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Morphometric analysis of collagen and inflammatory cells in periodontal disease

Morfometrijska analiza kolagena i inflamatornih ćelija u periodontalnoj bolesti

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Abstract

Background/Aim. Periodontal disease affects gingival tissue and supporting apparatus of the teeth leading to its decay. The aim of this study was to highlight and precisely determine histological changes in the gum tissue. **Methods.** Gingival biopsy samples from 53 healthy and parodontopathy-affected patients were used. Clinical staging of the disease was performed. Tissue specimens were fixed and routinely processed. Sections, 5 μm thin, were stained with hematoxylin and eosin, histochemical Van-Gieson for the collagen content, Spicer method for mast-cells and immunochemical method with anti-CD68 and anti-CD38 for the labelling of the macrophages and plasma-cells. Morphometric analysis was performed by a M42 test system. **Results.** While the disease advanced, collagen and fibroblast volume density decreased almost twice in the severe cases compared to the control ones, but a significant variation was observed within the investigated groups. The mast-cell number increased nearly two times, while the macrophage content was up to three times higher in severe parodontopathy than in healthy gingival tissue. However, the relative proportion of these cells stayed around 6% in all cases. Plasma-cells had the most prominent increase in the number (over 8 times) compared to the control, but again, a variation within investigated groups was very high. **Conclusion.** Gingival tissue destruction caused by inflammatory process leads to significant changes in collagen density and population of resident connective tissue cells. Although inflammatory cells dominated with the disease advancing, a high variation within the same investigated groups suggests fluctuation of the pathological process.

Key words:
peridontal diseases; gingiva; histological techniques; collagen; macrophages; plasma cells.

Apstrakt

Uvod/Cilj. Parodontopatija utiče na tkivo desni i potpornog aparata zuba i dovodi do njegovog propadanja. Cilj ovog istraživanja bio je da se istaknu i precizno odrede histološke promene u tkivu desni. **Metode.** Korišćeni su uzorci biopsija gingiva kod 53 zdrave osobe i osoba sa parodontopatijom. Izvršena je klinička gradacija oboljenja. Uzorci tkiva su fiksirani i rutinski obrađeni. Preparati debljine 5 μm bojeni su hematoksilin-eozin metodom, histohemijski po Van-Gizonu za sadržaj kolagena, Špicerovom metodom za mastocite i imunohemijski sa anti-CD68 i anti-CD38 za obeležavanje makrofaga i plazmocita. Morfometrijska analiza je izvršena upotrebom M42 test sistema. **Rezultati.** Dok je bolest napredovala, volumenska gustina kolagena i broj fibroblasta u teškim slučajevima smanjili su se skoro dva puta u odnosu na kontrolu, ali su uočene značajne razlike u ispitivanim grupama. Broj mastocita povećao se skoro dva puta, dok je sadržaj makrofaga bio i do tri puta veći u teškoj parodontopatiji u odnosu na zdravu gingivu. Međutim, relativni udeo tih ćelija ostao je oko 6% u svim slučajevima. Porast broja plazma ćelija bio je najizrazitiji (preko 8 puta) u odnosu na kontrolu, ali ponovo, varijacije u okviru ispitivanih grupa bile su veoma visoke. **Zaključak.** Destrukcija gingivalnog tkiva izazvana zapaljenskim procesom dovodi do značajnih promena u gustini kolagena i broju ćelija vezivnog tkiva. Iako inflamatorne ćelije dominiraju sa napredovanjem oboljenja, velike varijacije u okviru istih ispitivanih grupa sugerišu promenljivost patološkog procesa.

Ključne reči:
periodontalne bolesti; gingiva; histološke tehnike; kolagen; makrofagi; plazma ćelije.

