J Clin Periodontol 2004; 31: 1110–1116 doi: 10.1111/j.1600-051X.2004.00612.x

Journal of Clinical Periodontology

Alterations of gene expression in human neutrophils induced by smoking cessation

Toshiya Morozumi, Takehiko Kubota, Noriko Sugita, Manami Itagaki and Hiromasa Yoshie

Division of Periodontology, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Morozumi T, Kubota T, Sugita N, Itagaki M, Yoshie H: Alterations of gene expression in human neutrophils induced by smoking cessation. J Clin Periodontol 2004; 31: 1110–1116. doi: 10.1111/j.1600-051X.2004.00612.x. © Blackwell Munksgaard, 2004.

Abstract

Objective: The purpose of the present study was to investigate the effect of smoking cessation on the peripheral neutrophil mRNA expression levels for inflammatory cytokines, chemokine, growth factor and matrix metalloproteinase (MMP).

Material and Methods: Sixteen male smokers (aged 22–39 [25.3 ± 4.0] years), with no clinical signs of periodontal and systemic diseases, were recruited. The experiment was performed before (baseline) and at 1, 4 and 8 weeks after smoking cessation. The status of smoking and smoking cessation was verified by exhaled carbon monoxide (CO) concentration and serum cotinine concentration. Neutrophils were isolated from each subjects' peripheral blood, then the cell was stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). The mRNA expression levels for interleukin (IL)-1β, IL-8, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF) and MMP-8 were analyzed by semiquantitative reverse transcription-polymerase chain reactions. The same experiment was performed on 11 non-smoking controls (four female and seven male), aged 23–27 (24.4 ± 1.2) years.

Results: Eleven of 16 smokers successfully completed smoking cessation for 8 weeks. At 1 day after smoking cessation, there was a statistically significantly lower CO concentration than at baseline ($p<0.01$). Also, cotinine concentration markedly decreased at the second measurement, which was taken at 1 week. All of the analyzed mRNA expression levels of neutrophils from smokers were statistically significantly lower than that in non-smokers ($p<0.01$: IL-1β, IL-8, VEGF; $p<0.05$: TNF-α, MMP-8). The MMP-8 mRNA levels were statistically significantly increased at 8 weeks after smoking cessation compared with the baseline ($p<0.05$). Although the other mRNA expression levels were also elevated gradually from the baseline, they did not reach the statistically significant levels at 8 weeks after smoking cessation.

Conclusion: The results showed that the neutrophil transcript levels in smokers were generally lower than those in non-smokers, which could be related to an impairment of neutrophils by smoking effects. The significant increase of MMP-8 mRNA levels were associated with the effects of smoking cessation, while recovery of the other mRNA levels seemed to require a bit longer period beyond 8 weeks after smoking cessation.

Key words: gene expression; neutrophils; smoking cessation

Accepted for publication 15 March 2004

Cigarette smoking is the leading cause of several systemic diseases including cancers, pulmonary, cardiovascular and gastrointestinal diseases (Bartecchi et al. 1994). There is further evidence that smoking is one of the most significant risk factors in the development and progression of periodontal disease (Bergström & Preber 1994). Also, several clinical and epidemiological studies indicate that smokers respond poorly to a variety of periodontal therapies (Rivera-Hidalgo 2003). Therefore, quitting smoking is absolutely essential for the prevention of diseases, health enhancement and improving the healing potential before beginning treatments of diseases, including periodontitis (Kinane & Chestnutt 2000).

Recently, studies related to the effects of smoking cessation are enlightening. It is known that smoking cessation diminishes the risk of lung cancer, myocardial infarction and stroke (US

Department of Health and Human Services 1988). Additionally, the airway inflammation and the progression of chronic obstructive pulmonary disease are reduced by quitting smoking (Fletcher & Peto 1977). In periodontal tissue, it has been reported that smoking cessation appears to return the periodontal healing response to the same level as in non-smokers, and also that responses of former smokers are comparable with those of non-smokers (Kaldahl et al. 1996, Grossi et al. 1997). In addition, recent studies have demonstrated that the gingival microcirculation which includes gingival blood flow (GBF), gingival crevicular fluid (GCF), and suppressed gingival bleeding on probing, increases in the early stages of smoking cessation (Nair et al. 2003, Morozumi et al. 2004). These findings provide the evidence that quitting smoking is vital for both systemic and periodontal health. Although evidence of epidemiological studies is accumulating, little information is available about the biological effects of cigarette smoking cessation on the immune system.

Neutrophils are considered to play a pivotal role in host defense against micro-organisms as primary phagocytic cells, and in acute phases of inflammatory reactions in periodontal diseases (De Nardin 1996). Smoking damages neutrophil chemotaxis and/or phagocytosis (Eichel & Shahrik 1969), and stimulates the oxidative burst (Ryder et al. 1998). Smoke exposure impairs f-actin kinetics, resulting in the damage of the neutrophil cytoskeleton (Ryder et al. 2002). Morphological changes related to smoking have been demonstrated in neutrophils (Lannan et al. 1992). Also, neutrophils are known to synthesize and release immunoregulatory cytokines and inflammatory mediators, which play a role in periodontal tissue destruction (American Academy of Periodontology 1999).

