologically-active fibronectin was determined in a similar manner as described above. However, instead of sensitizing the microtiter plate with anti-fibronectin, the plate was coated with swine skin gelatin (5 μ g/ml) taking advantage of the natural affinity of fibronectin for that substrate (Selmer et al. 1984). Samples having a volume of less than 0.1 μ l were classified as *not detectable* and were not used in the fibronectin analysis. This occurred only in the healthy/least disease sites and was consistent regardless of the overall classification of the patient.

Statistical analysis

For statistical analysis, the subjects were divided into 4 groups, i.e., control, gingivitis, adult periodontitis, and maintenance. The one-way analysis of variance combined with the Scheffe test was used to compare fibronectin levels between subject of differing disease categories. Student *t*-tests were performed to examine fibronectin levels in diseased and non-diseased sites in each subject category. All analyses of data were performed using programs in the Michigan Interactive Data Analysis System (MIDAS).

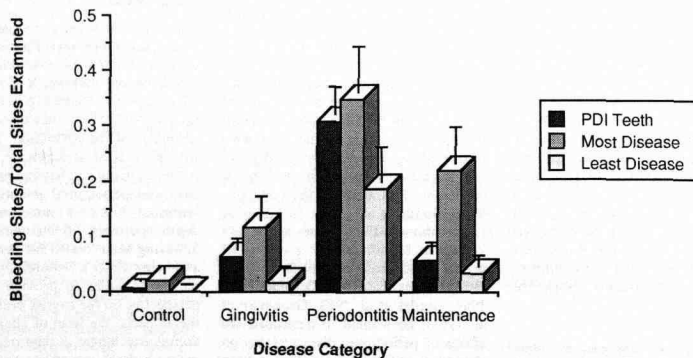


Fig. 1. Percentage of bleeding sites in GCF-sampled sites compared to total mouth values (PDI teeth). The mean % (\pm standard error of the mean) of the bleeding sites in the sampled areas (most disease and least disease) compared to that of the periodontal disease index teeth.

Results**Clinical parameters****Bleeding sites**

After sampling the GCF in the 4 selected sites (2 least diseased and 2 most diseased), those sites, as well as the 6 teeth of the periodontal disease index (PDI), were examined for bleeding and loss of attachment. Fig. 1 shows, for each disease category, the % of bleeding sites in the sampled sites for the sites

having most disease, for sites having least disease and sites for the PDI teeth. In each case, the values for the PDI teeth were intermediate between the least and most diseased sites sampled, thus confirming the initial visual selection of those sites for sampling.

Clinical attachment levels.

The attachment levels of the sampled sites shown in Fig. 2. As shown above for

the bleeding sites, the mean attachment loss of the PDI teeth represented the intermediate values between the most and least diseased sampled sites.

Periotron™ readings.

In this study, the Periotron™ was not used in the standard fashion. Rather than drying the site and collecting the fluid expressed in a defined time period, we collected the pre-existing static fluid, which actually reflected the actual en-

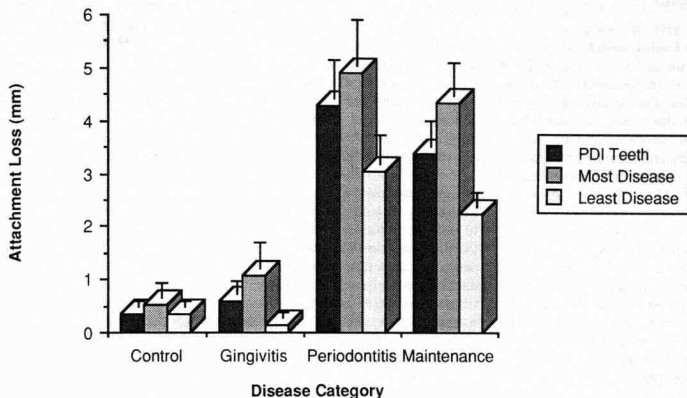


Fig. 2. Clinical attachment of sampled sites compared to total mouth values (PDI teeth). The mean clinical attachment (\pm standard error of the mean) of the sampled sites (most disease and least disease) compared to that of the periodontal disease index teeth.

