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Interaction Between Fibronectin Fragments and Immunoglobulin G in Gingival Crevicular Fluid of Patients with Periodontal Disease

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SUMMARY

Introduction Fibronectin (FN) can interact with immunoglobulin G (IgG) molecules affecting the process of physiological elimination and causing abnormal deposition of immune complexes. The aim of the study was to analyze interaction between FN fragments and IgG molecules with different glycosylation profiles in gingival crevicular fluid (GCF) of patients with periodontal disease and healthy controls.

Material and Methods The study included 30 patients with moderate and advanced periodontitis and 22 healthy subjects. IgG and FN content in GCF were measured as well as the presence of FN and galactose expression on IgG molecules.

Results IgG content in GCF was five times higher in patients with moderate ($p<0.01$) and eight time higher in patients with advanced periodontitis ($p<0.001$) compared to healthy subjects. Also, hypogalactosylated forms of IgG were found in higher concentration in GCF of patients with advanced periodontitis compared to moderate periodontitis and healthy subjects ($p<0.05$). FN fragments of molecular mass 48 - 53 kDa were the most commonly found fragments in all three groups. Furthermore, in patients with advanced periodontitis, fibronectin fragments were attached to IgG molecules.

Conclusion IgG and FN fragments form complexes in GCF in patients with periodontal disease and healthy subjects.

Keywords: IgG; hypogalactosilation; fibronectin; gingival crevicular fluid; periodontitis

INTRODUCTION

Periodontitis is an infectious disease of bacterial etiology that leads to destruction of teeth supporting tissues and eventually teeth loss. Clinical significance of periodontal disease is high due to its influence on different organs and tissues as well as general health [1].

Periodontal disease is caused by resident microorganisms of oral cavity which pathogenic potential is expressed under certain conditions primarily when immune homeostasis is impaired [2, 3]. Progression of the disease is related to direct effect of bacteria on host tissue, as well as the activation of several autocrine and paracrine factors that amplify local inflammatory response leading to tissue damage. It has been found that patients with periodontitis produce systemic and local antibodies against bacteria causing periodontitis, unlike healthy persons that do not generally produce high level of such antibodies [4]. IgG antibodies predominate in gingival fluid of patients with periodontal disease suggesting that this class of antibodies is the most important in pathogenesis of periodontitis [5]. Also, there has been detected a change in glycoforms of IgG in oral fluids and predominant glycan molecules with reduced content of terminal galactose [6]. These IgG molecules with reduced galactose content are attributed to have proinflammatory potential and pathogenic effect in large number of inflammatory and autoimmune diseases [7, 8].

Fibronectin is multifuctional glycoprotein that in its insoluble form occurs as a component of cell surfaces, basement membrane and extracellular matrix, including extracellular matrix of periodontal tissues. In soluble form, it can be found as a constituent of plasma and other body fluids, including gingival fluid [9, 10]. Fibronectin, present in the gingival fluid, originates from plasma and connective tissue [11]. It can be attached to immunoglobulins, including IgG, under various physiological and pathological conditions in humans. Fibronectin, both in plasma and tissue is primarily attached to polymerized immunoglobulins in the form of homopolymers (aggregated Ig) or in the form of specific antigen-antibody complexes (immune complexes) [12]. The attachment is achieved via Fc region of immunoglobulin in immune complexes and IgG antibodies to the protein GroEL.

It has been shown that *A. actinomicetemcommitans* cross-reacts with fibronectin via the antigen-binding site

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[13, 14]. Localization of fibronectin in gingival tissue allows binding of immune complexes from circulation and gingival fluid *in situ* that can lead to inflammation and irreversible tissue damage [12, 15].

The aim of this study was to investigate connection between fibronectin and IgG in various glycosylated forms in the gingival fluid of patients with periodontal disease and healthy subjects.

MATERIAL AND METHODS

Patients

The study included 22 adults with healthy periodontium age 28.3 years (15 males, 7 females) in the control group and 30 adult patients with clinical signs of periodontal disease age 34.4 years (15 males and 15 females). The research was conducted at the Clinic of Periodontology and Oral Medicine, School of Dental Medicine in Belgrade. All patients received diagnosis and clinical stage of disease was determined. The diagnosis was made based on the degree of inflammation and destruction of periodontal tissues using standard clinical parameters: gingival bleeding index (GBI), gingival index (GI) Loe–Silness, plaque index (PI) Silness–Loe, depth of periodontal pockets (PDI) and the level of epithelial attachment (CAL). Based on these parameters, patients were divided into two groups: mild/moderate inflammatory changes (moderate periodontal disease) which included 14 patients and severe inflammatory changes (advanced periodontal disease) which consisted of 16 patients. Patients with moderate periodontal disease had PDI≤5 mm, CAL≤4 mm and GBI≥1 whereas patients with advanced periodontal disease had PDI>5 mm, CAL≥5 mm and GBI≥1. Healthy patients did not have any spot with CAL or PDI>3 mm, and their GBI was 0. Pregnant patients and those with certain systemic diseases known to alter immune response and glycosylation of IgG or those who received antibiotics in the last 6 months were excluded from the study. All patients signed informed consent for sample collection.

Gingival fluid

Gingival fluid was collected from four areas in the deepest pockets whereas controls were sampled from areas where PI and GBI were zero. After isolation with cotton rolls to avoid contamination with saliva and gentle air-drying supragingival plaque was removed using curettes. Gingival fluid was collected using commercial filter paper strips (Periopaper, Pro Flow, Amityville, NY, USA) that were placed for 1 minute at the bottom of the pocket or gingival sulcus in healthy subjects. Immediately after samples were collected, the volume of gingival fluid was measured using Periotron apparatus, model 600 (Harico Electronics Ltd., Canada). Then after, samples were transferred to plastic tubes filled with protease inhibitor cocktail in 1 ml of PBS (phosphate buffered saline), pH 7.2. After gentle mixing, the samples were frozen at -70°C until use.

Quantification of IgG

The content of IgG in the gingival fluid was determined by "dot blot" procedure. Samples of gingival fluid were deposited on nitrocellulose membrane (Amersham Biosciences) by vacuum aspiration in Bio Dot apparatus (Bio-Dot Apparatus, Bio Rad). IgG subclasses were identified by their reactivity with mouse monoclonal antibodies specific for human γ1, γ2, γ3 and γ4 (Nordic, Netherlands) and visualized with secondary antibody marked with peroxidase (Nordic, Netherlands), using DAB (3,3'-diaminobenzidine) as peroxidase substrate. IgG subclasses concentrations were determined by densitometric tracing of visualized samples. For standard curves, homogeneous monoclonal IgG of all four subclasses, isolated and highly purified from serum of patients with multiple myeloma were used. The total concentration of IgG was calculated as the sum of individual subclasses of IgG.

