Apoptotic Activity of Gingival Crevicular Fluid from Localized Aggressive Periodontitis

Huwaida Makhoul*, Jill Bashutski*, Sindhu Halubai, Darya Dabiri, Erika Benavides and Yvonne L. Kapila

***Both authors contributed equally to the manuscript**

Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI 48109-1078

Abstract

Introduction: The aim of this study was to examine a potential link between apoptotic biomarkers in gingival crevicular fluid (GCF) and periodontal destruction in four cases of localized aggressive periodontitis (LAP), diagnostically enhanced by cone beam computed tomography. **Case series:** This study examined the GCF in four patients diagnosed with LAP (formerly localized juvenile periodontitis) at a routine periodontal examination. The LAP diseased sites had attachment loss ranging from 5-12 mm. A total of 62 samples of GCF were collected from diseased sites and from contralateral, matched healthy sites with minimal or no attachment loss. All samples were assayed for apoptotic markers, including Fas/FasL, DNA fragmentation, and nitric oxide. The GCF samples were analyzed utilizing enzyme-linked immunosorbent assays for DNA fragments and nitric oxide levels, whereas Western blotting was used for Fas/FasL analyses. Our results showed a significant increase in the apoptotic markers Fas/FasL and DNA fragmentation when comparing GCF from diseased versus non-diseased sites in patients with LAP. **Conclusion:** To our knowledge, this is the first report of apoptotic biomarkers associated with patients diagnosed with LAP. Finding significantly increased levels of these markers in localized areas may help us understand the pathophysiology associated with this specific form of periodontitis, and, furthermore, may provide a basis for a quantifiably prognostic test when attempting to treat this disease.

Key words: Apoptosis; localized aggressive periodontitis; gingival crevicular fluid; biomarkers; DNA fragmentation

Introduction:

Localized aggressive periodontitis (LAP) is an uncommon condition characterized by rapid and severe attachment loss and bone resorption of the first molars and incisors (Armitage, 1999). Because of the aggressive nature of this disease, we hypothesized that high levels of inflammation and programmed cell death or apoptosis would underlie this condition.

Factors important to the pathogenesis of LAP include genetic and microbiological factors, however the role of specific host immune-mediated responses and markers of inflammation are not known. Specific markers of inflammation have been associated with periodontal disease progression and severity. One of these markers includes nitric oxide, which specifically indicates apoptosis. Nitric oxide is a free radical with a short half-life that is synthesized by a family of three nitric oxide synthase (NOS) isoenzymes, including inducible nitric oxide synthase (iNOS) (Sharma *et al.*, 2007). iNOS is generally quiescent in healthy tissues, however it is promptly expressed in response to pro-inflammatory stimuli like bacterial lipopolysaccharides (LPS) (Blix and Helgeland, 1998; Choi *et al.*, 2007; Kim *et al.*, 2005). Once iNOS is activated, large amounts of nitric oxide are produced for a period of time. Nitric oxide can function both as an apoptotic and as an anti-apoptotic molecule depending on its concentration and its interactions with other cellular molecules (Pawate and Bhat, 2006; Pichika and Homandberg, 2004). Furthermore, at high levels nitric oxide functions as a pro-inflammatory mediator (Sharma *et al.*, 2007). Also,

Correspondence to: Yvonne L. Kapila
Department of Periodontics and Oral Medicine
School of Dentistry, University of Michigan
Ann Arbor, Michigan 48109-1078
Telephone: (734) 615-2295, Fax: (734) 763-5503
E-mail: ykapila@umich.edu

high concentrations of nitric oxide have been linked to cell and tissue injury (Alexander and Damoulis, 1994; Li and Wogan, 2005). Of significance, high levels of nitric oxide and i-NOS have been reported in mechanisms of inflammation, and in patients with inflammatory diseases, including patients with periodontal disease (Alexander and Damoulis, 1994; Chan *et al.*, 2001; Choi *et al.*, 2007; Gaspirc *et al.*, 2002; Gutierrez-Venegas *et al.*, 2005; Jang and Murrell, 1998; Kim *et al.*, 2004; Lappin *et al.*, 2000; Lin *et al.*, 2007; Pichika and Homandberg, 2004; Reher *et al.*, 2007; Seminara *et al.*, 2007) and in rodent models of periodontal disease (Di Paola *et al.*, 2004; Gyurko *et al.*, 2003; Leitao *et al.*, 2005), underscoring the importance of this inflammatory mediator in the pathogenesis of periodontal disease. Despite this information, limited knowledge is available about the role of nitric oxide in aggressive forms of periodontitis.