We have previously reported significantly elevated mRNA expression for interleukin (IL)-8, supervillin and vascular endothelial growth factor (VEGF) in human neutrophils stimulated with lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*P. gingivalis*) (Sugita et al. 1998, Morozumi et al. 2001). We have also shown that IL-1β and VEGF mRNA levels were significantly higher in *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated neutrophils from both aggressive and chronic periodontitis patients (Kubota et al. 2001). However, whether cigarette smoking alters the gene expression levels in neutrophils has not been elucidated in detail yet. Furthermore, few studies have been reported concerning the effects on neutrophil functions by quitting smoking, much less the alteration of mRNA expressions in neutrophils related to smoking cessation. The process still remains under discussion: the relationship between the non-smoking period and alteration of mRNA expression is not discussed enough and is worth investigating.

The aims of the present study were: (1) to quantify and compare mRNA expression levels in neutrophils between smokers and non-smokers, and (2) to evaluate their alterations induced by smoking cessation for 8 weeks. We investigated, in the present study, mRNA levels for human neutrophil products: IL-1β, IL-8, tumor necrosis factor (TNF)-α, VEGF and matrix metalloproteinase (MMP)-8. These have been known to be produced by neutrophils and could be the representative cytokines or enzymes reflecting various neutrophil functions in periodontal diseases (Lloyd & Oppenheim 1992, Liede et al. 1999).

Material and Methods

Subjects

The study population was as follows: the test group consisted of 16 smokers (all male) in the age range of 22–39 years and the control group consisted of 11 non-smokers (seven males and four females) in the age range of 23–27 years. All subjects had no history or current signs of systemic diseases. The means and standard deviations (SD) in the ages of smokers and non-smokers were 25.3 ± 4.0 and 24.4 ± 1.2 years, respectively. The mean and SD pack year of smokers was 9.5 ± 6.8. Both of the groups were recruited from dental students of the Faculty of Dentistry, Niigata University, Niigata, Japan. Subjects who had taken antibiotics, anti-inflammatory drugs or immunosuppressive drugs during the previous 8 weeks were excluded from the study. Subjects were selected on the basis of reported smoking habits, namely, smokers who claimed to have smoked at least 10 cigarettes per day for the past 5 years at the minimum. Non-smokers were defined as people who had never smoked. Group allocation was subsequently confirmed according to exhaled carbon monoxide (CO) and serum cotinine levels. Smokers were required to demonstrate a CO level of >8 p.p.m. and a cotinine level of >14 ng/ml. Non-smokers were required to show a baseline CO level of <7 p.p.m. and cotinine level of <5 ng/ml.

Experimental protocol

In order to evaluate the changes of mRNA levels for neutrophil products over time by smoking cessation, blood was collected from smoker subjects over an 8-week period at the following times: before smoking cessation (baseline), and then at 1, 4 and 8 weeks after smoking cessation. Additionally, an exhaled CO measurement was performed eight times: at baseline, at 1, 3 and 5 days, and at 1, 2, 4 and 8 weeks after smoking cessation. In the control group, these measurements (mRNA levels and CO) were performed only once. The blood collection and the CO measurement were carried out between 11:00 and 11:30, and 16:00 and 18:00 hours, respectively. In the event of illness or health problems, subjects were withdrawn from the study. Subjects were instructed to relax for 30 min, and were forbidden to eat anything for 2 h prior to taking measurements. Also prior to measurements, their physical condition was evaluated to determine if they were in normal health. The mean arterial blood pressure and heart rate were recorded using a sphygmomanometer (Kentarou BP-203RV II®, COLIN Co., Aichi, Japan) attached to the left arm, and then body temperature was taken by clinical thermometer (Electronic Thermometer®, TERUMO Co., Tokyo, Japan). The investigation was approved by the local ethical committee of the Niigata University Graduate School of Medical and Dental Sciences, and all subjects signed informed consent to participate in the study.

Status of smoking

Status of smoking and quitting smoking was verified by exhaled CO and serum cotinine concentrations. The Micro Smokerlyzer® (Bedfont Scientific Ltd., Kent, UK), which is an exhaled CO monitor used during smoking cessation programs to give the smoker visible proof of the damaging CO levels, was used to obtain the measurement of expired CO concentration before every

experiment. In brief, subjects were asked to hold their breath for 15 s after a deep breath, then to slowly exhale all of it through a cardboard tube into the machine's mouthpiece. The peripheral blood samples for the serum cotinine measurement were left at room temperature for 1 h, and then centrifuged at $2000 \times g$ for 20 min. Serum cotinine levels were analyzed by BML General Laboratory (Saitama, Japan).