Expression, molecular localization and quantification of terminal sugar on total IgG in gingival fluid

IgG from gingival fluid was isolated by binding to Protein G Sepharose (Amersham Biosciences) from samples of healthy controls, patients with moderate and advanced periodontal disease. Lectin blotting was used for detection of galactose (Gal) and N-acetylglucosamine (GlcNAc) on heavy and light chains of IgG molecules isolated after electrophoresis of samples on polyacrylamide gel (SDS-PAGE) under reduction conditions. After protein transfer to nitrocellulose membrane, terminal galactose (Gal) and N-acetyl galactosamine (GlcNAc) were detected by biotinylated plant lectins from *Ricinus communis* (RCA-I) and *Griffonia (Bandeiraea) simplicifolia* (GS-II). The reaction was visualized using streptavidin-peroxidase. The expression of Gal and GlcNAc on heavy and light chains of IgG was evaluated using densitometric tracing of visualized samples. The total content of Gal, GlcNAc in total IgG was evaluated based on the relationship GS-II / RCA-I.

Analysis of fibronectin fragments

Western immunoblot procedure was used to determine fibronectin fragments in gingival fluid. After electrophoresis of gingival fluid samples, separated fractions were transferred onto nitrocellulose membrane and incubated with monoclonal primary/secondary antibody (conjugated peroxidase) and chloronaphtol as peroxidase substrate. For fibronectin fragments detection in gingival fluid, monoclonal antibody (MAB 1940) specific for EIIIA domain of fibronectin (Chemicon International, USA) was used. Striping process of removing primary and secondary antibodies after Western blot was used for the analysis of co-precipitation of fibronectin with IgG during isolation of IgG from gingival fluid. Further procedure was identical to that described for Western blot where isolate was incubated with monoclonal anti-fibronectin antibody.

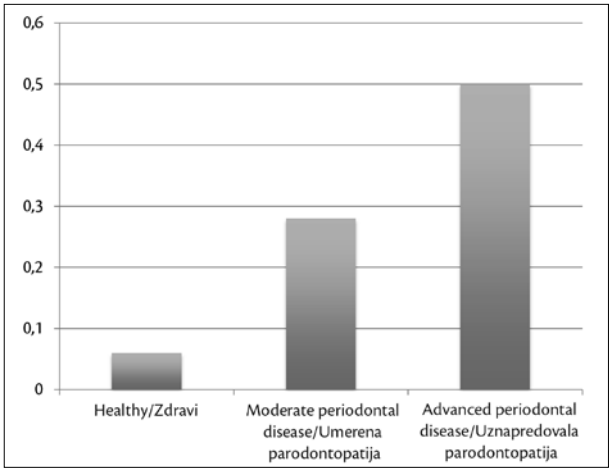
Statistical analysis

For statistical analysis Student t-test was used. Values of $p \leq 0.05$ were considered statistically significant.

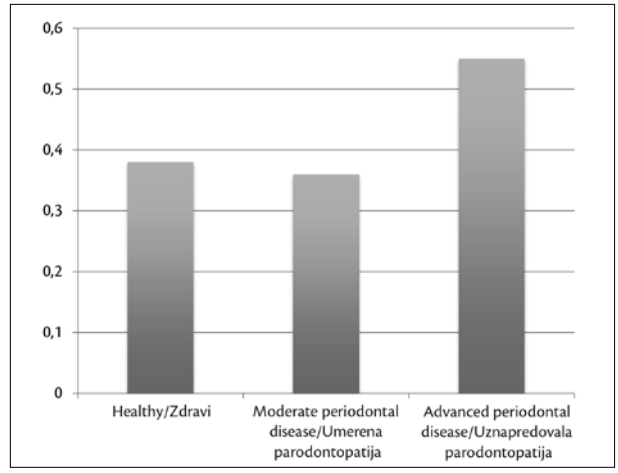
RESULTS

The total concentration of IgG in the gingival fluid of healthy subjects was 0.06±0.01 mg/ml, while in patients with moderate periodontitis it was 4.7 times higher (0.28±0.13 mg/ml). In patients with advanced form of periodontal disease the total concentration of IgG was even 8.3 times higher (0.50±0.09 mg/ml) than in healthy subjects and 1.5 times higher than in patients with moderate form of disease (Graph 1). The differences in concentrations of total IgG in the gingival fluid between healthy subjects and both groups of patients with periodontal disease were statistically significant ($p<0.01$; $p<0.001$), while statistical significance was not found between the two groups of patients (moderate vs. advanced periodontitis) ($p=0.07$).

The analysis of gingival IgG isolated by lectin immunoblot showed differences in the degree of galactosylation of total IgG between healthy controls and patients with periodontal disease, as well as between the two groups of patients with periodontal disease. Lectin relationship GS-II / RCA-I was significantly higher (50%) in the group of patients with advanced form of periodontal disease than in the group with moderate form of periodontal disease and control group (Graph 2). This result indicates reduced expression of galactose, ie. increased content of hypogalatosyled form of IgG, in the group of patients with advanced form of disease [6]. The average level of galactose in total IgG isolated from gingival fluid was significantly lower ($p<0.05$) in the group with advanced form of periodontal disease compared to other two groups.



Graph 1. Levels of IgG in gingival fluid of healthy subjects and patients with periodontitis; healthy – moderate periodontitis ($p<0.01$); healthy – advanced periodontitis ($p<0.001$)
Grafikon 1. Koncentracije ukupnih IgG u gingivalnoj tečnosti zdravih i osoba s parodontopatijom; zdrav parodoncijum – umerena parodontopatija ($p<0,01$); zdrav parodoncijum – uznapredovala parodontopatija ($p<0,001$).



Graph 2. Galactose content expressed as GS-II/RC-I ratio. Higher ratio reflects lower galactose content. Healthy – moderate/advanced periodontitis ($p<0.05$).
Grafikon 2. Sadržaj galaktoze u IgG gingivalne tečnosti izražen kroz odnos GS-II i RCA-I, gde veći odnos održava manji sadržaj galaktoze, tj. više hipogalaktozilovanih IgG; zdrav parodoncijum – umerena/uznapredovala parodontopatija ($p<0,05$).

