Original

IL-1 β , TNF- α , and IL-6 levels in gingival fluid and serum of patients with ischemic stroke

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Abstract: It has been documented that an increase of immune cells in periodontitis may stimulate the process of atherosclerosis and lead to ischemic stroke. The aim of this study was to investigate whether there is any significant difference in serum and gingival crevicular fluid (GCF) cytokine concentrations between patients with early ischemic stroke (IS) and a control group. Eighty-six patients were consecutively enrolled for this study, 50 with ischemic stroke and 36 as a control group with no previous stroke. Only dentulous patients were included. In each patient, GCF was collected from the deepest periodontal pocket. Blood serum samples were also taken from all subjects. The collected material was analyzed for cytokine concentrations using the cytometric bead array technique. Cerebral ischemia was found to be correlated with higher serum levels of IL-1 β (13.01 ± 13.87 vs. 7.83 ± 2.11) and IL-6 (15.82 ± 16.64 vs. 6.64 ± 2.5) compared to the control group. The levels of IL-1, IL-6 and TNF- α in GCF were higher in patients with ischemic stroke but the difference was not significant. In conclusion, ischemic stroke was found to be associated with higher levels of IL-1 β and IL-6 in serum. The cytokine concentrations in GCF did not appear to be directly correlated with those in serum in patients with ischemic stroke. (J Oral Sci 58, 509-513, 2016)

Keywords: stroke; periodontal disease; cytokines.

Introduction

Periodontal disease is a form of chronic infection considered to contribute to the development of systemic inflammation. Although it is initiated by bacteria, its pathogenesis and progression are immune-mediated. The host overacts to infectious stimulation by secreting increased amounts of pro-inflammatory cytokines, such as IL-1 β , TNF- α and IL-6, and matrix metalloproteinases (MMPs), which are tissue-destructive proteolytic enzymes (1). The locally produced pro-inflammatory mediators may enter the circulation and exert systemic effects. Increases in both the number of peripheral leukocytes and the levels of cytokines and acute-phase proteins have been found in patients with periodontal disease, but are not always significant (2,3). It has also been shown that this increase in leukocytes is aggravated by the severity and extent of the disease. Therapy may lead to a decrease in the number of leukocytes (4,5). Cytokine levels in the healthy systemic circulation are minimal and at times undetectable without the use of hypersensitive methodology (3). Pro-inflammatory mediators circulating in peripheral blood vessels may stimulate atherosclerosis or destabilize atherosclerotic plaque, thus increasing the likelihood of ischemic stroke (IS) (6). The aim of the present study was to compare cytokine concentrations in gingival fluid and serum between patients with IS and a control group matched for age and dental status.

Materials and Methods

The study subjects were 86 patients who were divided into two groups: IS patients and controls. The IS group included 50 randomly selected patients (18 women and 32 men; age range 38-96 years), who had been hospital-

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Table 1 Characteristics of the study subjects

	IS group	Control group
Gender	W 18, M 32	W 23, M 13
Age	68.29 ± 11.62	65.8 ± 10.94
Arterial hypertension (n/N)	35/46	22/36
Diabetes (n/N)	15/46	8/36
Dyslipidemia (n/N)*	17/34	4/36
Smoking (n/N)	17/43	7/36
Coronary heart disease (n/N)*	22/46	7/36
API*	96.44 ± 13.27	78.30 ± 29.61
SBI	33.42 ± 30.67	27.86 ± 28.39
Number of teeth (n)	13.14 ± 8.38	15.72 ± 8.17
Periodontal pocket depth (mm)-source of GCF	4.81 ± 1.22	5.00 ± 1.54

n = 50. **P* < 0.05; IS group vs. control group, Mann-Whitney *U* test. Data are presented as mean ± standard deviation. n: number of patients, N: total amount of patients.

ized due to IS at the Neurology Department, Medical University of Gdansk (Poland). The stroke subtype was based on the WHO criteria (7) and confirmed clinically and radiologically (CT or MRI brain scans). All tests were performed up to 48 h from the onset of IS symptoms. The control group comprised 36 individuals (23 women and 13 men; age range 40-84 years) who had been attending to undergo periodic blood tests. None of these individuals had a history of stroke. Edentulous individuals, those with autoimmune diseases, those that had received antibiotic or anti-inflammatory treatment within 1 month before the study, and those who had undergone surgical procedures in the previous 12 months were excluded. All patients or their families were interviewed and information in the available medical records was also used. The study protocol and procedures were approved by the Independent Ethics Committee of the Medical University of Gdansk (NKEBN/783/2005/2006). Written informed consent was obtained from each study participant.