Isolation of neutrophils

We have previously established an experimental method that begins with neutrophil isolation and results in semiquantitative analysis by reverse transcription-polymerase chain reaction (RT-PCR) (Kubota et al. 2001, Morozumi et al. 2001). In brief, neutrophils were separated from peripheral blood by Histopaque® (Sigma Chemical Co., St Louis, MO, USA) gradient centrifugation according to the manufacturer's instructions. The cells were washed with phosphate-buffered saline (PBS) and suspended in RPMI 1640-HEPES (GIBCO BRL, Gaithersburg, MD, USA). The viability was over 98%, as judged by the tripan blue dye exclusion test. The purity was morphologically above 95%. The aliquots containing 5×10^6 neutrophils were suspended in 480 μl of RPMI 1640-HEPES in microtubes and pre-incubated for 5 min at 37°C in 5% CO_2. Then 20 μl of 10^{-6} M FMLP (final concentration: 4×10^{-8} M in a total volume of 500 μl) was added to each cell culture and incubated at 37°C in 5% CO_2 for 15 min. After incubation, the neutrophils in the respective tubes were washed with 1 ml of PBS and subjected to the RNA extraction procedure. Total RNA was extracted using guanidine–isothiocyanate–phenol–chloroform extraction method (ISOGEN-LS, Nippon Gene, Toyama, Japan) and treated with RNase-free DNase (DNase I-RT grade, Nippon Gene) to remove residual contaminating DNA fragments.

Semiquantitative analysis by RT-PCR

Neutrophil first strand cDNA was synthesized as previously described (Kubota et al. 2001, Morozumi et al. 2001). In brief, 1 μg of total RNA was reverse transcribed in duplicate by incubating samples for 2 h at 37°C in 20 μl of a reaction mixture containing 20 U moloney murine leukemia virus reverse transcriptase, 40 U RNase block ribonuclease inhibitor, 4 μl of 5 × first-strand buffer; 0.5 M Tris-HCl (pH 8.3), 0.75 M KCl, 0.03 M $MgCl_2$, 1 μl of dNTP mixture (25 mM each nucleoside triphosphate) (Stratagene, La Jolla, CA, USA) and 1 μl of 50 μM random nonamer (TaKaRa Shuzo, Kyoto, Japan).

Gene-specific oligonucleotide primer pairs are shown in Table 1. The primer pairs were designed with primer analysis software (Oligo® Ver.5.1: NBI, Annapolis, MD, USA), and custom-synthesized by Sigma-Genosys (The Woodlands, TX, USA). Semiquantitative analysis by RT-PCR was then performed. The PCR reaction mixture (total volume of 100 μl) containing 2 μl of cDNA, 50 μl of Premix Ex Taq (TaKaRa Shuzo) and 0.5 μM of each 5′ and 3′ oligonucleotide primer, was covered with two drops of silicone oil (GIBCO BRL) and subjected to PCR amplification using a PCR Thermal Cycler MP (TaKaRa Shuzo). The thermocycling was as follows: 94°C for 5 min, 60°C for 5 min, 72°C for 2 min, 94°C for 1 min, employed for 40 cycles. Five microliters of the PCR-amplified product were taken every two cycles from the 16th to the 40th cycle to assess the rate of amplification. In each experiment, duplicate cDNA samples were used for semiquantitative RT-PCR. Each RNA sample without RT was subjected to PCR as a negative control from which no yield was obtained, confirming the absence of genomic contamination.

The reaction mixture was fractionated on 2% agarose gels (NuSieve® 3:1: BioWhittaker Molecular Applications, Rockland, ME, USA) and stained with ethidium bromide. Then electrophoresis was performed on the samples at 50 V for 2 h. The gels were scanned, digitized and the density of the amplified bands were analyzed with NIH image software and calculated by a modification of the formula described by Chelly et al. (1988).

Statistical analysis

The data obtained from the experiments are presented as means and SD. Differences of mRNA abundance in neutrophils of smokers and non-smokers were compared by the paired Mann–Whitney *U*-test. Alterations between baseline and time intervals in smokers were evaluated by repeated measures analysis of variance (ANOVA) with post hoc contrasts.