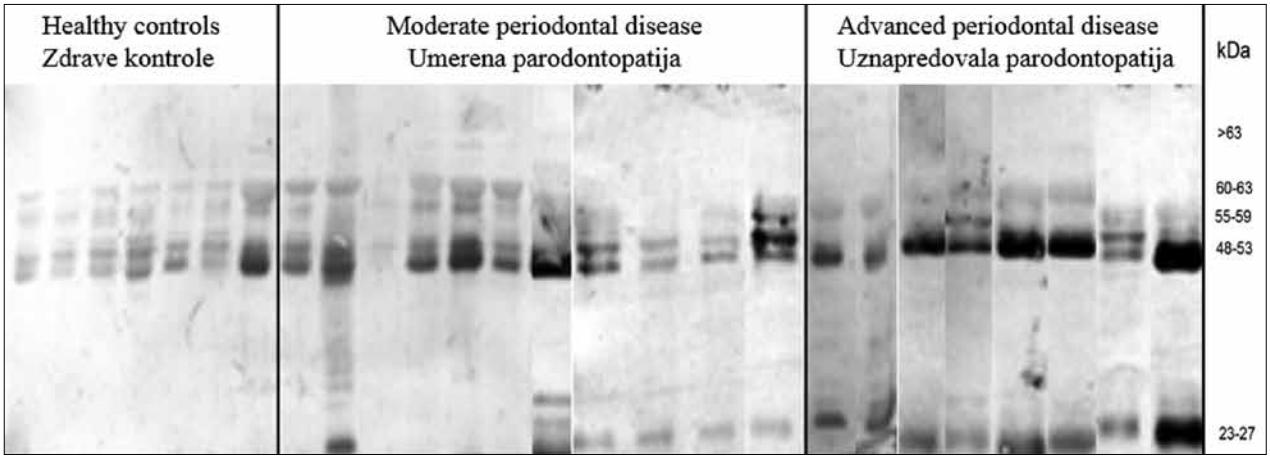
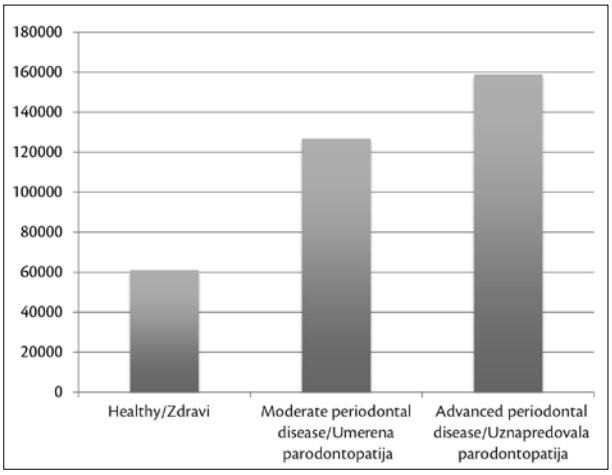
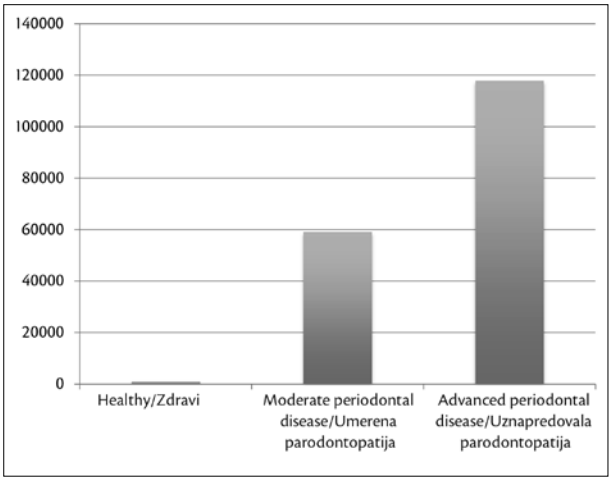


Figure 1. Western blot analysis of fibronectin fragments in gingival fluid of healthy subjects and patients with moderate and advanced periodontitis using monoclonal anti-EIIIA anti-fibronectin antibody (representative samples).
Slika 1. Western blot analiza gingivalne tečnosti pacijenata sa zdravom gingivom i pacijenata s umerenim i uznapredovalim oblikom parodontopatije, izvedena monoklonskim anti-EIIIA anti-fibronektinskim antitelom. Reprezentativni primeri obrasca raspodele fibronektinskih fragmenata za tri analizirane grupe.



Graph 3. Content of 48-53 kDa fragments in gingival fluid of healthy subjects and patients with moderate and advanced periodontitis; healthy – moderate periodontitis (p=0.02); healthy – advanced periodontitis (p=0.0005).
Grafikon 3. Sadržaj fibronektinskih fragmenata molekularne mase 48–53 kDa u gingivalnoj tečnosti zdravih osoba i ispitanika s umerenom i uznapredovalom parodontopatijom; zdrav parodoncijum – umerena parodontopatija (p=0,02); zdrav parodoncijum – uznapredovala parodontopatija (p=0,0005).



Graph 4. Content of 23-27 kDa in gingival fluid of healthy subjects and patients with moderate and advanced periodontitis
Grafikon 4. Raspodela fibronektinskih fragmenata molekularne mase 23–27 kDa u gingivalnoj tečnosti zdravih osoba i ispitanika s umerenom i uznapredovalom parodontopatijom

The application of anti-fibronectin antibody (EIIIA) detected few fractions of different molecular weight in the gingival fluid of each patient in all groups (Figure 1). It was possible to see five groups of fibronectin fragments in the following ranges of molecular weight: over 63 kDa, 60-63 kDa, 55-59 kDa, 48-53 kDa and 23-27 kDa. In addition, fibronectin fragment of molecular weight 48-53 kDa was the most common in all three groups of patients. Although 48-53 kDa fragments were detected in healthy subjects and patients with periodontal disease, the content of this fragment was different in different groups and increased with increased level of gingival inflammation. Fragments 48-53 kDa were over 2.1 times more prevalent in patients with moderate periodontal disease than in healthy controls (p=0.02), and 2.6 times more frequent in patients with advanced disease than in healthy subjects (p=0.0005). At the same time, even though its content was 25% higher in patients with advanced disease compared to patients with moderate periodontal disease, this difference was not statistically significant (p=0.4) (Graph 3). Interesting finding in the distribution of fibronectin fragments were fragments of low molecular weight of 23-27 kDa in the gingival fluid of patients with periodontal disease, which were not detected in healthy controls. Also the content of these fragments was increased in patients with advanced periodontal disease compared to moderate form (p=0.12) (Graph 4).

