Original Research
Periodontics

Characteristics of neutrophil extracellular traps in patients with periodontitis and gingivitis

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Abstract: We sought to compare the characteristics and clinical significance of neutrophil extracellular traps in gingival samples from patients with periodontitis and those with gingivitis. The clinical indexes of gingival samples from patients with periodontitis and gingivitis were measured; the expression of TNF-alpha and IL-8 was measured by real-time fluorescent quantitative PCR; and the expression of TLR-8 and MMP-9 was measured by western blotting assays. Chemotaxis, phagocytosis and phagocytic activity of neutrophils were measured. Compared with the healthy group, the expression of TNF-α and IL-8 in the periodontitis group and the gingivitis group increased significantly ($p < 0.05$), and TNF-α in the gingivitis group was significantly lower than that in the healthy group ($p < 0.05$). The expression of IL-8 in the periodontitis group was significantly higher than that in the periodontitis group ($p < 0.05$). Furthermore, the expression of TLR-8 and MMP-9 in the periodontitis group was different from that in the gingivitis group and the healthy group, and the expression of TLR-8 and MMP-9 in the gingivitis group was significantly different from that in the healthy group ($p < 0.05$). In addition, the neutrophil mobility index in healthy people was 3.02 ± 0.53, that in the periodontitis group was 2.21 ± 0.13, and that in the gingivitis group was 2.31 ± 0.12. In conclusion, the chemotaxis of neutrophils in gingival samples of patients with periodontitis and gingivitis was decreased, the phagocytotic ability and activity of neutrophils were reduced, and the release of the extracellular trap-releasing inducible factors TNF-alpha and IL-8 also declined.

Keywords: Periodontitis; Gingivitis; Neutrophils; Extracellular Traps.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Periodontitis is an infectious disease caused by plaque microorganisms and their metabolites.[1] The main clinical manifestations are loss of periodontal attachments and gradual destruction and absorption of the alveolar bone.[2] In this process, the immune response to the pathogenic microorganism plays an important role. The gum is one of the periodontal tissues (the gum, periodontal ligament, alveolar bone, and cementum) and is directly exposed to the mouth and consists of keratinized epithelium and connective tissue covering the alveolar bone and roots. Gum disease is confined to the gum tissue, with the most common being chronic marginal

gingivitis. Plaque microorganisms are the initiating factor of gingivitis and periodontitis. Among them, non-adherent gingival plaque is closely related to the occurrence and development of periodontitis and is considered to be the "development frontier" of periodontitis, which is related to the rapid destruction of alveolar bone. A large number of activated immune cells not only trigger the host's immune response to periodontal pathogen infection but also release a cascade of inflammatory factors, including various acute phase reaction proteins and cytokines, leading to the destruction of periodontal soft tissue and hard tissue.[3] Neutrophils have a rod-shaped nucleus or lobulated nucleus and fine particles. The cytoplasm is colourless or reddish. It has chemotaxis, phagocytosis and bactericidal action. When the body is attacked by pathogens, neutrophils are under the action of chemokines. They quickly gather near the site of infection, enter the infected tissue through the vessel wall, and kill pathogenic microorganisms[4] by phagocytosis and the release of bactericidal particles. Neutrophil extracellular reticular traps (NETs) are a new defence mechanism,[5] and unlike apoptosis and necrosis, NETs release a variety of bactericidal substances to remove pathogenic microorganisms, including neutrophil primary granule release, such as protease or secondary particle bacteriostatic peptide LL-37, etc.; tertiary particles include protease-containing matrix metalloproteinase 9 (MMP-9), etc.[6,7] Many factors can stimulate neutrophils to produce neutrophil extracellular traps. For example, plasma TNF-α and interleukin-8 can also stimulate neutrophil release of NETs.[8,9]

Methodology

General information

Between July 2015 and September 2016, we randomly selected 27 patients (14 males and 13 females) with periodontitis (CP), 17 patients (7 males and 10 females) with gingivitis (CG) and 20 patients (8 males and 12 females) with no periodontal disease (PH). Subjects were 24–60 years old with an average age of 33.24 ± 6.93 years. Periodontitis and gingivitis were diagnosed according to the diagnostic criteria of the Periodontal Disease Association.