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Introduction

Periodontal disease represents a significant health problem today and many morphological studies performed so far have yield a lot of evidence about general histological changes within gingival tissue. During the decades of the research a chain of principal pathophysiological events in the disease have been revealed which had, in essence, progressive nature. Both the tissue arrangement and the cellular composition display particular pattern during evolution of periodontal disease. In early stages, gingival inflammation predominates due to activation of involved cells and secretion of pro-inflammatory cytokines in contact with bacterial products of gingival plaque. In this phase, monocytes, macrophages and some other cells (including fibroblasts) play crucial role [1]. However, in chronic gingival lesions the cellular pattern is changed with predomination of lymphocytes, primarily of plasma-cell type [2, 3]. All these processes result in the final event, a loss of collagen, which ultimately causes the loss of gingival tissue and thoughtlessness [4, 5]. Although we know much about principal mechanism underlying the initiation and the development of gingival destruction, some issues concerning pathogenesis of periodontal disease are less investigated. The exact distribution of collagen fibers within different anatomical sites of periodontal tissue remains unresolved for fine details. Furthermore, few reports are available in the literature on *in situ* quantification of infiltrating inflammatory cells [6–8]. The role of some inflammatory cells such as macrophages, mast-cells as well as plasma-cells is investigated, but the results are somewhat controversial [9, 10]. Therefore the aim of our study was to investigate the changes in collagen distribution and quantity, the connection between volume density of inflammatory cells and the clinical stage of the periodontal disease as well as the distribution and quantification of the less investigated types of inflammatory cells – mast cells.

Methods

In the study gingival biopsy samples from 53 patients aged 14–60 years were used. Ethical approval for the research protocol was issued by the Institutional Ethics Committee and the informed consent from the study participants were obtained. The first act in this study was clinical examination performed in order to determine the condition of the periodontal apparatus. Community periodontal index of treatment needs [11], Muhlemann-Sulcus bleeding index [12] as well as assessment of the periodontal pocket depth were used to classify the patients. According to the periodontal disease classification [13] the patients were divided into four groups: the control group (12 healthy donors), the group 1 (10 patients with gingivitis), the group 2 (14 patients with moderate periodontal disease) and the group 3 (17 patients with severe periodontal disease). All gingival tissue samples were fixed in 10% buffered formalin for 48 hours, routinely processed and embedded in paraffin. Sections 5 μm thin were stained with classic hematoxylin/eosin for verification of the pathological changes, Van-Gieson for highlight of the collagen fibers and histochemical Spicer method for identification of the mast cells. Routine immunohistochemical staining was performed with anti-CD68 (Sigma-Aldrich, USA) and anti-CD38 (Dako, Denmark) antibodies for visualization of macrophages and plasma cells respectively. Quantification of collagen fibers and morphometric analysis of the labeled cells was performed using the M42 test system calibrated for proper magnification. All the values were calculated *per* unit area (1 mm^2).

The results were presented in Tables and Figures as mean ± standard deviation. Statistical analysis was performed with SPSS software. Estimation of statistical significance between the mean values was performed by independent Student's *t*-test and Mann-Whitney U-test. The level of significance of statistical differences was set at $p \leq 0.05$ with the double-sided approach.

Results

According to the design of the study, our results comprised of histological examination, characterization of collagen and morphometric analysis of fibroblasts, mast-cells, macrophages and plasma cells.

Histological examination

Microscopic examination of the human gingiva in the control group showed a well-known structure of mucous membrane. Stratified epithelium was subdivided into 3 sections: oral, sulcular and junctional. Underlying *lamina propria* showed thin and short collagen fibers in the papillary layer and thick, dense and long fibers in the reticular layer. In the group 1 only junctional epithelium showed signs of lymphocyte and polymorphonuclear leukocyte infiltration. Scarce inflammatory fields located just below this epithelium were present in, sometimes edematous, *lamina propria* with rare fragmentation and lysis of the collagen fibers. Histological characteristics of moderate and severe periodontal disease were quite different from the gingivitis cases. The increased number and extent of inflammatory lesions, lysis of collagen fibers as well as the loss of fibroblast were verified. In rare cases it was almost impossible to discern the moderate and severe form of the disease because the extent of the pathological changes was inconsistent. Nevertheless, severe forms of the disease showed confluent fields of inflammation sometimes located in the deeper gingival tissue and sometimes much nearer to the papillary layer or extended to either oral or junctional gingiva with the significant degradation of the collagen fibers. In two cases of severe illness focal destruction of the basal *lamina* with infiltration of immune cells into the gingival epithelium occurred. Fields of irregular and fairly regular re-deposition of collagen fibers by the fibroblasts were identified in two cases of severe periodontal disease which suggest reparation process. All these findings showed cyclic nature of periodontal disease, with the phases of deterioration and the phases of reparation of the gingival tissue.