Results

As participants in this smoking cessation program, 11 of the 16 smokers aged 22–39 years (25.9 ± 4.7) kept systemic

Table 1. Synthetic oligonucleotide primers used for reverse transcriptase polymerase chain reaction (RT-PCR)

Primers		Sequence	Length (mer)	Location		Length of PCR product (base pairs)
IL-1β:	forward	5′-CATCAGCACCTCTCAAGCAG-3′	20	6683–6702	[a]	192
	reverse	5′-ATAGCCGTACTCAAAAACCT-3′	20	6855–6874		
IL-8:	forward	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′	24	102–125	[b]	292
	reverse	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′	25	369–393		
TNF-α:	forward	5′-TCCAGCTGGAGAAGGGTGAC-3′	20	2262–2281	[c]	196
	reverse	5′-TGAGCCAGAAGAGGTTGAGG-3′	20	2438–2457		
VEGF:	forward	5′-GCCTTGCCTTGCTGCTCTAC-3′	20	132–151	[d]	320
	reverse	5′-TATGTGCTGGCCTTGGTGAG-3′	20	432–451		
MMP-8:	forward	5′-AGCCAAATGAGGAAACTCTGGACA-3′	24	325–349	[e]	515
	reverse	5′-TGAGTAGTTGCTGGTTTCCCTGAAA-3′	25	817–841		
β-actin:	forward	5′-GCGAGAAGATGACCCAGATCATGTT-3′	25	388–412	[f]	300
	reverse	5′-GCTTCTCCTTAATGTCACGCACGAT-3′	25	663–687		

The sequence:
[a]for interleukin (IL)-1β (Bensi et al. 1987), [b]for IL-8 (Matsushima et al. 1988), [c]for tumor necrosis factor (TNF)-α (Marmenout et al. 1985), [d]for vascular endothelial factor (VEGF) (Leung et al. 1989), [e]for matrix metalloproteinase (MMP)-8 (Kubota et al. 1996) and [f]for β-actin (Ponte et al. 1984).

health and successfully quit smoking for 8 weeks. Five smokers dropped out during the course of the study.

CO and cotinine concentrations

The exhaled CO concentration in smokers was statistically significantly higher than that in non-smokers at baseline only (baseline versus non-smokers, 31.08 ± 5.96 versus 1.65 ± 0.52, $p < 0.01$).

The CO concentration in smokers was statistically significantly low at 1 day after smoking cessation, compared with the baseline (1 day versus baseline, 3.42 ± 1.51 versus 31.08 ± 5.96, $p < 0.01$). Meanwhile, the serum cotinine concentration markedly decreased after 1 week at the second measurement, and showed below 5 ng/ml (lower limit of quantitation) in some of the smokers. Although statistical analysis was not performed because the numbers of detectable subjects differed in each period, there seems to be a decreasing tendency in cotinine concentration after smoking cessation. The cotinine concentrations at baseline fulfilled the reference level (< 13.7 ng/ml) as a smoker (Jarvis et al. 1987). Also, cotinine was not detected in non-smoker samples. These data proved that quitting smoking was completed successfully in this study.

Comparison of mRNA expression levels between smokers and non-smokers

Fig. 1 shows the results of semiquantitative analysis of the mRNA expression levels relative to β-actin for a number of neutrophil products in smokers and non-smokers. The analyzed mRNA expression levels of neutrophils from smokers were all statistically significantly lower than that in non-smokers ($p < 0.01$: IL-1β, IL-8; VEGF; $p < 0.05$: TNF-α, MMP-8), while β-actin mRNA expression was almost the same in all of the samples. Untreated and unstimulated neutrophils expressed low levels for all gene products included in this study, except for β-actin mRNA, and showed similar levels of expression to those described previously (Morozumi et al. 2001).

Alteration of mRNA expression during smoking cessation

The mRNA levels relative to β-actin for IL-1β, IL-8, TNF-α, VEGF and MMP-8 at each period of quitting smoking in smokers are shown in Table 2. The MMP-8 mRNA levels were statistically significantly increased at 8 weeks after smoking cessation compared with the baseline ($p < 0.05$). While the other mRNA expression levels also elevated gradually from the baseline, they did not reach the statistically significant levels. Fig. 2 shows images of a gel electrophoresis used for the semiquantitative RT-PCR analysis for MMP-8 and β-actin in representative samples. The greater amplification of MMP-8 in the 8-week period compared with baseline is demonstrated.