Oral examination

Dental examinations of IS patients were carried out in hospital when they were semi-prone or supine. The control group was examined in a laboratory while the individuals were sitting upright. Patients were examined in the daylight using dental mirrors, and their dental and periodontal tissue status was checked, taking into consideration the number of teeth and periodontal pockets depth (PD). The presence of dental plaque (API-Approximal Plaque Index) and periodontal pocket bleeding (SBI-Sulcus Bleeding Index) was assessed, and a two-sided probe was employed for measurement of periodontal pocket depth.

GCF and serum collection

The source of gingival fluid for examination was the deepest periodontal pocket without any signs of bleeding. Gingival fluid was collected using a strip of paper (Peri-

opaper, Pro Flow, Amityville, NY, USA) and 5 ml of venous blood was collected into a glass test tube and left for 30 min. Blood samples were centrifuged at 21°C for 15 min (Eppendorff 5804 r centrifuge, Hamburg, Germany), and the serum and strips with GCF were stored at -80°C (Polar 550H, AS Biomedica, Massa Martana, Italy).

Cytokine assay

The concentrations of the cytokines IL-1 β , IL-6 and TNF- α in GCF and serum were estimated using the cytometric bead array technique (CBA) and a Human Inflammatory Cytokines Kit (Becton Dickinson Co. Franklin Lakes, NJ, USA) in the Department of Immunology, Medical University of Gdansk. The test was conducted using a Flow Cytometer LSRII (BD, Franklin Lakes, NJ, USA) and accompanying software, FacsDIVA (BD).

Statistical analysis

All calculations were performed by Statistica, version 8.0 (StatSoft, Cracow, Poland), and Microsoft Excel spreadsheets. All presented variables were compared using the Mann-Whitney *U* test and Spearman's R Correlation Test. Differences at *P* < 0.05 were considered to be significant.

Results

Study population

Table 1 shows the demographic structure of the examined population as well as the dental status and prevalence of main cardio- and cerebrovascular risk factors.

Dental characteristics

Patients with IS were characterized by a smaller number of teeth, but the difference was not statistically significant in comparison with the control group. The average depth of periodontal pockets (PDs), which was the source of GCF, did not exceed 5 mm and was similar in both the IS

Table 2 Average cytokine concentrations in GCF and serum

	<i>n</i>	IS	<i>n</i>	Control group	<i>P</i> value
GCF					
IL-1 β	42	206.29 $\pm$ 327.02	27	114.37 $\pm$ 222.58	0.873
IL-6	43	7.64 $\pm$ 3.83	27	6.76 $\pm$ 2.44	0.461
TNF- α	43	74.78 $\pm$ 108.89	27	30.31 $\pm$ 51.37	0.241
Serum					
IL-1 β *	40	13.01 $\pm$ 13.87	34	7.83 $\pm$ 2.11	0.004
IL-6*	42	15.82 $\pm$ 16.64	34	6.64 $\pm$ 2.5	0.000035
TNF- α	42	12.86 $\pm$ 7.33	34	10.26 $\pm$ 4.38	0.074

**P* < 0.05; IS group vs. control group, Mann-Whitney *U*-test. Data are presented as mean $\pm$ standard deviation. pg/mL, *n*: number of patients.