Collagen fibers analysis

In the control group collagen fibers did not show any visible changes. In the gingivitis group the findings were similar, but perivascular spaces were deprived of collagen content. Slight tissue edema and the scarce regions of thinned collagen fibers were the only difference comparing with the control group. In the groups 3 and 4 a large variation of collagen content (high standard deviations) was observed and this was not so much related to the clinical stage, but mostly to the phase of the disease. It was obvious that the quantity and quality of collagen fibers were inversely related to the extent of the inflammatory lesions. In the severe cases, inside the confluent inflammatory fields, collagen fibers were absent and located only on its rim in the form of thin, dense bundles preventing the penetration of inflammatory cells into the healthy part of the gingiva (Figure 1A). Quantitative analysis (Table 1) showed a significant decrease of the collagen volume density especially in the moderate and severe cases compared to the control and the gingivitis group. Large variations of collagen content were observed in the two parodontopathy groups.

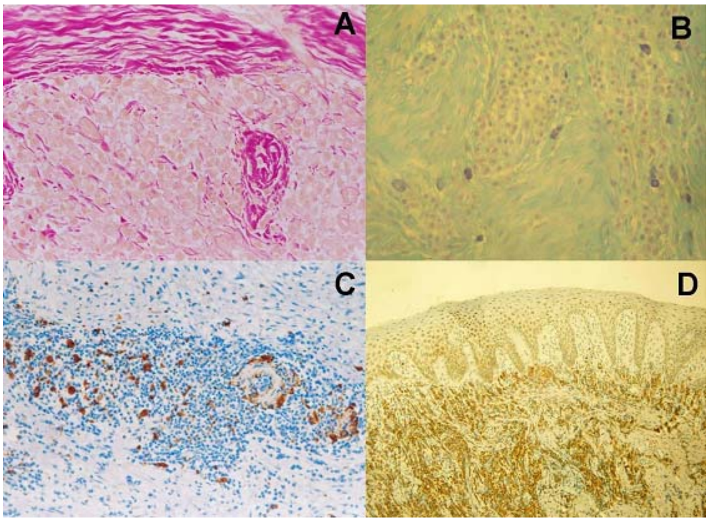

Fig. 1 – A) Bundles of collagen fibers surrounding the inflammatory focus. Inside the focus fibers are present in traces, (Van-Gieson, ×400); B) Mast cells on the periphery of the inflammatory foci (Spicer, ×400); C) Macrophages inside the inflammatory foci and around the field of collagen fibers degradation (Anti-CD68, ×200); D) Plasma-cells delineating the healthy papillary and the affected reticular layer of *lamina propria* (Anti-CD38, ×400).

Table 1

Morphometric data of collagen and inflammatory cells volume density

Variable	Healthy gingiva (n = 12)	Gingivitis (n = 10)	Moderate parodontopathy (n = 14)	Severe parodonthopathy (n = 17)
Collagen volume density	57.33 ± 5.27	46.48 ± 9.34^{1}	32.72 ± 14.59$^{1, 2}$	26.93 ± 13.71$^{1, 2}$
Fibrocytes/Fibroblasts (n/mm^2)	539.11 ± 171.34	396.77 ± 192.23^{1}	367.08 ± 264.57^{1}	303.32 ± 236.06^{1}
percent	55.22 ± 6.25	23.51 ± 9.18^{1}	15.49 ± 12.74$^{1, 2}$	13.25 ± 11.42$^{1, 2}$
Mast cells (n/mm^2)	62.56 ± 21.87	94.56 ± 27.32$^{1, 2}$	141.41 ± 31.14$^{1, 2}$	134.13 ± 29.93$^{1, 2}$
percent	6.35 ± 2.25	5.56 ± 1.76	5.97 ± 2.07	5.85 ± 2.37
Macrophages (n/mm^2)	58.74 ± 18.56	116.55 ± 54.78^{1}	147.73 ± 81.14^{1}	157.41 ± 77.56^{1}
percent	6.02 ± 0.83	6.90 ± 2.28	6.23 ± 2.48	6.86 ± 2.85
Plasma cells (n/mm^2)	77.63 ± 61.11	356.42 ± 224.84^{1}	788.53 ± 861.90$^{1, 2}$	731.34 ± 651.74$^{1, 2}$
percent	12.67 ± 4.52	21.09 ± 14.04^{1}	33.26 ± 15.49^{1}	31.93 ± 11.75^{1}
All cells (n/mm^2)	976.32 ± 284.45	1688.46.46 ± 602.41^{1}	2359.43 ± 1404.57^{1}	2289.73 ± 1345.74^{1}
percent	27.52 ± 1.16	20.71 ± 7.32^{1}	18.73 ± 9.47^{1}	16.96 ± 8.42^{1}