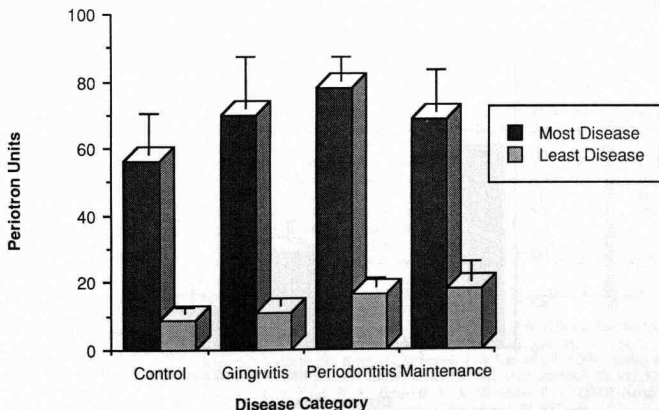


Fig. 3. Mean Periotron readings in various disease categories. Comparison of the mean Periotron readings (\pm standard error of the mean) of most and least disease sites in various disease categories.

environment of the sulcus at the time of sampling. Also, the volume of fluid that could be collected in the standardized manner often resulted in less material than was needed in the fibronectin assay. As shown in Fig. 3, the mean amount of fluid collected in the 30-s period from the most diseased sites, regardless of the overall diagnosis of the patient, did not significantly differ. This was also true for the least diseased sites.

Fibronectin levels

Fibronectin levels in plasma.

Fibronectin levels in plasma were measured as shown in Table 1. The levels of fibronectin in the gingivitis and maintenance patients' plasma are significantly ($p \leq 0.01$) lower than the control level of 294 ± 54 (mean \pm standard error). There was no significant difference between the control and periodontitis values.

Fibronectin levels in GCF.

When the GCF fibronectin levels were compared, we did not find any significant differences in the content of the least diseased sites when different patient categories were compared (Fig. 4). In all of these cases, the volume of sample collected was so small that they were beyond the limits of detection, which was not the case with the most disease categories (see Fig. 3). However, if the fibronectin concentrations of the most diseased sites are examined, we found the highest levels of fibronectin ($106 \pm 28 \mu\text{g/ml}$) in the GCF of the con-

Table 1. Relationship between periodontal disease status and plasma fibronectin concentration*

Group	n	Fibronectin concentration ($\mu\text{g/ml} \pm \text{SEM}$)	Significance ^b
control	8	294.8 \pm 19.3	-
gingivitis	5	226.6 \pm 17.6	$p < 0.01$
periodontitis	9	296.6 \pm 12.5	n.s.
supervision	5	233.6 \pm 12.2	$p < 0.01$

*Concentration determined by microELISA using hyperimmune anti-FN.

^bSignificance determined by one-way ANOVA, combined with Scheffe analysis.

trol group. There were virtually undetectable ($3.7 \pm 2 \mu\text{g/ml}$) levels of fibronectin in the most diseased sites of gingivitis patients. Periodontitis patients had levels intermediate ($52 \pm 12 \mu\text{g/ml}$) between control and gingivitis. Following periodontal therapy, patients in maintenance had values intermediate ($23 \pm 11 \mu\text{g/ml}$) between gingivitis and periodontitis patients.

Biological activity.

The ability to bind to gelatin was used to assess the biological activity of the GCF fibronectin. While such measurements could not be made in the least disease categories because of minimal volumes of GCF collected, in few instances did any of the GCF samples from the most disease categories contain biologically active fibronectin, as measured by the gelatin ELISA.

Discussion

Fibronectin has previously been demonstrated by other investigators to be present in the gingival crevicular fluid

(Tynelius-Bratthall et al. 1986). The results of this study suggest that there is a difference in the fibronectin content of the GCF when it is examined as a function of the periodontal disease activity of the patient, as well as of the individual gingival site. The highly permeable junctional epithelium of the sulcus has been shown by numerous investigators to allow the passage of species of molecular weights as high as 700,000 daltons both into and out of the connective tissue (Schroeder 1977). A molecule of fibronectin, even with a high molecular weight of 450,000 daltons should have little difficulty passing through this barrier. In this study, volumes of crevicular fluid were too low ($< 0.1 \mu\text{l}$) in most of the healthy sites to be measured. However, with the occurrence of marginal inflammation in control subjects sufficient to permit the expression of larger volumes of fluid, the fibronectin content was found to be approximately 1/3 of plasma levels. Since this study was only a preliminary survey of fibronectin levels in the gingival crevicular fluid, it is not possible at this time