The inclusion criteria were as follows: at least 20 teeth in the mouth, 4–6 teeth with severe periodontitis in all patients with periodontitis; no periodontal treatment in the preceding 6 months; no smoking history in the preceding 6 months; no history of head and neck radiotherapy; no antibiotics, phenytoin sodium, cyclosporine, calcium channel blockers, oral contraceptives, atropine and other drugs within the month; and no serious systemic diseases or serious infections in other areas. Pregnant or lactating women were excluded. All study participants provided informed consent.

Experimental methods

Determination of clinical indicators

The researchers who received professional training in periodontal examination and passed the standard conformance test (Kappa = 0.73) used the Williams probe to detect periodontal clinical indicators in all subjects, including the gingival index (GI), detection of bleeding on probing (BOP), depth of detection (PD), and clinical attachment level (CAL). Among them, PD and CAL were detected at 6 sites on the buccal side and at the distal, middle and proximal sites of the lingual side.

Collection of peripheral blood samples

Two millilitres of peripheral venous blood from all subjects was extracted and placed in an anticoagulation tube containing K2EDTA. Peripheral blood samples were collected by centrifugation (4,000 r/min, 6 min) at 4°C. The supernatant was placed in a sterile Eppendorf tube and stored at -80°C until use.

Extraction of total RNA from peripheral blood samples and RT-PCR determination

One millilitre of TRIzol was added to the serum and repeatedly pipetted, and the cellular lysate was transferred to a 1.5-mL Eppendorf tube, left for 5 min, and fully lysed. Two hundred microlitres of chloroform was added per 1 mL of TRIzol, mixed for 15 s, and left at room temperature for 3 min. After centrifugation at 12,000 *g* for 15 min at 4°C, the upper aqueous phase was aspirated and transferred to another new Eppendorf tube. Then, 0.5 mL of isopropanol was added per 1 mL of TRIzol, mixed, and left at room

temperature for 10 min. After centrifugation at 42,000 *g* at 4°C for 10 min, the supernatant was discarded, and RNA was precipitated on the bottom of the tube. One millilitre of 75% ethanol was added per 1 mL of TRIzol and mixed vigorously. After centrifugation at 4°C at 7,500 g for 5 min, the supernatant was discarded. RNA was precipitated at room temperature for 5–10 min, dissolved in 30 μL of EPC-treated water, and stored at -80°C for use. RNA purity and concentration were measured by spectrophotometry at A260 and A260/280. Total RNA was extracted using a total RNA rapid extraction kit and reverse transcribed with Super M-MLV reverse transcriptase using miRNA-specific reverse transcription primers (Table 1). RT-PCR was performed in a real-time quantitative system. The amplification conditions are shown in Table 2, the primer design is shown in Table 3, and the reaction system is shown in Table 4.

Table 1. Reverse transcription system.

Reagent	Concentration
5×PrimeScript Buffer	4 μL
1×PrimeScript RT Enzyme Mix I	1 μL
Oligo dT Primer (50 μM)	1 μL (25 pmol)
Random 6 mers (100 μM)	1 μL (50 pmol)
Total RNA	L μL
RNase Free dH2O	12 μL

Table 2. PCR conditions.

Step	Temperature	Time	Frequency
Pre-denaturation	95°C	10 min	
Transsexual	94°C	30 s	40 cycles
Annealing/extension	60°C	1 min	
Melting curve analysis			
1	95°C	15 s	
2	60°C	1 min	
3	94°C	15 s	
4	60°C	15 s	

Western blotting assays

The expression of TLR-8 and MMP-9 in cells was detected by western blotting assays. The protein was resolved by 4%–10% polyacrylamide gel electrophoresis, and the gel was transferred to a PVDF membrane using SDS polyacrylamide gel electrophoresis. After blocking with 5% non-fat dry milk for 1 h at room temperature, the membrane was first incubated with 1:500 diluted primary antibody overnight, and after washing with TBS-T buffer, the membrane was incubated with secondary antibody for 1 h. Following rinsing with TBS-T solution, enhanced chemiluminescence was carried out. The PVDF membrane was placed in a developing solution in a dark room and exposed to an X-ray film. Protein densitometry was performed using Image 1.6 software.