Markers of inflammation such as nitric oxide have been valuable in assessing tissue destruction associated with periodontal disease; however, apoptotic markers have only recently been explored in this context. Furthermore, no studies have examined apoptotic markers in aggressive forms of periodontal disease. Apoptosis is a highly integrated process mediated by intricate signaling pathways to control programmed cell death. Apoptosis is central to many cell and tissue processes, including embryonic development and inflammation, and its dysregulation is associated with tumorigenesis. Apoptosis can be activated by specific cell death receptors and ligands, including the well described Fas/Fas-ligand complex (Salvesen and Dixit, 1999; Scaffidi *et al.*, 1999; Schmitz *et al.*, 2000; Thornberry, 1998). This activation process then turns on a cascade of cysteine proteases, called caspases, which induce other downstream effector proteins, including the tumor suppressor protein p53, to ultimately render apoptosis (Brozovic *et al.*, 2006; Gamonal *et al.*, 2001; Muller *et al.*, 1997). This cell death process also results in DNA fragmentation, which is another hallmark of apoptosis. Gamonal *et al.* reported that DNA fragmentation was present in biopsies from diseased periodontal tissues when compared to healthy tissues (Gamonal *et al.*, 2001). Brozovic *et al.* reported that *Porphyromonas gingivalis* can stimulate epithelial cell apoptosis via Fas-FasL up-regulation and activation of caspase-3 and caspase-8 (Brozovic *et al.*, 2006). Gamonal *et al.* (2001) found that adults with chronic periodontitis exhibited the apoptotic markers caspase-3, Fas, FasL, and p53.

It is noteworthy that an examination of the apoptotic process as sampled from the gingival crevicular fluid (GCF) in patients with aggressive periodontitis has not been undertaken, and limited data are available on this process even for chronic periodontitis cases. The aim of our study was to determine the apoptotic profile of four LAP patients, focusing on the relationship between the levels of Fas, FasL, DNA fragmentation and nitric oxide in diseased versus healthy sites.

Materials and methods

This study examined the GCF from four patients diagnosed with LAP at a routine periodontal examination. Demographic information of the patients is presented in Table 1. A diagnosis of LAP was made according to currently published diagnostic guidelines (Armitage, 1999). The LAP diseased sites had attachment loss ranging from 5-12 mm. A total of 62 samples of GCF were collected from diseased sites and from contralateral, matched healthy sites with minimal or no attachment loss.

GCF collection and processing

Gingival crevicular fluid samples were collected with sterile filter paper strips (OraflowPeriopaper® gingival fluid collection strips) for 30 seconds after cotton roll isolation of the area. GCF strips were immediately transferred to a sterile microcentrifuge tube containing 10 µl of a protease inhibitor cocktail (Protease inhibitor cocktail set II and III; Calbiochem, La Jolla, CA), and stored at -70°C until further processing. GCF was eluted with 135 µl of lysis buffer (100 µl of 1 mM phenylmethanesulphonylfluoride in 1 ml of standard radio-immunoprecipitation assay buffer).

Apoptotic cell death detection by ELISA

Histone-associated DNA fragments (mono- and oligonucleotides) were determined by Cell Death Detection ELISA^{PLUS} ELISA kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Briefly, equal volumes (20 µl) of GCF from each healthy and diseased site were placed in a streptavidin-coated microplate and incubated with a mixture of anti-histone biotin and anti-DNA peroxidase. After addition of the peroxidase substrate (ABTS), the absorbance was measured at 405 nm.

Nitric oxide determination

Equal volumes (12.5 µl) of GCF from each healthy and diseased site were mixed with an equal volume of Griess reagent (Sigma) and aliquoted into 96 well clusters and incubated at room temperature for 10 min before measuring the absorbance at 540 nm. Sodium nitrite was used as a standard to measure the amount of nitric oxide released into the GCF. Nitric oxide was expressed as micromoles of nitric oxide.