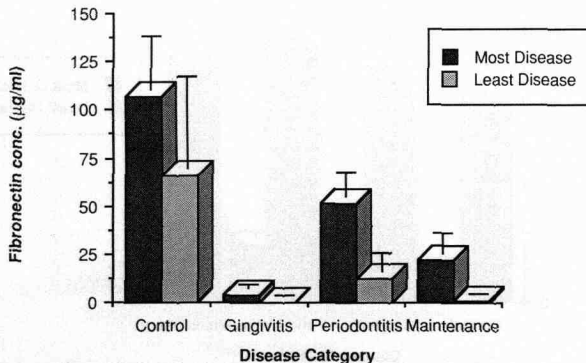


Fig. 4. Fibronectin concentrations in GCF. Comparison of the mean concentrations of total fibronectin concentration (by anti-fibronectin ELISA; \pm standard error of the mean) of the most and least disease sites in various disease categories.

to characterize the actual mechanism(s) responsible for different concentrations of fibronectin in sulci having different disease severity. This phenomenon, however, might be discussed in the context of a number of distinct mechanisms known to influence fibronectin levels.

The most trivial of explanations, i.e., that capillary permeability increases as a function of inflammation, is not sufficient, since there was not a consistent relationship between disease severity and fibronectin content of the sulcus. If this were merely a function of increased capillary permeability, the most diseased sites would be expected to have the highest levels of fibronectin. Since the relationship between the fibronectin level and the stage of disease was not linear, the resident flora and its role in influencing the fibronectin content of the GCF should be considered. There are numerous reports of the ability of various microbial species to bind fibronectin (Porvaznik et al. 1982, Babu et al. 1983, Imai et al. 1984, Stanislawski et al. 1985). The opsonic qualities of fibronectin facilitate the phagocytosis of certain microbial species, which include *Strep. mutans*, *Strep. sanguis*, *Strep. pyogenes*, and *Staph. aureus*. The presence of such bacterial "sponges" might help explain the depletion of fibronectin in certain sites. Other reports have indicated that certain members of the plaque flora possess enzymes capable of degrading fibronectin (Wikstrom & Linde 1986). They include *B. gingivalis*, *B. intermedius*, and *B. loeschei*, which are organisms thought to predominate in more advanced diseased sites (Loesche et al. 1985, Slots 1979). The presence of degradative enzymes would explain, not only the depletion of fibronectin, but the lack of biologically active fibronectin in the samples. Thus, the degree of depletion of fibronectin from the GCF might be related to the proportions and amounts of specific micro-organisms in the crevicular flora.

One should also consider host factors that might deplete fibronectin content. The leukocyte infiltrate into the sulcus during inflammatory episodes might be responsible for proteolytic degradation of exogenous fibronectin (McDonald & Kelley 1980). In addition, depletion of fibronectin might occur during the tissue repair process (Mosesson & Amrani 1980). Fibronectin is known to bind strongly to fibrin, actin, and denatured collagen, probably mediating the non-specific opsonization of cellular debris

and its removal through phagocytic activity. Therefore, in the actively diseased gingival sulcus, tissue destruction and repair might be expected to be associated with significant amounts of fibronectin consumption.

The assays used to quantify fibronectin in the samples may bias our perception of the actual phenomenon. Our first assay, which measured the "total" fibronectin in the specimen, used an anti-fibronectin antibody to detect the fibronectin molecules. However, it is quite possible that the assay detected only fragments of fibronectin that possessed the correct antigenic epitope(s). Thus, even if the fibronectin were degraded, certain levels of fibronectin would still appear to be present, even if it were no longer biologically active. The results of the "total" fibronectin assay may have little meaning in the absence of knowing the molecular weight of the species being detected, i.e., has it been degraded to a lower molecular weight? The second assay, binding to gelatin, is a truer measure of biological activity, and indicated that there was little, if any, biologically active fibronectin in the specimens obtained from the gingival sulcus, regardless of the disease activity associated with the site.