To analyze connection between IgG and fibronectin in the gingival fluid, anti-fibronectin antibodies were "stripped" from immunoblots and immunoblots were then incubated with monoclonal antibodies specific for heavy chains of IgG. It was shown that fractions that have previously reacted with anti-fibronectin antibodies in the range of molecular weights 48-53 kDa after stripping reacted with monoclonal antibody for heavy chains of IgG. This reaction was barely visible in the gingival fluid of healthy subjects while it was intensive in gingival fluid of patients with periodontal disease, especially those with advanced form of disease (Figure 2a). This finding indicates that IgG and fibronectin could be in some kind of non-specific association in the gingival fluid of patients with periodontal disease. This was also confirmed by analysis of IgG iso-

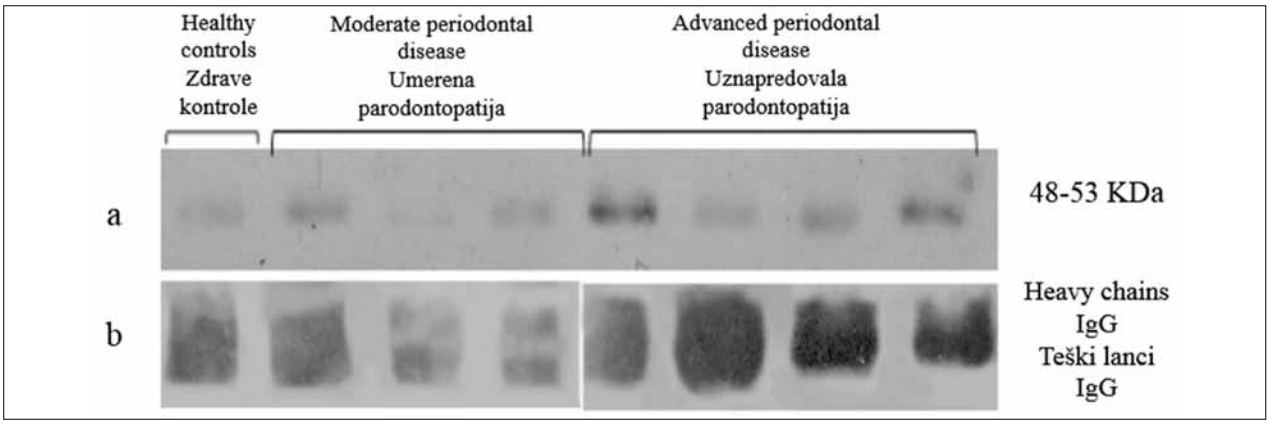


Figure 2. a) Western immunoblotting of gingival fluid samples with monoclonal anti-gamma antibodies; b) Western immunoblotting of IgG isolated from gingival fluid reactive with anti-fibronectin antibody.
Slika 2. a) Imunoblot uzoraka gingivalne tečnosti dobijen monoklonskim antigama antitelima; b) imunoblot IgG izolovanih iz gingivalne tečnosti i reaktivnih s antifibronektinskim antitelom.

lated from gingival fluid of all tested subjects. Specifically, when IgG was isolated by electrophoresis and exposed to anti-fibronectin antibody, this antibody detected fraction that corresponded to heavy chain of IgG (Figure 2b). Since precipitation on the protein G-sepharose selectively extracts only IgG from protein mixtures, reactivity of obtained isolates with anti-fibronectin antibody indicates that fibronectin co-precipitated with IgG, which is possible only if there is physical connection between these molecules.

DISCUSSION

An important aspect of glycosylation of IgG in periodontal disease may be connection between some hypogalatosyled glycoforms with structures that reflect degree of gum inflammation. One such structure is fibronectin, a multifunctional glycoprotein that exhibits reactivity with macromolecules, cells and bacteria. In our study, the degree of fragmentation of fibronectin in the gingival fluid of patients with healthy periodontium and patients with periodontal disease was assessed. Fibronectin fragments were characterized using the anti-fibronectin antibody specific for EIIIA domain. This domain characterize cellular fibronectin, therefore these fragments reflect tissue destruction [10].

The results of the current study showed that the anti-EIIIA anti-fibronectin antibody detected several fractions of different molecular weights in the gingival fluid of each patient in all groups. This indicates that local degradation of fibronectin that takes place in physiological conditions (healthy controls) is intensified in gingival inflammation (periodontitis). Talonpoika et al. [11, 16] observed that degree of fibronectin degradation in gingival fluid increased with periodontal inflammation, and decrease after the treatment is applied; therefore, it was assumed that fibronectin fragments could be of importance for periodontal disease dynamics. Fibronectin fragments found in the gingival fluid of healthy individuals most likely originate from plasma and could be formed by human leukocytes that are able to degrade fibronectin under physiological conditions [17]. As most of observed fragments were also present in the gingival fluid of patients with periodontal disease, it is possible that fibronectin degradation in gingival tissue was done by endogenous proteinases, since plasma is filtered out from circulation to periodontal pockets or gingival sulcus [16]. In periodontal pockets, fibronectin could be also degraded by proteinase of oral bacteria [18]. Fibrinogenolitic activity of oral bacteria is registered in saliva and gingival fluid, so it is possible that fibronectin degradation in gingival fluid in periodontal disease is performed by action of endogenous and exogenous proteinase [16]. On the other hand, fragments of low molecular weight, observed in gingival fluid in periodontal disease only, but not in healthy controls, could only originate from local degradation. This was confirmed in the study of Talonpoika et al. [16], who found that gingival fluid contained some smaller fibronectin fragments compared to those found in plasma. Fibronectin fragments can express different effects than unbroken fibronectin molecule, which is the reason why they are observed in periodontal disease in the context of pathophysiology of this disease [19]. Huynh et al. [10] demonstrated that fibrinogen fragments of molecular weights of 40 kDa, 68 kDa and 120 kDa are associated with severity of periodontal lesions and represent markers of the status of gingival tissue.

Fibronectin is shown to link with immunoglobulins, including IgG under physiological and pathological conditions [12, 20]. Fibronectin, in plasma and tissue is primarily linked to polymerized immunoglobulins either in the form of homopolymers (aggregated Ig) or in the form of specific antigen-antibody complexes (immunocomplexes). The purpose of this binding is unclear, but it is speculated that it may have physiological and pathological consequences. Given that fibronectin binds to the Fc region of an immunoglobulin in immune complexes, it is assumed that it mediates physiological elimination (clearance) of immune complexes from circulation [13]. On the other hand, tissue localization of fibrinogen allows *in situ* binding of immune complexes from circulation that could result in inflammation and irreversible tissue damage. Bond between immunoglobulin and fibronectin was observed in nephropathy, pulmonary fibrosis, multiple myeloma and certain autoimmune diseases [21-24]. In oral diseases this association has not been tested yet.

Our results indicate that there is bond between IgG and fibronectin in the gingival fluid of patients with periodontal disease. Those fractions that reacted with anti-fibronectin antibodies in immunoblotting also reacted with antibodies for heavy chains of IgG after stripping. The reaction occurred in the range of molecular weight of 48-53 kDa. This reaction was barely visible in the gingival fluid of healthy persons but it was intensified in the gingival fluid of patients with periodontal disease, especially those with advanced forms of the disease. This finding suggests that IgG and fibronectin could be in some kind of non-specific link in the gingival fluid of patients with periodontal disease. Such possibility was confirmed by the analysis of IgG isolated from gingival fluid of all tested subjects. Namely, when isolated IgG were contacted with anti-fibronectin antibody, this antibody detected fraction of molecular weight that corresponded to heavy chains of IgG. Since precipitation procedure from protein mixtures on the protein G-sepharose extracted only IgG, reactivity of obtained isolates with anti-fibronectin antibody indicates co-precipitation of fibronectin with IgG, which is possible only if there is physical connection between these molecules.