Preparation of leukocyte suspension

After extracting 3 mL of venous blood from all subjects, we used heparin as an anticoagulant to precipitate red blood cells, and white blood cells were taken. After washing, a 1×10^6/mL white blood cell suspension was prepared in RPMI 1640 containing 10% bovine serum.

Determination of neutrophil chemotaxis

Using the agarose glass plate method, agarose solution was prepared with 1640 solution, and after the addition of 10% inactivated AB serum, the glass plate

Table 4. PCR system.

Reagent	Concentration
5×PrimeEx Taq TM II (2×)	12.5 μL
PCR forward primer (10 μM)	1 μL (0.4 μM)
PCR reverse primer (10 μM)	1 μL (0.4 μM)
DNA template	2 μL
dH2O (sterilized distilled water)	8.5 μL
Total	25 μL

Table 3. Sequences of primers used in the study.

RNA	Upstream	Downstream
TNF-α	5′-TGTTCTTACACCCCCTCCCCTTTT-3′	5′-TATAAGTGTCAGCCGGCTGAGAA-3′
IL-8	5′-GGCCGAUUGUGAACAUGGATT-3′	5′-UCCAUGUUCACAAUCGGCCGC-3′
β-actin	5′-TGGCACCCAGCACAATGAA-3′	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

was poured. Each group consisted of three circular holes arranged in a straight line with a diameter and a pitch of 2.5 mm. Five microlitres of a white cell suspension (1×10^6/mL) was added in triplicate to the mesopores, *Escherichia coli* was added to the side wells for 24 h, 5 μL of the filtrate was used as the chemokine, and the other side was treated with 5 μL of the 1640 solution. The agarose glass plate was placed in a 37°C incubator, incubated with saturated humidity and 5% CO_2 for 4 h, removed, and fixed with methanol and formaldehyde. After agarose gel electrophoresis, the gel was stained with Wright and measured under an optical microscope. The distance a cell moved from the edge of the hole to the hole on either side was measured.

Determination of the phagocytic function of neutrophils

Staphylococcus aureus was grown on agar slants for 24 h, colonies were washed with sterile isotonic saline, washed twice with PBS, and suspended at 5×10^7/ml by the specific concentration method. Three drops of heparinized blood were placed on the concave slide, and three drops of *Staphylococcus aureus* suspension were added. After thorough mixing, they were placed in a sealed wet box. After incubation at 37°C for 30 min, the box was shaken once every 10 min; 1 drop was taken with a capillary suction tube, pushed onto a slide on a glass slide, fixed in methanol, stained with Giemsa, and counted by light microscopy.

Data processing

Data are expressed as the mean ± standard deviation. One-way analysis of variance was used for comparisons between groups. $p < 0.05$ was considered to indicate a significant difference.

Results

Clinical indicators

The clinical indicators in the groups are shown in Table 5. The periodontal clinical indexes of periodontitis and gingivitis samples were significantly higher than those of healthy subjects ($p < 0.05$). Comparison of periodontitis samples with gingivitis samples showed significant differences in the blood index, depth of detection and CAL ($p < 0.05$) but no difference in the gingival index ($p > 0.05$).

RNA expression measurement results

The purity of extracted total RNA was measured by agarose gel electrophoresis, and the results are shown in Figure 1. As seen from the figure, RNA was of high purity and can be used for PCR amplification.

The expression levels of RNA in each group were measured by RT-PCR. The results are shown in Table 6. The results showed that the expression of TNF-alpha

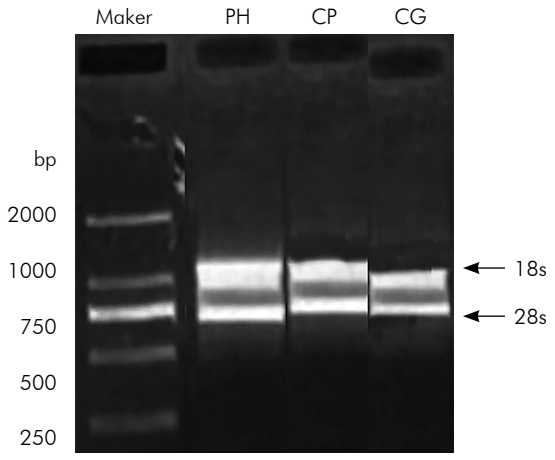

Figure 1. RNA agarose gel electrophoresis.