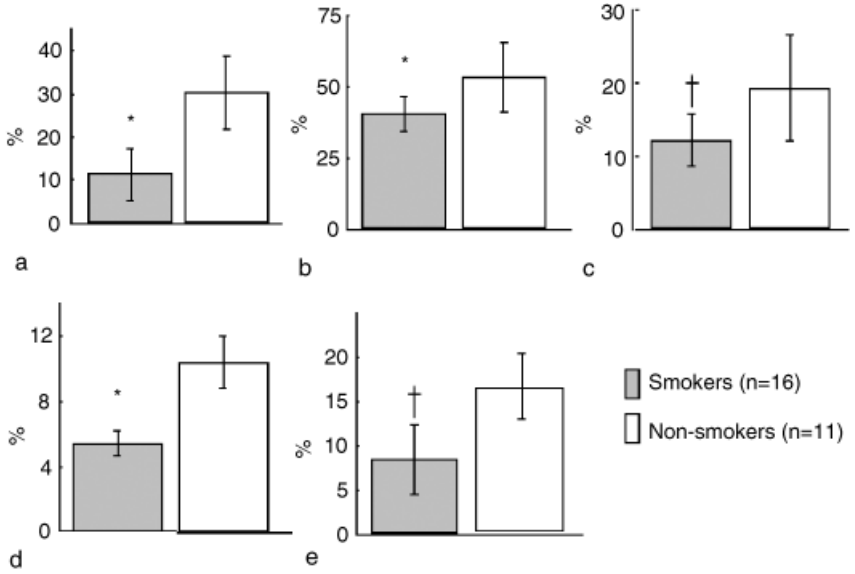

Fig. 1. Semiquantitative analysis of the mRNA expression levels relative to β-actin for a number mRNA species between smokers and non-smokers (mean $\pm$ SD). Graphs (a)–(e) show the mRNA levels of interleukin (IL)-1β (a), IL-8 (b), tumor necrosis factor-α (c), vascular endothelial growth factor (d) and matrix metalloproteinase-8 (e). The *mark and the †mark represent significantly low levels in the smokers compared with the non-smokers at $p < 0.01$ and $p < 0.05$, respectively.

Discussion

The objectives of the present study were to quantify and compare the mRNA expression levels for a number of mRNA species in neutrophils between smokers and non-smokers, and to evaluate alterations of the mRNA levels by quitting smoking for 8 weeks. We demonstrated that the mRNA expression levels were generally lower in smokers than in non-smokers. The mRNA level of MMP-8 was significantly increased in the 8-week smoking cessation period.

Neutrophils in GCF are generally being stimulated with resident periodontal pathogens such as *P. gingivalis*, *Actinobacillus actinomycetemcomitans* and/or *Bacteroides forsythus*, etc. (Delima & Van Dyke 2003). We have previously reported that a number of differentially displayed transcripts were obtained by RNA fingerprinting by arbitrarily primed PCR methods, from human neutrophils stimulated with LPS of several periodontal pathogens including *P. gingivalis* (Morozumi et al. 2001). We have also shown that differential gene expressions were induced in neutrophils from patients with generalized aggressive periodontitis compared with healthy subjects or chronic periodontitis patients, when stimulated with the non-specific chemotactic ligand, FMLP (Kubota et al. 2001).

To assess differences in susceptibility to periodontitis between smokers and non-smokers, we have here analyzed gene expressions of neutrophils stimulated with FMLP. FMLP is a synthetic molecule used to mimic the effect of bacterial peptide on host-defense cells (Perez et al. 1991) and is known to induce numerous neutrophil functions such as chemotaxis, enzyme release and oxidant-free radical production (Perez et al. 1991). Neutrophil functions are sometimes impaired in periodontal diseases (Biasi et al. 1999), hence, the FMLP-induced transcripts could be related to impaired functions of these neutrophils.

In the present study, we demonstrated that the analyzed mRNA expression

Table 2. Transcript levels relative to β-actin during a non-smoking period

Weeks	IL-1β	IL-8	TNF-α	VEGF	MMP-8
baseline	17.6 ± 7.5	31.4 ± 11.4	13.3 ± 9.4	7.0 ± 5.0	5.4 ± 3.6
1	18.8 ± 6.4	33.0 ± 11.2	14.6 ± 9.1	8.8 ± 5.3	7.2 ± 4.9
4	18.4 ± 5.1	36.1 ± 5.8	15.4 ± 7.9	10.1 ± 4.1	9.4 ± 7.1
8	20.4 ± 3.0	34.9 ± 11.4	15.8 ± 6.1	11.0 ± 5.4	14.3 ± 6.5*

$n = 11$. Values are expressed as mean ± SD. IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial factor; MMP, matrix metalloproteinase.
*Significantly high compared with the baseline ($p < 0.01$).

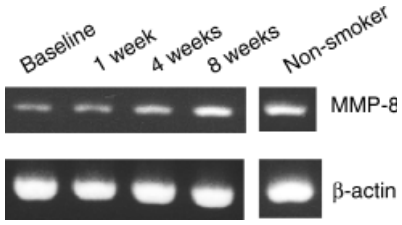

Fig. 2. An image of agarose gel electrophoresis for matrix-metalloproteinase (MMP)-8 and β-actin mRNA. Representative cases of an 8-week smoking quitter and a non-smoker are shown. Bands show mRNA expressions of polymerase chain reaction (PCR) products for MMP-8 and β-actin at 32 PCR cycles. A weak MMP-8 mRNA expression is seen at baseline, and gradually increased the expression level at 1, 4 and 8 weeks. The intensity of the MMP-8 mRNA of the smoker at 8 weeks was at the same level as the non-smoking subject. Note that β-actin bands were similar in both samples. The electrophoresis was carried out on 2% agarose gel at 50 V for 2 h.