Western blot assays for Fas and FasL

Equal volumes (10 µl) of GCF from each sample were loaded onto 4-20% polyacrylamide gels and electrophoretically resolved by standard methods. After electrophoresis, gels were transferred to PVDF membranes (Immobiline-P, Millipore, Billerica, MA) by a semi-dry transfer method. The immunoblots were probed with the primary antibodies, anti-Fas rabbit

Table 1. Demographics of patients with localized aggressive periodontitis.

Patient	Gender	Ethnicity	Age (years)
1	Female	African American	45
2	Male	African American	49
3	Male	African American	36
4	Male	African American	26

antibody and anti-FasL rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), then incubated with a secondary anti-rabbit antibody conjugated to peroxidase (Santa Cruz Biotechnology). Bound antibody was detected using the West-Pico ECL detection system (Pierce Biotechnology, Rockford, IL).

Statistics

The GCF data were pooled per patient based upon disease status and the average values per patient group were calculated along with the standard deviation. A paired *t*-test was conducted to compare the diseased to healthy patients. For the DNA fragmentation assay, the absorption values versus probing depths were graphed on an individual site basis and a trend line of linear regression was chosen for the best-fit line from the scatter plot. The equation and the R squared value were calculated. For numerical data, independent sample *t*-tests were also applied. A p value of less than 0.05 was considered statistically significant.

Results

Standard periodontal intraoral photographs, complete series of digital intraoral radiographs, and cone beam computed tomography (CBCT) scans of the patients were used to aid in the diagnosis. Furthermore, clinical examination revealed that the amount of plaque and calculus on the teeth was not commensurate with the level of attachment loss noted. Additionally, previous standard full mouth radiographic series did not mirror the current advanced clinical attachment loss noted on affected teeth (data not shown). The average probing depth of the healthy sites was 2.86 ± 0.77 mm compared with an average of 6.35 ± 1.11 mm in the diseased sites.

Because rapid and aggressive tissue loss is a hallmark of LAP, we hypothesized that this robust loss of tissue may be associated with high levels of cell death or apoptosis that could be assessed by measuring DNA fragmentation, Fas and FasL, and nitric oxide levels. A paired *t*-test was conducted to compare the diseased sites to healthy sites. On average, the diseased sites had a trend towards higher values than healthy sites ($p = 0.07$; *Figure 1A*). When analyzed at the individual patient level, DNA fragmentation levels were statistically higher in diseased sites compared to healthy sites in three of four patients evaluated. DNA fragmentation ELISA data showed that the levels of DNA fragments in GCF correlated with deeper probing depths (diseased sites) in LAP subjects (*Figure 1D*). There was a weak linear correlation between DNA fragmentation and probing depths ($R^2 = 0.29$).

Similarly, nitric oxide levels were elevated in GCF derived from sites with deep probing depths that exhibited advanced attachment loss compared to contralateral healthy sites with shallow probing depths (*Figure 1B*). In one patient, there was a statistically significant elevation of nitric oxide levels in diseased versus healthy sites ($p < 0.003$); however, this difference was not statistically significant overall.

In agreement with these other apoptotic markers, Fas and FasL levels in the GCF obtained from sites with deep probing depths were significantly higher than in GCF derived from the healthy sites with shallow probing depths as assessed by Western immunoblotting (*Figure 1C*).

Discussion

This report documents for the first time that apoptotic biomarkers, including DNA fragmentation, nitric oxide and Fas/FasL, correlate with advanced diseased sites in patients with LAP. Although this is only a cross-sectional observation of four cases, it documents the association of these two processes at a site level.