The values are given as mean ± standard deviation of the number or percent of the cells *per* mm^2 of gingival tissue; 1 – $p < 0.05$ *vs* healthy control; 2 – $p < 0.05$ *vs* gingivitis *vs* healthy gingiva

Distribution, number and density of fibrocytes/fibroblasts

In our study the staining method did not allow us to discern these two cell types, so they were observed as a unique cell population. In healthy gingiva fibrocytes/fibroblasts they were distributed primarily within the papillary layer while poorly present in the reticular layer. In the gingivitis group these cells can be observed mainly in the regions with preserved collagen fibers, while they were absent from the inflammatory fields. Stereological analysis of the fibrocytes/fibroblasts showed a gradual loss of their number and a significant decrease in the relative proportion of these cells in a pathologically altered gingiva (Table 1). The difference in the absolute number of fibrocytes/fibroblasts in the examined groups was not significant because of a high variation, but their differences in relative proportion (the number of fibrocytes/fibroblasts per overall number of cells) were highly significant in the parodontopathy groups compared to the control as well as gingivitis group.

Distribution, number and density of mast-cells

Mast-cells of healthy gingiva were mainly located in the perivascular spaces, almost always as a single cell. In gingivitis, the number of mast cells increased particularly within reticular layer of the *lamina propria* and around the inflammatory focuses. In the groups 3 and 4, the total number of mast cells was further increased and their main location was on the edge of the inflammatory fields (Figure 1B). Stereological and statistical analysis (Table 1) showed that the number of mast cells was significantly higher in the gingivitis group compared to healthy gingiva and also in the parodontopathy groups compared to the gingivitis group. On the other hand, their relative proportion was not changed and these cells comprised ~6% of total cells in all the examined groups (Table 1). Additionally, across all the four investigated groups mast cells remained with the similar granular content.

Distribution, number and density of macrophages

Macrophages are scattered throughout the healthy gingival *lamina propria* as rare, isolated cells with dominant location below the sulcular and junctional epithelium. In gingivitis tissue specimens they were positioned mainly between the papillary and reticular layer of *lamina propria* and they were not numerous inside the fields of inflammation. In parodontopathy cases, macrophages were always present inside the inflammatory foci, but their number was greater around the collagen fibers facilitating their degradation (Figure 1C). Only in severe cases they can be verified in the stratified epithelium, also. Stereological examination showed that, similarly to mast cells, the absolute number of macrophages was increasing with advancing of the pathological process, but relative contribution to the total cell population remained the same, around 6% (Table 1).

Distribution, number and density of plasma-cells

In healthy gingiva, plasma cells were rare, primarily distributed in small groups, around the vasculature, within reticular layer of the *lamina propria*. In gingivitis, plasma cells density was related to inflammatory foci if they were the dominant cell population. In moderate and severe cases of periodontal disease, plasma cells almost completely expel other cells within inflammed focuses, but were absent from the spaces between the foci. The papillary region and tissue near and around collagen fibers contained very rare, scattered plasma cells. When the severe inflammatory process was located deeper in the gingival lamina propria, plasma cells seemed to demarcate healthy papillary and affected reticular layer (Figure 1D). Morphometric analysis showed that the number of these cells was increased in both, absolute value and relative contribution (Table 1) in parodontopathy cases and the difference was statistically significant compared to the gingivitis and the control group. The relative increase in the plasma cell number was, in one part, in correlation with the decrease in the fibrocytes/fibroblasts number. In the other part, their relative contribution in the overall cell population was the consequence of the overgrowth of the other two types of inflammatory cells (mast-cells, macrophages). Hence, plasma-cells represented the dominant cell population of inflammatory foci in the periodontal disease.