Table 3 Correlation between PD and concentrations of cytokines in GCF

PD		<i>n</i>		R Spearman	<i>P</i> value
IS					
4.81 $\pm$ 1.22	IL-1 β	42	206.29 $\pm$ 327.02	0.245	0.117
4.81 $\pm$ 1.22	IL-6	43	7.64 $\pm$ 3.83	0.168	0.279
4.81 $\pm$ 1.22	TNF- α	43	74.78 $\pm$ 108.89	-0.071	0.649
Control group					
5.00 $\pm$ 1.54	IL-1 β *	27	114.37 $\pm$ 222.58	0.416	0.030
5.00 $\pm$ 1.54	IL-6	27	6.76 $\pm$ 2.44	-0.135	0.500
5.00 $\pm$ 1.54	TNF- α	27	30.31 $\pm$ 51.37	-0.203	0.307

**P* < 0.05; PD vs. GCF cytokine concentrations, Spearman's rank correlation test. Data are presented as mean $\pm$ standard deviation, mm, pg/mL, *n*: number of patients.

and control groups (*P* > 0.05).

GCF analysis

The analysis of the GCF cytokine concentrations revealed a higher average level of IL-1 β , IL-6, and TNF- α in the IS group than in the control group, but the difference was not statistically significant (Table 2). PD was positively correlated with the concentration of IL-1 in gingival fluid in the control group (Table 3). Analysis of the cytokine concentrations in serum demonstrated significantly higher levels of IL-1 β and IL-6 in the IS group than in the control group.

Discussion

Our present study demonstrated that IS patients had non-significantly higher levels of the cytokines IL-1 β , IL-6, and TNF- α in GCF and significantly higher levels of IL-1 β and IL-6 in serum in comparison with the control group. The levels of IL-1 β and TNF- α were higher in GCF than in serum in all IS patients, and level of IL-6 was similar in GCF and serum.

A wide variety of recent studies have evaluated dental and periodontal status as risk factors for IS ischemic stroke. Roquer et al. reported that 9.7% of all patients with stroke had suffered a previous infection within the month before stroke onset, and that previous infection was more frequent in patients with IS than in those with intracerebral hemorrhage (8). Our study excluded patients who had had recent infections in order to rule out any inflammatory factors from sources other than periodontal disease. We also excluded those who had

undergone antibiotic or anti-inflammatory treatment within the month immediately to ensure that the cytokine concentrations had not been modified by medication. Thrombolytic treatment of IS may also affect cytokine levels. Chamorro et al. reported that treatment of IS patients with unfractionated heparin depressed the levels of anti-inflammatory cytokines in blood (9). We examined patients in the early state of IS who had received drugs that could have influenced the results. Age and major cardio- and cerebrovascular risk factors such as diabetes and smoking can also alter the concentrations of cytokines (10-13). However, the aim of the present study was to focus on any differences in GCF and serum cytokine concentrations between IS patients and controls. Statistical analysis showed that there were no significant inter-group differences in age, incidence of diabetes, and smoking. Patients in the early stage of IS had significantly higher API. Poor oral hygiene could have been explained by the functional disability of patients during hospitalization. SBI did not differ between the groups.

It has been documented by many authors that periodontal disease affects the levels of cytokines in gingival fluid and serum (14-16).

Lee et al. examined the concentration of IL-1, IL-6, and TNF- α in GCF. They were unable to confirm any relationship between IL-6 and TNF- α and the presence of periodontal disease. In this study the degree of alveolar bone loss was correlated with a higher level of IL-1 in GCF (17). Zaremba et al. considered that in patients with chronic periodontitis (CP) the loss of connective attachment did not necessarily impact the ability of bacteria to