Previous reports on apoptosis in periodontal disease were limited to *in vitro* examinations (Kato *et al.*, 1995; Morimoto *et al.*, 1999) and *in vivo* studies of chronic periodontitis. *In vitro* studies demonstrate that several periodontal pathogens induce apoptosis of inflammatory and host cells, which could be the source of apoptotic factors released into human GCF. *Aggregatibacter actinomycetemcomitans,* the bacterial pathogen associated with LAP, induces apoptosis of a mouse macrophage cell line, T cells, B lymphocytes, human gingival epithelial cells, and bone-like cells (Kato *et al.*, 1995; Kato *et al.*, 2000; Mangan *et al.*, 1991; Morimoto *et al.*, 1999; Ohguchi *et al.*, 1998; Okinaga *et al.*, 2007). It is interesting that the concentration of *A. actinomycetemcomitans* needed to induce apoptosis of neutrophils and lymphocytes is much higher than that needed for monocytes (Kelk *et al.*, 2003). Similarly, *P. gingivalis* induces apoptosis of lymphocytes and fibroblasts (Geatch *et al.*, 1999; Graves *et al.*, 2001; Imatani *et al.*, 2004). However, *P. gingivalis* blocks or delays apoptosis of human monocytes and polymorphonuclear leukocytes (Ozaki and Hanazawa, 2001; Preshaw *et al.*, 1999). The role of *P. gingivalis* in the apoptosis of gingival epithelial cells is contradictory