Table 5. Clinical indicators of the study population.

Group	Periodontal index			
	GI	BOP positive (%)	PD	CAL
PH	0	0	1.66 ± 0.21	1.66 ± 0.21
CP	2.25 ± 0.23	37.43 ± 0.21	4.34 ± 0.30	4.91 ± 0.20
CG	2.26 ± 0.15	28.34 ± 0.22	2.25 ± 0.12	2.21 ± 0.13
F	49.932	50.043	29.943	48.039
p-value	< 0.05	< 0.05	< 0.05	< 0.05

and IL-8 in the periodontitis group and the gingivitis group increased significantly compared with the healthy control group ($p < 0.05$), the expression of TNF-alpha in the gingivitis group was significantly lower than that in the periodontitis group ($p < 0.05$), and the expression of IL-8 was significantly higher than that in the periodontitis group ($p < 0.05$).

Western blotting results

The plasma expression levels of TLR-8 and MMP-9 were determined by western blotting assays. The results are shown in Figure 2 and Table 7. The expression of TLR-8 and MMP-9 in the periodontitis group was different from that in the gingivitis group and the healthy control group, and the difference between the gingivitis group and the healthy control group was significant ($p < 0.05$). The expression of TLR-8 and MMP-9 in the periodontitis group was higher than that in the gingivitis group, but there was no significant difference ($p > 0.05$). This result indicated that the neutrophil chemotactic function in patients with periodontitis or gingivitis was significantly lower than that in healthy subjects.

Neutrophil chemotaxis

The neutrophil shift index was 3.02±0.53 in healthy subjects, 2.21 ± 0.13 in the periodontitis group, and 2.31 ± 0.12 in the periodontitis group (Table 8). There were significant differences between the periodontitis group and the gingivitis group and the healthy control group ($p < 0.05$, $p < 0.05$). This indicated that the chemotactic function of neutrophils in patients with periodontitis and gingivitis is significantly lower than that in healthy subjects.

Determination of neutrophil phagocytosis

The phagocytic ability and activity of neutrophils were 392.03 ± 20.04 and 89.34 ± 10.12, respectively, in healthy subjects; 360.32 ± 18.23 and 80.02 ± 10.21, respectively, in the periodontitis group; and 359.32 ± 20.11 and 78.93 ± 17.32, respectively, in the gingivitis group (Table 9). There were significant differences between the periodontitis group and the gingivitis group and the healthy control group ($p < 0.05$, $p < 0.05$). The results showed that the phagocytic ability and activity of neutrophils in patients with periodontitis and gingivitis were significantly lower than those in healthy subjects.

Table 6. RNA expression measurement results.

Group	TNF-α (ng/mL)	IL-8 (ng/mL)
PH	0	0
CP	40.21 ± 10.03	102.32 ±1 5.34
CG	26.31 ± 5.02	114.92 ± 20.11
F	49.323	59.182
p-value	< 0.05	< 0.05

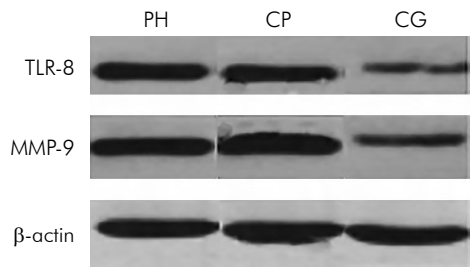

Figure 2. Western blotting results.

Table 7. TLR-8 and MMP-9 protein expression levels.

Group	TLR-8	MMP-9
PH	0.91 ± 0.04	0.89 ± 0.12
CP	0.89 ± 0.03	0.82 ± 0.04
CG	0.45 ± 0.11	0.52 ± 0.02
F	89.043	93.045
p-value	< 0.05	< 0.05

Table 8. Neutrophil movement index results.

Group	Mobile index
PH	3.02 ± 0.53
CP	2.21 ± 0.13
CG	2.31 ± 0.12
F	90.485
p-value	< 0.05

Table 9. Neutrophil phagocytosis results.