Results of this study also indicated that plasma levels of fibronectin in gingivitis and maintenance patients were significantly reduced when compared to the control levels. There was no apparent correlation with age or sex, as has

been shown in other studies to affect plasma fibronectin levels (Stathakis et al. 1981). Various systemic diseases have been characterized by abnormal fibronectin levels in the plasma and other body fluids. For example, in synovial fluid and pannus tissue of arthritics, these levels are found to be elevated (Scott et al. 1981a; Scott et al. 1981b). Other diseases, most typically inflammatory diseases, sepsis, trauma, and severe burns, have been characterized by depressed levels of plasma fibronectin (Mosher & Williams 1978, Brodin et al. 1984, Deigaard et al. 1984, Gerdes et al. 1983, Grossman et al. 1983). In our study, depressed levels of plasma fibronectin were found in patients having gingivitis and those on maintenance recall programs, both situations where the disease activity was minimal. The basis for this relationship is unclear at this time; however, it provides impetus for further research of this phenomenon.

Zusammenfassung

Konzentration von Fibronectin im Serum und Sulkusfluid während verschiedener Stadien der Parodontalerkrankung.

Zusammenfassung: Während frühere Studien gezeigt haben, daß Fibronectin (FN) im gingivalen Sulkusfluid (GCF) vorhanden ist, wurde über seine Qualität und Quantität noch nicht berichtet. Da diese Information für laufende Studien zum Gebrauch von FN für gingivales Reattachement relevant ist, führten wir diese Messungen durch und ver-

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glichen die Plasmaspiegel von gesunden Probanden mit denen von Patienten mit Gingivitis und Parodontitis sowie von Patienten, die sich einer Erhaltungstherapie unterzogen. Die Plasma- und GCF-Proben wurden von vier Stellen bei jedem Patienten gewonnen. Ein Periortron-Gerät wurde zur Quantifizierung der Proben benutzt. Die FN-Konzentrationen wurden in einem Mikro-ELISA mittels Hyperimmun-Anti-FN-Antikörper bestimmt. Bei der Quantifizierung diente gereinigtes FN als Referenzsubstanz. Die funktionelle Aktivität jeder Probe wurde durch Untersuchung der natürlichen Bindung von FN an Gelatine bestimmt. Probanden mit Gingivitis und die mit Erhaltungstherapie hatten reduzierte Plasmalevel an Fibronectin. Während im GCF von gesunden Stellen, unabhängig von der Patientengruppe, wenig Fibronectin gefunden wurde, zeigte die Untersuchung der am stärksten erkrankten Stellen in jeder Gruppe, daß die Konzentration von FN im GCF am höchsten bei gesunden Verhältnissen und reduziert bei Vorliegen einer gingivalen Entzündung war. In keinem Fall war GCF FN biologisch aktiv.

Résumé

Teneur du sérum et du fluide gingival en fibronectine à différents stades de la maladie parodontale

La présence de fibronectine (FN) dans le fluide gingival (GCF) a déjà été mise en évidence, mais il n'y a pas comptés rendus quantitatifs ni qualitatifs. Etant donné que cette information est nécessaire pour les études en cours sur l'utilisation de FN pour obtenir la réattache gingivale, nous avons pratiqué ces mesures et comparé les niveaux plasmatiques chez des sujets en bonne santé, chez des patients avec gingivite et parodontite et chez des patients recevant un traitement de maintien. Les échantillons de plasma et de GCF ont été recueillis dans 4 sites chez chacun des sujets, en utilisant un Periortron pour permettre la détermination quantitative des échantillons. Les concentrations de FN ont été établies par une analyse microELISA avec un anticorps anti-FN hyperimmun. On utilisait une FN purifiée comme référence pour les déterminations quantitatives. L'activité fonctionnelle de chacun de ces échantillons a été évaluée en examinant l'affinité naturelle de FN pour la gélatine. Le niveau de fibronectine plasmatique était significativement réduit chez les sujets avec gingivite et chez ceux qui suivaient un traitement de maintien. Alors qu'on ne pouvait déceler que peu de fibronectine dans le GCF des sites sains, indépendamment de la catégorie à laquelle appartenait le patient, l'examen des sites les plus atteints dans chacun des groupes a montré que la santé gingivale coïncidait avec la concentration la plus élevée de FN dans le GCF, et que cette concentration était réduite lorsqu'il y avait une inflammation gingivale. La FN du GCF n'a en aucun cas été trouvée biologiquement active.

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Address:

Dennis E. Lopatin
300 North Ingalls Bldg., Room 1192SE
School of Dentistry
The University of Michigan
Ann Arbor Michigan 48109-0402
USA

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