migrate into blood vessels (18). More importantly, a high value of the Sulcus Bleeding Index (SBI) was considered to indicate the presence of an active inflammatory process. However, periodontal pocket depth is correlated with alveolar bone loss, which always involves activation of immune cells in response to inflammatory factors (19,20). It is also important to note that loss of connective tissue attachment does not prove disease activity, and patients examined by Zaremba et al. had mainly a chronic form of periodontal disease. Orozco et al. showed that the synthesis of IL-1 in gingival fluid increases with the progression of inflammation. Large amounts of locally synthesized IL-1 enter the bloodstream and can cause fever, as well as increased synthesis of acute-phase proteins by the liver. These authors observed a decrease in the concentration of IL-1 after the treatment of periodontal disease (16). On the basis of a study of 208 patients with IS, Grau et al. found that the severity of periodontal disease increased the risk of IS, whereas missing teeth and the presence of plaque and caries did not. They considered the risk of stroke to be higher in patients with severe periodontitis who had lost fewer than 10 teeth; periodontal disease was still associated with a higher risk of stroke in patients with more than five remaining teeth (15). A number of studies have shown that periodontal disease as a risk factor for stroke affects mainly men and individuals under 60 years of age. In the Oral Infections and Vascular Disease Epidemiology Study (INVEST), a significant association was observed between the degree of tooth loss and the prevalence of carotid artery plaque; such plaque was present in 46% of patients with 0-9 missing teeth and approximately 60% of those with more than 10 missing teeth (21). In our present study there were no significant differences in cytokine concentrations between males and females with ischemic stroke and controls and also no significant inter-group differences in the number of teeth. Chiu have stated that periodontitis can be an independent risk factor of ischemic stroke only in younger patients and men. This may be attributable to a decreasing role of periodontitis as a risk factor for ischemic stroke with increasing age and differences in health awareness between the sexes. Periodontitis has been associated with cerebral ischemia caused by large-artery atherosclerosis, supporting the hypothesis of a possible link between periodontitis and atherogenesis or complications of atherosclerosis (22).

The effect of ischemia-related cerebral tissue inflammation on the serum levels of cytokines has been challenged by Emsley et al. (23), who found that the concentrations of IL-1 β and IL-10 in patients with IS at the time of admission to hospital and 5-7 days after admission were similar

to those in a control group, and had no relationship to the status of the patients. Authors also found contribution of TNF- α in cerebral ischemia; this is controversial since our results are opposed to research of Ferrarese et al. (24). Ferrarese et al. observed an increase in the concentrations of TNF- α , IL-6, and IL-8 in serum of cerebral ischemia patients after *in vitro* activation of LPS, as compared to a control group. They suggested that the leukocytes of such patients have enhanced capability for cytokine production in response to a second stimulus (24). In contrast, Emsley et al. considered that cells in the peripheral blood do not play a significant role after cerebral ischemia and they are relatively resistant to LPS stimulation. The evidence is demonstrated by lower concentrations of IL-1 β , TNF- α , IL-6 in blood serum. Lower cytokine production is significantly associated with poorer clinical status and mortality (23). Vila et al. studied the levels of IL-10 in patients after IS and found no differences in comparison to a control group. However, they noted that the IL-10 concentration was higher in patients in a stable condition, or in those who improved within 48 hours after onset of IS (25). Elneihoum et al. determined the serum concentration of TNF- α in IS patients within 3 days after onset in comparison with that in a healthy group (26). TNF- α was found in 18% of the IS patients and 17% of the healthy subjects, and its level did not differ significantly between the groups. In our present study, the serum concentration of TNF- α was higher in the IS group; in the IS patients, the concentrations in GCF and serum were similar. According to Fassbender et al., the level of TNF- α was not increased and did not show any variations during the evolution of IS. Elneihoum et al. (26) and Fassbender et al. (27) explained the lack of any increase of TNF- α in serum of patients with cerebral ischemia as being due to high dilution of the cytokine in peripheral blood. However, this seems to contradict the increase in the serum IL-6 concentration reported by Fassbender et al. In patients with IS there are a number of accompanying factors that affect the immune system. The presence of different strains of bacteria is one factor that could modify cytokine synthesis. Tamura et al. demonstrated that LPS isolated from strains of the bacteria *Porphyromonas gingivalis* and *Prevotella intermedia* stimulated fibroblasts to synthesize cytokines to a greater degree than LPS from other bacteria (28). However, they did not examine bacterial strains from periodontal pockets and their impact on cytokine levels.

Further studies will be needed to clarify whether early treatment of periodontitis should be an element of primary and secondary prevention of IS. Also, depending on clinical status, it will also be important to incorporate

such dental treatment into the standard management of patients with IS.

Conflict of interest

The authors have no conflicts of interest to declare.

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