Pathological immunoglobulin glycosylation could be the reason for their increased adhesiveness to cell matrix proteins, and consequent tissue damage in certain pathological conditions in humans [25]. By examining the molecular basis of IgA deposition in glomeruli in IgA nephropathy, Kokubo et al. [25] showed that removal of carbohydrates from IgA1 molecules resulted in self-aggregation and increased adhesion to fibronectin. Based on these literature data, it can be concluded that glycans have protective role because they prevent accumulation of non-immunological glomerular IgA1.

CONCLUSION

The results of the current study indicate that advanced form of periodontal disease is associated with accumulation of hypogalactosyled forms of IgG attached to extracellular matrix protein, fibronectin, in the gingival fluid.

ACKNOWLEDGMENT

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Kompleksi fibronektinskih fragmenata i imunoglobulina G u gingivalnoj tečnosti osoba obolelih od parodontopatije

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KRATAK SADRŽAJ

Uvod Fibronektin može da interreaguje s molekulima imunoglobulina G (IgG) i utiče na normalan klirens ili poremećeno deponovanje imunskih kompleksa. Cilj ovog rada je bio da se ispita veza između fibronektina i IgG različitih glikoformi u gingivalnoj tečnosti osoba obolelih od parodontopatije i parodontalno zdravih ispitanika.

Materijal i metode rada U studiju je uključeno 30 pacijenata s umerenom i uznapredovalom parodontopatijom i 22 parodontalno zdrave osobe. U gingivalnoj tečnosti određivan je sadržaj IgG i fibronektina dot blot i imunoblot tehnikama. IgG iz gingivalnih tečnosti su afinitetno izolovani i analizirani na prisustvo fibronektina i ekspresiju galaktoze.

Rezultati Sadržaj IgG u gingivalnoj tečnosti osoba s umerenom parodontopatijom bio je oko pet puta veći u odnosu na sadržaj IgG kod zdravih osoba ($p<0,01$), dok je kod uznapredovalih oblika bio oko osam puta veći ($p<0,001$). Takođe, hipogalaktozilovane forme IgG su većoj meri postojale kod osoba sa uznapredovalom parodontopatijom u odnosu na zdrave i osobe s umerenom parodontopatijom ($p<0,05$). U sve tri analizirane grupe dominirali su fibronektinski fragmenti od 48 do 53 kDa. Uočeno je da su IgG izolovani iz gingivalne tečnosti vezani za fragmente fibronektina, pri čemu su IgG osoba sa uznapredovalom parodontopatijom, imali najveću količinu ovih vezanih fragmenata.

Zaključak Dobijeni rezultati pokazuju da IgG gingivalne tečnosti zdravih i osoba s parodontopatijom formiraju komplekse sa fibronektinom.

Ključne reči: IgG; hipogalaktozilacija; fibronektin; gingivalna tečnost; parodontopatija

UVOD

Parodontopatija je infektivno oralno oboljenje bakterijske etiologije u kojem dolazi do destrukcije svih potpornih tkiva zuba, što kao krajnji rezultat ima gubitak zuba. Kao takvo, ali i po posledicama koje može imati na razne organe i tkiva u organizmu i celokupno zdravlje ljudi, ovom oboljenju se pridaje veliki klinički značaj [1].

Parodontopatiju izazivaju mikroorganizmi koji se nalaze u usnoj duplji, čiji patogeni potencijal dolazi do izražaja pod određenim uslovima, pre svega u uslovima poremećene imunološke homeostaze [2, 3]. Progresija ovog oboljenja je u vezi sa direktnim efektima bakterija na tkivo domaćina, kao i s aktivacijom niza autokrinih i parakrinih faktora, koji pojačavaju lokalne zapaljenjske reakcije i dovode do oštećenja tkiva.

Kod pacijenata obolelih od parodontopatije stvaraju se sistemska i lokalna antitela na parodontopatogene, za razliku od parodontalno zdravih osoba, kod kojih se uglavnom ne stvara visok nivo takvih antitela [4]. U parodontalnim lezijama dominiraju IgG-pozitivne plazma ćelije, što ukazuje na to da su imunoglobulini klase G najznačajniji imunoglobulini u gingivalnoj tečnosti osoba obolelih od parodontopatije [5]. Za pacijente s parodontopatijom tipična je i promena glikoformi IgG u oralnim tečnostima, pri čemu preovlađuju molekuli sa glikanima u kojima je smanjen sadržaj terminalne galaktoze [6]. Ovakvim IgG molekulima, sa smanjenim sadržajem galaktoze, pripisuju se proinflamatorni potencijal i patogeni efekat u velikom broju zapaljenjskih i autoimunskih bolesti [7, 8].

Fibronektin je multifunkcionalni glikoprotein, koji se u nerastvorljivom obliku javlja kao komponenta ćelijskih površina, bazalnih membrana i vanćelijskog matriksa, uključujući i vanćelijski matriks parodontalnog tkiva, a u rastvorljivom obliku kao sastavni deo plazme i drugih telesnih tečnosti, uključujući i gingivalnu tečnost [9, 10]. Fibronektin, koji se nalazi u gingivalnoj tečnosti, potiče iz plazme i vezivnog tkiva [11]. Za fibronektin se zna da se može vezivati za imunoglobuline, uključujući i IgG, pod fiziološkim i patološkim uslovima kod ljudi. Fibronektin, kako onaj u plazmi, tako i onaj u tkivima, prevashodno se vezuje za polimerizovane imunoglobuline, bilo da su u obliku homopolimera (agregirani Ig) ili specifičnih antigen–antitelo kompleksa (imunokompleksa) [12]. Veza se ostvaruje preko Fc regiona imunoglobulina u imunokompleksima, ali je za IgG antitela na GroEL protein.

Pokazano je da *A. actinomicetemcommitans* sa fibronektinom reaguje unakrsno, što znači preko antigen-vezujućeg mesta [13, 14]. Lokalizacija fibronektina u gingivalnom tkivu omogućava vezivanje *in situ* imunokompleksa, kako onih iz cirkulacije, tako i onih u gingivalnoj tečnosti, što za posledicu može da ima zapaljenje i nepovratno oštećenje tkiva [12, 15].

Cilj ovog rada je bio da se ispita veza između fibronektina i IgG različitih glikoformi u gingivalnoj tečnosti pacijenata obolelih od parodontopatije i parodontalno zdravih osoba.