levels of peripheral neutrophils were generally lower in smokers than in non-smokers. Previous studies demonstrated that higher levels of TNF-α in whole blood, IL-6 in plasma and IL-4 in peripheral blood mononuclear cells (PBMCs) were presented in smokers (Byron et al. 1994, Tappia et al. 1995). As the neutrophil products, we analyzed, are known to be pro-inflammatory cytokines (IL-1β, TNF-α), a chemokine (IL-8), a growth factor (VEGF) or a proteinase (MMP-8) relating to periodontal pathogenesis, it is somewhat surprising that all these mRNA levels in smokers were found to be low. Recent studies, however, have shown that nicotine exerts a negative immunoregulatory effect through modulation of the cytokine production such as IL-2 and TNF-α in PBMC (Madretsma et al. 1996), or IL-1β in gingival mononuclear cells (Bernzweig et al. 1998). Further, salivary MMP-8 or IL-1 receptor antagonist in GCF was reported to be present at lower levels in smokers (Liede et al. 1999, Rawlinson et al. 2003).

It seems difficult to make a direct comparison among these reports because of a difference between mRNA and protein; variations in experimental methodology, kinds of cells/tissues and smoking levels. The mechanisms behind these decreased mRNA expressions might be related to various host responses or more profound influences on cellular metabolism. It can be assumed that the effect of the low mRNA levels in smokers' neutrophils was a result of the long-standing use of tobacco. For example, cigarette smoke-derived compounds such as nicotine could impair neutrophil functions relating to gene expression as well as impairment of chemotaxis, phagocytosis and f-actin kinetics (Eichel & Shahrik 1969, Ryder et al. 2002). Also, it should be considered that smoking's effects are more complex than just increasing the nicotine, the other chemicals and noxious stimuli related to smoking (Kinane & Chestnutt 2000), which could be factored into the down-regulation of the neutrophil mRNA expression.

In this study, the neutrophil MMP-8 mRNA levels significantly increased at 8 weeks after smoking cessation. The other mRNA expression levels (IL-1β, IL-8, TNF-α and VEGF) were also gradually increased; however, they did not reach the significant levels at 8 weeks after smoking cessation. MMP-8 (neutrophil collagenase or collagenase-2) is one of the key enzymes associated with connective tissue destruction in periodontitis (Reynolds & Meikle 1997, Liede et al. 1999). We have previously reported that the levels of MMP-8 mRNA were significantly higher in gingival tissues in periodontitis-affected patients (Kubota et al. 1996). Thus it seems surprising that the MMP-8 mRNA levels were significantly lower in neutrophils obtained from smokers and a significant increase was observed at 8 weeks after smoking cessation. Saari et al. (1990) reported that cigarette smoke exert conflicting actions (activation and inactivation/fragmentation to MMP-8). However, we should consider that the mRNA expression level does not always reflect the protein amounts directly, especially in neutrophils, which store MMP-8 in their secondary granules (Birkedal-Hansen 1993, Kubota et al. 1996). Possible explanation is that MMP-8 is thought to be upregulated by IL-1α, β or TNF-α both at transcriptional (Koshy et al. 2002) and at post-transcriptional levels (Hanemaaijer et al. 1997); therefore, an increase of MMP-8 transcripts in neutrophils by quitting smoking possibly related to a transcriptional activation by an increase of some cytokines such as IL-1β and TNF-α. Because these cytokines also gradually increased after smoking cessation, MMP-8 could be synergistically increased at statistically significant level by the 8 weeks. Another possibility is that smoking cessation could recover neutrophil viability/metabolism or decrease damage to neutrophils, resulting an increase in the mRNA synthesis.

It is uncertain how long a period is required for the complete recovery of these neutrophil transcriptional functions after smoking cessation. Some researchers have demonstrated the beneficial effects of short-term (2-month) smoking reduction or cessation in smokers without systemic diseases. Rennard et al. (1990) reported a decrease in the number of alveolar macrophages and neutrophils, a reduction in the concentration of neutrophil elastase α-antiprotease complex in alveolar lavage fluid by smoking reduction. Also, a decrease of number of macrophages in sputum has reported at 2 month after smoking cessation (Swan et al. 1992). On the other hand, we have previously reported that both GBF and GCF significantly increased at 3 days and at 5 days after smoking cessation, respectively (Morozumi et al. 2004). These reports could be served as valuable information for recovery of host metabolism induced by smoking cessation. According to these reports, the neutrophil metabolic function could be recovered quite quickly (within several months) after smoking cessation.

In conclusion, the present investigation revealed that the transcript levels of peripheral neutrophils in smokers are generally lower than those in non-

smokers. This may be related to an impairment of some neutrophil functions caused by cigarette smoking. The MMP-8 mRNA levels were significantly recovered at 8 weeks after smoking cessation, while recovery of the other neutrophil transcripts seemed to require substantially longer period beyond 8 weeks.