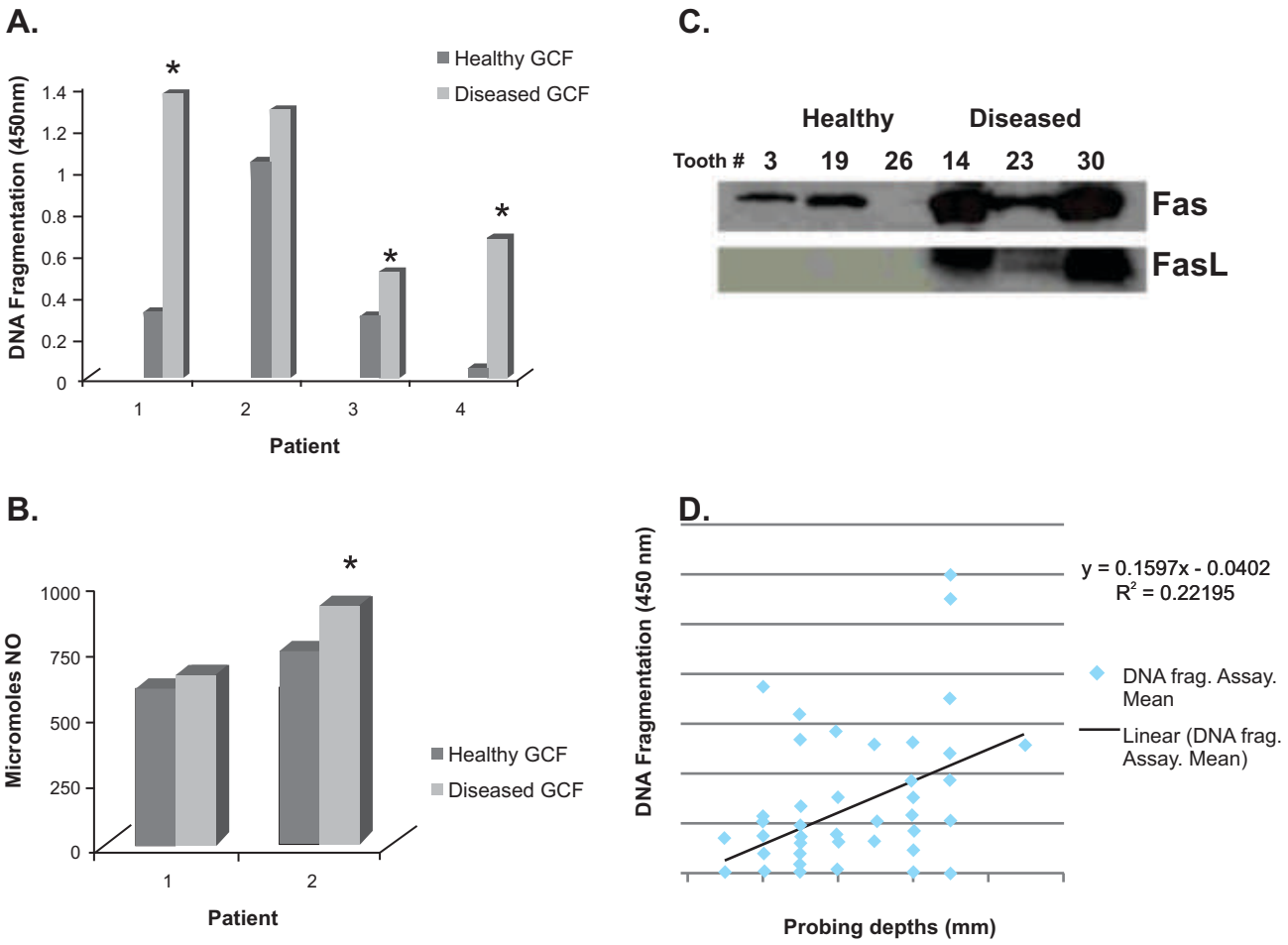

Figure 1: Apoptotic biomarkers in the gingival crevicular fluid (GCF). A. DNA fragmentation ELISA values for GCF collected from healthy and diseased GCF tooth sites from 4 patients. *Values for diseased sites were statistically higher than those for healthy sites in three of four patients with significance at a level of p < 0.003. When analyzed using a paired t-test, there was a trend towards higher levels in the diseased sites versus the healthy sites (p = 0.07). B. Nitric oxide (NO) ELISA values for GCF collected from healthy and diseased GCF sites in two patients. In one patient, there was a statistically significant elevation of nitric oxide levels in diseased versus healthy sites (p < 0.003); however, this difference was not statistically significant overall. C. Western blot analysis from a representative patient for Fas and Fas ligand (FasL) protein levels in GCF collected from tooth sites as indicated. D. Scatter plot of the correlation between DNA fragmentation values and probing depth measurements.

(Brozovic *et al.*, 2006; Yilmaz *et al.*, 2004). Other oral bacteria, including *Treponema denticola, Fusobacterium nucleatum* and *Bacteroides forsythus,* also induce apoptosis of human T lymphocytes, human peripheral blood monocytes and polymorphonuclear leukocytes, human gingival fibroblasts, and oral epithelial cells (Hasebe *et al.*, 2004; Jewett *et al.*, 2000; Lee *et al.*, 2004). Interestingly, LPS from *F. nucleatum* has not been shown to induce apoptosis of human macrophage-like cells (Grenier and Grignon, 2006). These data suggest that periodontal pathogens induce apoptosis of inflammatory and host cells, and these cells could be the source of apoptotic factors released into human GCF.

Studies that have examined apoptosis *in vivo* have focused on histological examination of tissue specimens from either mouse models of periodontal disease or inflamed gingival tissues harvested from periodontitis or gingivitis patients. A few studies have also examined human GCF and serum samples in this context. In the case of mouse models of periodontal disease, *P. gingivalis* and *E. coli* LPS induce apoptosis of inflammatory cells, fibroblasts, bone-lining cells, and periodontal ligament cells (Ekuni *et al.*, 2005; Li and Amar, 2007). Studies on human gingival biopsies from inflamed or diseased sites show apoptosis of epithelial cells, fibroblasts, junctional keratinocytes, and cells within the perivascular infiltrate (Jarnbring *et al.*, 2002; Koulouri *et al.*, 1999; Tonetti *et al.*, 1998; Vitkov *et al.*, 2005). One of these studies included tissue samples from early onset periodontitis patients, which was an

earlier term used to describe aggressive periodontitis. A study performed on peripheral blood monocytes from periodontitis patients found that approximately one third of the genes expressed by these cells were relevant to apoptosis (Papapanou *et al.*, 2007). Also, a study sampling GCF from periodontitis patients found that one of its components delayed neutrophil apoptosis (Gamonal *et al.*, 2003). Lastly, a study that sampled GCF from periodontitis patients could not detect the apoptotic markers, soluble Fas and bcl-2 in this fluid using ELISAs (Mogi *et al.*, 1999).

These *in vivo* data support the concept that apoptotic mechanisms are a feature of periodontal disease. Also, it is noteworthy that an examination of the apoptotic process as sampled from the GCF in aggressive periodontitis patients has not been undertaken, and there are limited data available on this process even for chronic periodontitis cases. Human GCF has been a focus of many studies on biomarkers as a means to understand disease pathogenesis and to aid in diagnosis. This case study is a first attempt to examine the GCF from patients with aggressive periodontitis to determine the feasibility of using apoptotic factors as biomarkers for this condition.

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