MATERIJAL I METODE RADA

Pacijenti

Ispitivanje je obuhvatilo 22 odrasle osobe sa zdravim parodoncijumom (15 muškaraca i sedam žena) prosečne starosti od 28,3 godine (kontrolna grupa) i 30 odraslih pacijenata s kliničkom slikom parodontopatije (15 muškaraca i 15 žena) prosečne starosti od 34,4 godine. Pacijenti su lečeni na Klinici za parodontologiju i oralnu medicinu Stomatološkog fakulteta Univerziteta u Beogradu, gde je postavljena dijagnoza i određen klinički stadijum bolesti. Dijagnoza je postavljena na osnovu stepena zapaljenja i oštećenja tkiva parodoncijuma korišćenjem standardnih kliničkih parametara: indeksa krvarenja gingive (IKG),

gingivalnog indeksa (GI) po Lou–Silnesu (*Löe–Silness*), plak-indeksa (PI) po Silnes–Louu (*Silness–Löe*), dubine parodontalnog džepa (DDŽ) i nivoa pripojnog epitela (NPE). Na osnovu ovih parametara, pacijenti su svrstani u dve grupe: jednu je činilo 14 ispitanika sa blagim, odnosno umerenim zapaljenjskim promenama parodoncijuma (umerena parodontopatija), a drugu grupu 16 ispitanika s izraženim zapaljenjskim promenama parodoncijuma (uznapredovala parodontopatija). Kod ispitanika s umerenom parodontopatijom utvrđene su sledeće vrednosti posmatranih parametara: DDŽ≤5 mm, NPE≤4 mm i IKG≥1. Kod ispitanika sa uznapredovalom parodontopatijom zabeležene su sledeće vrednosti: DDŽ>5 mm, NPE≥5 mm i IKG≥1. Kod zdravih ispitanika nisu uočena mesta sa NPE ili DDŽ većim od 3 mm, dok je vrednost IKG bila 0. U trenutku uzimanja uzoraka ni u jednoj grupi nije bilo trudnica, niti pacijenata s nekim sistemskim oboljenjem, za koje je poznato da imaju izmenjen imunološki odgovor i glikozilacioni status IgG, kao ni onih koji su primali antibiotsku terapiju u poslednjih šest meseci. Svi ispitanici su bili informisani o razlozima prikupljanja uzoraka i potom su dali pisani pristanak da se uzorci mogu koristiti u naučnoistraživačke svrhe.

Gingivalna tečnost

Gingivalna tečnost je sakupljana sa četiri mesta u najdubljim džepovima kod obolelih ispitanika, dok su kod zdravih uzorci uzimani s mesta na kojima su PI i IKG imali vrednost nula. Zone sakupljanja su izolovane papirnim vaterolnama, da bi se izbegla kontaminacija pljuvačkom, zatim su blago sasušene vazduhom, dok je supragingivalni plak uklonjen kiretama. Sakupljanje gingivalne tečnosti je obavljeno pomoću komercijalnih traka od filter papira (*Periopaper, Pro Flow, Amityville, NY*, SAD), koje su umetane na dno džepa ili gingivalnih sulkusa kod zdravih osoba i zatim ostavljane jedan minut. Odmah nakon uzimanja uzorka izmerena je zapremina gingivalne tečnosti aparatom Periotron, model 600 (*Harico Electronics LTD*, Kanada), a potom su uzorci preneti u plastične tube u kojima se nalazio koktel inhibitora proteaza u 1 ml PBS, pH 7,2. Posle blagog mešanja uzorci su zamrznuti na -70°C do upotrebe.

Kvantifikacija IgG

Sadržaj IgG u gingivalnoj tečnosti je određen „dot blot" postupkom. Uzorci gingivalne tečnosti nanošeni su na nitrocelulozu membranu (*Amersham Biosciences*), vakuumskom aspiracijom u Bio Dot aparatu (*Bio Dot Apparatus, Bio Rad*). IgG potklase u uzorcima su identifikovane na osnovu reaktivnosti s mišjim monoklonskim antitelima specifičnim za humane γ1, γ2, γ3 i γ4 teške lance (*Nordic*, Holandija) i vizuelizovane sekundarnim antitelom obeleženim peroksidazom (*Nordic*, Holandija), uz korišćenje DAB (3,3'-diaminobenzidina) kao supstrata peroksidaze. Koncentracije IgG potklasa su određivane denzitometrijskim trasiranjem vizuelizovanih uzoraka. Za konstruisanje standardnih kriva korišćeni su homogeni, monoklonski IgG sve četiri potklase, izolovani i visoko prečišćeni iz seruma bolesnika s multiplim mijelomom. Ukupna koncentracija IgG je izračunata kao zbir pojedinačnih potklasa IgG.

Ekspresija, molekularna lokalizacija i kvantifikacija terminalnih šećera na ukupnim IgG gingivalne tečnosti

IgG iz gingivalne tečnosti su izolovani afinitetno, vezivanjem za protein G sefarozu (*Amersham Biosciences*). IgG su izolovani iz uzoraka pacijenata kontrolne grupe, grupe s umerenom parodontopatijom i grupe sa uznapredovalom parodontopatijom. Lektinski blot je primenjen za detekciju galaktoze (Gal) i N-acetilglukozamina (GlcNAc) na teškim i lakim lancima izolovanih IgG molekula, nakon elektroforeze uzoraka na poliakrilamid gelu (SDS-PAGE) pod redukujućim uslovima. Nakon prenosa proteina na nitrocelulozu membranu, terminalna galaktoza (Gal) i N-acetil galaktozamin (GlcNAc) su detektovani biotinizovanim biljnim lektinima iz *Ricinus communis* (RCA-I) i *Griffonia (Bandeiraea) simplicifolia* (GS-II). Reakcija je vizuelizovana streptavidin-peroksidazom. Ekspresija Gal i GlcNAc na teškim i lakim lancima IgG je procenjivana denzitometrijskim trasiranjem vizuelizovanih uzoraka. Sadržaj Gal i GlcNAc u ukupnim IgG je procenjen na osnovu odnosa GS-II/RCA-I.

Analiza fibronektinskih fragmenata

Western imunoblot postupak je primenjen za određivanje fibronektinskih fragmenata u gingivalnoj tečnosti. Nakon elektoforeze uzoraka gingivalne tečnosti, razdvojene frakcije sa gelova su kapilarno prenete na nitrocelulozu membranu, zatim su uzorci inkubirani primarnim monoklonskim/sekundarnim antitelom (konjugovanom peroksidazom) i hloronaftolom kao supstratom peroksidaze. Za detektovanje fibronektinskih fragmenata u gingivalnoj tečnosti korišćeno je monoklonsko antitelo (MAB 1940) specifično za EIIIA domen fibronektina (*Chemicon International, CA*). Striping postupak (engl. *stripping*) uklanjanja primarnih i sekundarnih antitela nakon korišćenja Western blot testa primenjen je za analizu koprecipitacije fibronektina sa IgG tokom izolovanja IgG iz gingivalne tečnosti. Dalji postupak je istovetan već opisanom postupku za Western blot u kojem je izolat inkubiran monoklonskim anti-fibronektinskim antitelom.