Discussion

Parodontopathy represents a significant health problem for patients causing pain, discomfort and gums decay. Changes in gingival tissue include inflammation, collagen degradation and loss of protective function. In our study, with the progression of the disease, gingival collagen volume density decreases, with the loss of collagen fibers within inflammatory foci and its increase around the infiltrates. Our finding are similar to previous reports [4, 8, 14] and taking into account data from all studies, the mean collagen volume density in the healthy gingiva was in the range from 54% to 63%, in gingivitis cases from 38% to 46%, and in the specimens from severe parodontopathy from 25% to 27%. However, in our samples much greater variability in collagen content in the same study groups was found, approximately about 2–3 times (expressed as standard deviation from the mean) than in previous reports, being the highest in severe cases of the disease. Human and animal studies confirmed that, during the evolution of periodontal disease, alteration in collagen types occurred including collagen type I, III, IV, V and VI [14, 15]. Dynamism of these alterations in qualitative and quantitative terms could cause heterogeneity of exact collagen content in different phases of the disease. Further studies are required to better characterize the factors causing the novel observation in our study, e.g. progressive variability of collagen changes in different stages of parodontopathy.

Population of fibroblasts in our study is decreasing in both, absolute and relative numbers (relating to disease stages) and it is associated with the collagen volume density loss and the observed high variability of collagen content. Surprisingly, a few published papers dealt specifically with comparative morphometric analysis (quantification *in situ*) of these cells in the healthy gingiva and different stages of periodontal disease in humans. An old study referred equal proportion of fibroblast to other cell population in healthy gingival connective tissue [16] and our finding confirmed that report. Since then, researchers focused more on changes in fibroblast synthetic function [17, 18] and their characterization in a particular patient population such as diabetic patient with periodontal disease [19]. Seymour and Greenspan [3] described the frequent association of plasma-cells with fibroblasts suggesting that they were important in the pathogenesis of periodontal disease. Indeed, later studies confirmed the pleiotropic role of fibroblasts during the development of parodontopathy which encompassed several pieces of inflammatory cascades, key enzymatic processes in collagen synthesis and degradation and the regulation of their own life-cycle events [20].

Some studies addressed the role of mast cells in human periodontal disease, but a few of them dealt with morphometric analysis. Researchers found that the number of these cells and their degranulation is progressively increasing with advancing stages of the pathological process, placing them in the vicinity to mononuclear cells [21, 22]. However, others did not verify that findings and suggested that the mast cell population was progressively decreasing with weak migration to inflammatory foci [23]. In our specimens the absolute count of mast cell indeed increased, but in relative proportion they were greatly over-numbered by the rising population of plasma-cells. In addition, mast cells in our study were located around the inflammatory lesion showing weak histological signs of degranulation. Taking into account the presented evidence we could argue that mast cells are not the primary factor in gingival destruction pathways but, rather, contributing or secondary causes in evolution of the periodontal disease.

Morphometric analysis of macrophages in our study, in general, confirms previous findings. It is well-documented that the population of these cells increases in different pathological stages of periodontal disease and that they produced a variety of biologically active substances playing the crucial role in gingival tissue destruction and function of the other immune cells [24, 25]. In comparison to healthy gingiva, the number of macrophages increases ~2.5 times in severe parodontopathy cases and they were grouped within inflammatory foci as well as in the vicinity of collagen-destructing fibers which is similar to the results of the previous study [8]. The absolute number of these cells in our samples is, however less, compared to the mentioned report but it seems that some methodological differences could contribute to this variability. Firstly, we used manual cell counting approach instead of semi-automated imaging analysis and, secondly, our patient population is somewhat different, being, for example younger, with magnitude about a decade. Many risk factors influence the dynamics of periodontal disease including sociodemographic ones like age, gender, income and education [26]. All these factor could contribute to the heterogenity of study subjects in different researchers and consequently in results variability. The findings that the macrophage density in gingivitis was greater than in advanced periodontal destructions further documented the observed fluctuations [27].

The dominant cell population in our study are plasma cells which confirm a well-known fact about histological properties of the periodontal lesions [2, 28]. However, a few studies quantify plasma cells relative to other inflammatory cell subsets in gingival tissue during the different phases of the periodontal disease [8]. In general, evidence revealed a progressive increase in plasma cell density but in ~2 times less magnitude than in our samples. The above-discussed issue about research methods heterogeneity probably contributes to these differences. Taking into account the tissue distribution of plasma cells, we also confirmed that they are primary located within inflammatory lesions. One interesting finding is their placement in the demarcating zone between healthy papillary and affected reticular gingival tissue in the few most severe cases of disease.

Conclusion

Our study described histological and morphometric patterns of periodontal disease during its progression. The existance of severe inflammatory lesions deep in the gingival tissue could create conditions for underlying bone destruction. Integrating our findings with the existing knowledge in this topic highlights much greater fluctuations in disease onset and progression than previously thought.

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