We provide the first evidence describing the alteration of neutrophil gene expression induced by smoking cessation. Clarifying the mechanisms of cytokine networks, and regulation of immune response between neutrophils and local environment, are issues for future research.

Acknowledgments

The authors are grateful to Ms Beverly Britton and Mr Adam Townsend for their valuable support. We are also indebted to Dr Yukihiro Numabe, Department of Periodontology, School of Dentistry at Tokyo, The Nippon Dental University, for his advice and useful comments. This study was supported in part by Grant-in-Aid for Scientific Research (No. 14657552) from the Japanese Ministry of Education, Science, Sports and Culture, and the Fund for Scientific Promotion of Tanaka Industries Co., Niigata, Japan.

References

American Academy of Periodontology (1999) Position paper: tobacco use and the periodontal patient. *Journal of Periodontology* **70**, 1419–1418.

Bartecchi, C. E., MacKenzie, T. D. & Schrier, R. W. (1994) The human costs of tobacco use (1). *The New England Journal of Medicine* **330**, 907–912.

Bensi, G., Raugei, G., Palla, E., Carinci, V., Buonamassa, D. T. & Melli, M. (1987) Human interleukin-1 beta gene. *Gene* **52**, 95–101.

Bergström, J. & Preber, H. (1994) Tobacco use as a risk factor. *Journal of Periodontology* **65**, 545–550.

Bernzweig, E., Payne, J. B., Reinhardt, R. A., Dyer, J. K. & Patil, K. D. (1998) Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. *Journal of Clinical Periodontology* **25**, 246–252.

Biasi, D., Bambara, L. M., Carletto, A., Caramaschi, P., Andrioli, G., Urbani, G. & Bellavite, P. (1999) Neutrophil migration, oxidative metabolism and adhesion in early onset periodontitis. *Journal of Clinical Periodontology* **26**, 563–568.

Birkedal-Hansen, H. (1993) Role of matrix metalloproteinases in human periodontal diseases. *Journal of Periodontology* **64**, 474–484.

Byron, K. A., Varigos, G. A. & Wootton, A. M. (1994) IL-4 production is increased in cigarette smokers. *Clinical and Experimental Immunology* **95**, 333–336.

Chelly, J., Kaplan, J-C., Maire, P., Gautron, S. & Kahn, A. (1988) Transcription of the dystrophin gene in human muscle and non-muscle tissues. *Nature* **333**, 858–860.

De Nardin, E. (1996) The molecular basis for neutrophil dysfunction in early-onset periodontitis. *Journal of Periodontology* **67**, 345–354.

Delima, A. J. & Van Dyke, T. E. (2003) Origin and function of the cellular components in gingival crevice fluid. *Periodontology 2000* **31**, 55–76.

Eichel, B. & Shahrik, H. A. (1969) Tobacco smoke toxicity: loss of human oral leukocyte function and fluid-cell metabolism. *Science* **166**, 1424–1428.

Fletcher, C. & Peto, R. (1977) The natural history of chronic airflow obstruction. *British Medical Journal* **1**, 1645–1648.

Grossi, S. G., Zambon, J., Machtei, E. E., Schifferle, R., Andreana, S., Genco, R. J., Cummins, D. & Harrap, G. (1997) Effects of smoking and smoking cessation on healing after mechanical periodontal therapy. *Journal of the American Dental Association* **128**, 599–607.

Hanemaaijer, R., Sorsa, T., Konttinen, Y. T., Ding, Y., Sutinen, M., Visser, H., van Hinsbergh, V. W. M., Helaakoski, T., Kainulainen, T., Rönka, H., Tschesche, H. & Salo, T. (1997) Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-α and doxycycline. *Journal of Biological Chemistry* **272**, 31504–31509.

Jarvis, M. J., Tunstall-Pedoe, H., Feyerabend, C., Vesey, C. & Saloojee, Y. (1987) Comparison of tests used to distinguish smokers from nonsmokers. *American Journal of Public Health* **77**, 1435–1438.

Kaldahl, W. B., Johnson, G. K., Patil, K. D. & Kalkwarf, K. L. (1996) Levels of cigarette consumption and response to periodontal therapy. *Journal of Periodontology* **67**, 675–681.

Kinane, D. F. & Chestnutt, I. G. (2000) Smoking and periodontal disease. *Critical Reviews in Oral Biology and Medicine* **11**, 356–365.

Koshy, P. J. T., Lundy, C. J., Rowan, A. D., Porter, S., Edwards, D. R., Hogan, A., Clark, I. M. & Cawston, T. E. (2002) The modulation of matrix metalloproteinase and ADAM gene expression in human chondrocytes by interleukin-1 and oncostatin M. A time-course study using real-time quantitative reverse transcription-polymerase chain reaction. *Arthritis & Rheumatism* **46**, 961–967.