Statistička obrada podataka

Studentov t-test je primenjen za procenu razlika između ispitivanih grupa. Vrednosti p≤0,05 smatrane su statistički značajnim.

REZULTATI

Koncentracija ukupnih IgG u gingivalnoj tečnosti zdravih osoba bila je 0,06±0,01 mg/ml, kod ispitanika s umerenom parodontopatijom 4,7 puta veća (0,28±0,13 mg/ml), dok je kod pacijenata sa uznapredovalim oblikom parodontopatije bila 8,3 puta veća (0,50±0,09 mg/ml). Kod ispitanika sa uznapredovalim oblikom parodontopatije zabeležena je jedan i po put veća koncentracija ukupnih IgG nego kod pacijenata s umerenim oblikom oboljenja (Grafikon 1). Razlike u koncentraciji ukupnih IgG u gingivalnoj tečnosti između parodontalno zdravih i obe grupe ispitanika obolelih od parodontopatije bile su statistički

značajne (p<0,01; p<0,001), dok statistički značajna razlika nije zabeležena između dve grupe obolelih (p=0,07).

Analiza izolovanih gingivalnih IgG lektinskim imunoblotom pokazala je razlike u stepenu galaktozilacije ukupnih IgG između ispitanika kontrolne grupe i ispitanika obolelih od parodontopatije, kao i između dve grupe pacijenata. Slično prethodno objavljenim rezultatima, lektinski odnos GS-II/RCA-I je bio značajno veći (za 50%) u grupi sa uznapredovalim oblikom parodontopatije u odnosu na grupu s umerenom parodontopatijom i kontrolnom grupom ispitanika (Grafikon 2), što ukazuje na smanjenu ekspresiju galaktoze, tj. povećan sadržaj hipogalaktozilovanih formi IgG, u grupi pacijenata sa uznapredovalim oblikom oboljenja [6]. Prosečan nivo galaktoze u ukupnim IgG gingivalne tečnosti bio je statistički značajno manji (p<0,05) u grupi sa uznapredovalim oblikom parodontopatije u odnosu na druge dve grupe ispitanika.

Primenom antifibronektinskog antitela (EIIIA) otkriveno je nekoliko frakcija različite molekularne mase u gingivalnoj tečnosti svakog pojedinačnog pacijenta u svim ispitivanim grupama (Slika 1). Moguće je uočiti pet grupa fibronektinskih fragmenata u sledećim rasponima molekularnih masa: više od 63 kDa, 60–63 kDa, 55–59 kDa, 48–53 kDa i 23–27 kDa. Pri tome, fibronektinski fragment molekularne mase 48–53 kDa bio je najčešći u sve tri grupe ispitanika. Međutim, iako je fragment mase 48–53 kDa ustanovljen kod zdravih i ispitanika sa parodontopatijom, sadržaj ovog fragmenta se vidno razlikovao između ispitivanih grupa i povećavao sa stepenom zapaljenja gingive. Fragment mase 48–53 kDa bio je više od 2,1 put češći kod pacijenata s umerenom parodontopatijom nego kod zdravih ispitanika (p=0,02), odnosno 2,6 puta češći kod pacijenata sa uznapredovalim oblikom oboljenja u odnosu na zdrave osobe (p=0,0005). Istovremeno, iako je njegov sadržaj bio za 25% veći kod pacijenata sa uznapredovalom u odnosu na pacijente s umerenom parodontopatijom, ta razlika nije bila statistički značajna (p=0,4) (Grafikon 3). Upadljiv nalaz u raspodeli fibronektinskih fragmenata predstavlja i pojava fragmenata male molekularne mase (23–27 kDa) u gingivalnoj tečnosti osoba obolelih od parodontopatije, koja nije otkrivena ni kod jednog zdravog ispitanika. Pri tome je sadržaj ovih fragmenata bio izraženo povećan kod pacijenata sa uznapredovalom u odnosu na one s umerenom parodontopatijom (p=0,12) (Grafikon 4).

Da bi se analizirala veza između IgG i fibronektina u gingivalnoj tečnosti ispitanika, s imunoblotova dobijenih antifibronektinskim antitelima su „skinuta" antitela (striping), a potom su blotovi inkubirani monoklonskim antitelima specifičnim za teške lance IgG. Pokazalo se da su frakcije koje su prethodno reagovale sa antifibronektinskim antitelima u domenu molekularnih masa od 48 do 53 kDa reagovale nakon stripinga sa monoklonskim antitelima na teške lance IgG. Ta reakcija je bila jedva vidljiva u gingivalnoj tečnosti zdravih osoba, da bi se intenzivirala u gingivalnoj tečnosti pacijenata s parodontopatijom, posebno onih sa uznapredovalim oblikom bolesti (Slika 2a). Ovaj nalaz pokazuje da su IgG i fibronektin mogli biti u nekoj vrsti nespecifične veze u gingivalnoj tečnosti osoba obolelih od parodontopatije. Takvu mogućnost potvrđuje i analiza IgG izolovanih iz gingivalne tečnosti ispitanika. Naime, kada su izolovani IgG nakon elektroforeze dovedeni u kontakt s antifibronektinskim antitelom, pokazano je da ovo antitelo detektuje frakciju koja prema molekularnoj masi odgovara teškim lancima IgG (Slika 2b). S obzirom na to da se precipitacijom na protein G sefarozi iz proteinske smese selektivno izdvajaju samo IgG, reaktivnost dobijenih izolata s antifibronektinskim antitelom pokazuje da je fibronektin koprecipitirao sa IgG, što je moguće jedino ako postoji fizička veza između ovih molekula.

DISKUSIJA

Važan aspekt glikozilacije IgG u parodontopatiji može biti povezanost pojedinih hipogalaktozilovanih glikoformi sa strukturama koje odražavaju stepen zapaljenja gingive. Jedna od takvih struktura je fibronektin, multifunkcionalni glikoprotein koji ispoljava reaktivnost s makromolekulima, ćelijama i bakterijama. U ovom radu je ispitivan stepen fragmentacije fibronektina u gingivalnoj tečnosti osoba sa zdravim parodoncijumom i pacijenata obolelih od parodontopatije. Pri tome su okarakterisani fragmenti fibronektina primenom antifibronektinskog antitela specifičnog za tzv. EIIIA domen. Ovaj domen reprezentuje ćelijski fibronektin, pa fragmenti koji ga sadrže predstavljaju odraz tkivne destrukcije [10].