Group	Phagocytic ability	Phagocytic activity
PH	392.03 ± 20.04	89.34 ± 10.12
CP	360.32 ± 18.23	80.02 ± 10.21
CG	359.32 ± 20.11	78.93 ± 17.32
F	89.043	93.045
p-value	< 0.05	< 0.05

Discussion

Periodontal disease is a chronic inflammatory disease of the tooth-supporting tissue caused by plaque infection, including gingivitis and periodontitis. The main manifestation is periodontal support tissue destruction.[10]

In 2004, Warnatsch et al.[11] first proposed another method for killing pathogenic microorganisms, namely, neutrophil extracellular reticular traps (NETs), which differ from apoptosis and necrosis. NETs are closely related to human diseases and play a unique role in the development of disease.[12,13] In most cases, the production of NETs is conducive to the body's innate immunity,[14] but recent studies have found that the network is also involved in the pathological process of human disease.[15] When a mesh is produced, a large amount of autoantigens[16] are produced. If the removal is not timely, a large amount of antigens will accumulate, which may induce the body to produce a large amount of autoantibodies, leading to autoimmune diseases. Neural network research focuses on infectious diseases and autoimmune diseases, but it is also closely related to thrombotic diseases (acute myocardial infarction).[17,18]

NETs are a newly discovered neutrophil bactericidal mechanism that activates neutrophils to release chromatin and then combine with the gingival sulcus to form a new defence network against gingival plaque bacteria.[19] It has been reported[20,21] that under the induction of IL-8, LPS and interferon, the neutrophil nucleus is deformed, the chromatin is homogeneous, the nuclear membrane is broken, the nuclear material is directly in contact with the particles, forming a mixture, and finally the cell membrane is broken and NETs are released. This study found that compared with the healthy control group, the expression of TNF-α and IL-8 in the periodontitis group and the gingivitis group was significantly increased ($p < 0.05$), and TNF-α in the gingivitis group was significantly lower than that in the periodontitis group. The expression of IL-8 was significantly higher in the control group ($p < 0.05$) than that in the periodontitis group ($p < 0.05$).

MMP-8 secreted by neutrophils, also known as neutrophil collagenase, is one of the most important MMPs in inflammatory periodontal tissues. Type I collagen fibres are the main components of the periodontal ligament and alveolar bone. Studies have shown that the main role of MMP-8 is to destroy type I collagen fibres. MMP-8 is also present in normal periodontal tissues. In the absence of inflammation, the ratio of MMP-8 and its endogenous inhibitor (TIMP-1) is close to 1:1, and it is involved in normal metabolism of the extracellular matrix. However, when pathogens invade, the expression of MMP-8 is abnormally regulated by cytokines, which disrupts the balance between MMP-8 and TIMP-1, leading to damage to type I collagen in periodontal tissues. This study showed that there was a difference in the expression of TLR-8 and MMP-9 between the periodontitis group and the gingivitis group and the healthy control group, and the difference between the gingivitis group and the healthy control group was significant ($p < 0.05$). The expression of TLR-8 and MMP-9 in the periodontitis group was higher than that in the gingivitis group, but there was no significant difference ($p > 0.05$), indicating that the chemotactic function of neutrophils in patients with periodontitis and gingivitis is significantly lower than that in healthy controls.

Neutrophils are important cells for preventing periodontal infection. The study on the role of neutrophils in juvenile periodontitis shows that chemotaxis of neutrophils decreases and the phagocytosis function of neutrophils is normal. This report proves that the weakening of neutrophil chemotaxis makes juvenile patients with periodontitis susceptible to specific local bacteria and causes rapid destruction of the periodontal tissue.[22] This study indicated that there were significant differences between the periodontitis group and the gingivitis group and the healthy control group ($p < 0.05$). The results showed that the neutrophil chemotactic function of patients periodontitis and gingivitis was significantly lower than that of healthy controls. There were significant differences between the periodontitis group and the gingivitis group and the healthy control group ($p < 0.05$, $p < 0.05$). The results showed that the phagocytic ability and activity of neutrophils in patients periodontitis and gingivitis were significantly lower than those in healthy controls.

In summary, the chemotaxis of neutrophils in gingival samples of periodontitis and gingivitis decreased, the phagocytosis ability and activity of neutrophils were reduced, and the release of extracellular trap-releasing inducible factors TNF-alpha and IL-8 declined.

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