Rezultati su pokazali da je anti-EIIIA antifibronektinsko antitelo detektovalo nekoliko frakcija različite molekularne mase u gingivalnoj tečnosti svakog pojedinačnog pacijenta u svim ispitivanim grupama. Dobijeni nalazi u celini ukazuju na to da se lokalna degradacija fibronektina, koja se odigrava i u fiziološkim uslovima (zdravi ispitanici), intenzivira i produbljuje u uslovima zapaljenja tkiva gingive (ispitanici sa parodontopatijom). Talonpoika (*Talonpoika*) i saradnici [11, 16] su zapazili da se stepen degradacije fibronektina u gingivalnoj tečnosti povećava s parodontalnim zapaljenjem, kao i da se smanjuje nakon lečenja tog zapaljenja, pa su pretpostavili da bi neki od fibronektinskih fragmenata mogli biti važni za dinamiku parodontalne bolesti. Fragmenti fibronektina koji su ustanovljeni u gingivalnoj tečnosti osoba sa zdravim parodoncijumom su najverovatnije poreklom iz plazme, a mogli su nastati delovanjem enzima humanih leukocita, za koje se zna da su u stanju da degradiraju fibronektin u fiziološkim uslovima [17]. Kako se većina uočenih fragmenata nalazila i u gingivalnoj tečnosti obolelih od parodontopatije, moguće je da se degradacija fibronektina u gingivalnom tkivu odigrala endogenim proteinazama, budući da se plazma filtrira iz cirkulacije u parodontalne džepove ili gingivalni sulkus [16]. U parodontalnim džepovima fibronektin se mogao dodatno degradirati delovanjem proteinaza oralnih bakterija [18]. Fibronektinolitička aktivnost oralnih bakterija je zabeležena u pljuvački i gingivalnoj tečnosti, pa je moguće da su u degradaciju fibronektina u gingivalnoj tečnosti u parodontopatiji bili uključeni mehanizmi delovanja i endogenih i egzogenih proteinaza [16]. Pri tome su fragmenti male molekularne mase zapaženi u gingivalnoj tečnosti ispitanika s parodontopatijom, ali ne i kod zdravih ispitanika kontrolne grupe, mogli poticati samo od lokalne degradacije. To su pretpostavili i Talonpoika i saradnici [16], koji su uočili da gingivalna tečnost sadrži neke fibronektinske fragmente koji su manji od onih koji su nađeni u plazmi. Fibronektinski fragmenti mogu ispoljiti efekte koje nemaju intaktni molekuli, što je razlog da se u parodontopatiji posmatraju u kontekstu patofiziologije ovog oboljenja [19]. Huin (*Huynh*) i saradnici [10] su pokazali da su fibronektinski fragmenti molekularne mase od 40 kDa, 68 kDa

i 120 kDa povezani s težinom parodontalnih lezija i pripisali im značaj pokazatelja parodontalnog stanja tkiva gingive.

Za fibronektin se zna da se može vezivati za imunoglobuline, uključujući i IgG, i pod fiziološkim i patološkim uslovima [12, 20]. Fibronektin, kako onaj u plazmi, tako i onaj u tkivima, prevashodno se vezuje za polimerizovane imunoglobuline, bilo da su u obliku homopolimera (agregirani Ig) ili specifičnih antigen–antitelo kompleksa (imunokompleksa). Smisao ovog vezivanja je nejasan, ali se pretpostavlja da to može imati i fiziološke i patološke posledice. Budući da se fibronektin vezuje za Fc region imunoglobulina u imunokompleksima, pretpostavlja se da on posreduje u fiziološkoj eliminaciji (klirensu) imunokompleksa iz cirkulacije [13]. S druge strane, lokalizacija fibrinogena u tkivima pruža mogućnost vezivanja *in situ* imunokompleksa iz cirkulacije, što za posledicu može imati zapaljenje i nepovratno oštećenje tkiva. Veza između imunoglobulina i fibronektina uočena je u nefropatijama, fibrozi pluća, multiplom mijelomu i nekim autoimunskim bolestima [21-24]. U oralnim bolestima ova veza nije dosad ispitivana.

Dobijeni rezultati pokazuju da postoji spoj IgG i fibronektina u gingivalnoj tečnosti pacijenata s parodontopatijom. Oni su pokazali da su frakcije koje su u imunoblotingu prethodno reagovale sa antifibronektinskim antitelima nakon stripinga reagovale i s antitelima na teške lance IgG. Reakcija se javila u domenu molekularnih masa od 48 do 53 kDa. Ta reakcija je bila jedva vidljiva u gingivalnoj tečnosti zdravih osoba, ali je bila intenzivna u gingivalnoj tečnosti pacijenata s parodontopatijom, posebno onih sa uznapredovalim oblikom bolesti. Ovaj nalaz je pokazao da su IgG i fibronektin mogli biti u nekoj vrsti nespecifične veze u gingivalnoj tečnosti osoba obolelih od parodontopatije. Takvu mogućnost potvrđuje i analiza IgG izolovanih iz gingivalne tečnosti ispitanika. Naime, kada su izolovani IgG dovedeni u kontakt s antifibronektinskim antitelom, pokazalo se da ovo antitelo detektuje frakciju koja prema molekularnoj masi odgovara teškim lancima IgG. S obzirom na to da se precipitacijom na protein G-sefarozi iz proteinske smese selektivno izdvajaju samo IgG, reaktivnost dobijenih izolata s antifibronektinskim antitelom pokazuje da je fibronektin koprecipitirao sa IgG, što je moguće jedino ako postoji fizička veza između ovih molekula.

Dobijeni nalazi mogu biti značajni ako se stave u kontekst pretpostavke da bi izmenjena glikozilacija imunoglobulina mogla biti u osnovi njihove povećane adhezivnosti za proteine ćelijskog matriksa, a time i njihovog bitnog uticaja na tkivna oštećenja u pojedinim patološkim stanjima kod ljudi [25]. Ispitujući molekularnu osnovu deponovanja IgA u glomerule u glomerulskoj IgA nefropatiji, Kokubo i saradnici [25] su pokazali da uklanjanje ugljenih hidrata iz molekula IgA1 dovodi do samoagregiranja i povećanja prianjanja za fibronektin. Na osnovu navedenih podataka iz literature, može se zaključiti da glikani imaju zaštitnu ulogu jer sprečavaju neimunološku glomerulsku akumulaciju IgA1.

ZAKLJUČAK

Dobijeni rezultati pokazuju da se uznapredovali oblici parodontopatije dovode u vezu s akumulacijom hipogalaktozilovane forme IgG za koju je vezan protein vanćelijskog matriksa, fibronektin, u gingivalnoj tečnosti.

NAPOMENA

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