Kubota, T., Morozumi, T., Shimizu, K., Sugita, N., Kobayashi, T. & Yoshie, H. (2001) Differential gene expression in neutrophils from patients with generalized aggressive periodontitis. *Journal of Periodontal Research* **36**, 390–397.

Kubota, T., Nomura, T., Takahashi, T. & Hara, K. (1996) Expression of mRNA for matrix metalloproteinases and tissue inhibitors of metalloproteinases in periodontitis-affected human gingival tissue. *Archives of Oral Biology* **41**, 253–262.

Lannan, S., McLean, A., Drost, E., Gillooly, M., Donaldson, K., Lamb, D. & MacNee, W. (1992) Changes in neutrophil morphology and morphometry following exposure to cigarette smoke. *International Journal of Experimental Pathology* **73**, 183–191.

Leung, D. W., Cachianes, G., Kuang, W-J., Goeddel, D. V. & Ferrara, N. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306–1309.

Liede, K. E., Haukka, J. K., Hietanen, J. H. P., Mattila, M. H., Rönkä, H. & Sorsa, T. (1999) The association between smoking cessation and periodontal status and salivary proteinase levels. *Journal of Periodontology* **70**, 1361–1368.

Lloyd, A. R. & Oppenheim, J. J. (1992) Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immunology Today* **13**, 169–172.

Madretsma, G. S., Donze, G. J., VanDijk, A. P. M., Tak, C. J. A. M., Wilson, J. H. P. & Zijlstra, F. J. (1996) Nicotine inhibits the in vitro production of interleukin 2 and tumour necrosis factor-α by human mononuclear cells. *Immunopharmacology* **35**, 47–51.

Marmenout, A., Fransen, L., Tavernier, J., Van Der Heyden, J., Tizard, R., Kawashima, E., Shaw, A., Johnson, M. J., Semon, D., Müller, R., Ruysschaert, M-R., Van Vliet, A. & Fiers, W. (1985) Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor. *European Journal of Biochemistry* **152**, 515–522.

Matsushima, K., Morishita, K., Yoshimura, T., Lavu, S., Kobayashi, Y., Lew, W., Appella, E., Kung, H. F., Leonard, E. J. & Oppenheim, J. J. (1988) Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. *Journal of Experimental Medicine* **167**, 1883–1893.

Morozumi, T., Kubota, T., Sato, T., Okuda, K. & Yoshie, H. (2004) Smoking cessation increases gingival blood flow and gingival crevicular fluid. *Journal of Clinical Periodontology* **31**, 267–272.

Morozumi, T., Kubota, T., Sugita, N., Ohsawa, Y., Yamazaki, K. & Yoshie, H. (2001) Elevated mRNA expression for supervillin and vascular endothelial growth factor in human neutrophils stimulated with lipopolysaccharide from *Porphyromonas gingivalis*. *Journal of Periodontal Research* **36**, 160–168.

Nair, P., Sutherland, G., Palmer, R. M., Wilson, R. F. & Scott, D. A. (2003) Gingival bleeding

on probing increases after quitting smoking. *Journal of Clinical Periodontology* **30**, 435–437.

Perez, H. D., Kelly, E., Elfman, F., Armitage, G. & Winkler, J. (1991) Defective polymorphonuclear leukocyte formyl peptide receptor(s) in juvenile periodontitis. *Journal of Clinical Investigation* **87**, 971–976.

Ponte, P., Ng, S-Y., Engel, J., Gunning, P. & Kedes, L. (1984) Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Research* **12**, 1687–1696.

Rawlinson, A., Grummitt, J. M., Walsh, T. F. & Douglas, C. W. I. (2003) Interleukin 1 and receptor antagonist levels in gingival crevicular fluid in heavy smokers versus non-smokers. *Journal of Clinical Periodontology* **30**, 42–48.

Rennard, S. I., Daughton, D., Fujita, J., Oehlerking, M. B., Dobson, J. R., Stahl, M. G., Robbins, R. A. & Thompson, A. B. (1990) Short-term smoking reduction is associated with reduction in measures of lower respiratory tract inflammation in heavy smokers. *European Respiratory Journal* **3**, 752–759.

Reynolds, J. J. & Meikle, M. C. (1997) Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontology 2000* **14**, 144–157.

Rivera-Hidalgo, F. (2003) Smoking and periodontal disease. *Periodontology 2000* **32**, 50–58.

Ryder, M. I., Fujitaki, R., Johnson, G. & Hyun, W. (1998) Alterations of neutrophil oxidative burst by in vitro smoke exposure: implications for oral and systemic diseases. *Annals of Periodontology* **3**, 76–87.

Ryder, M. I., Wu, T. C., Kallaos, S. S. & Hyun, W. (2002) Alterations of neutrophil f-actin kinetics by tobacco smoke: implications for periodontal diseases. *Journal of Periodontal Research* **37**, 286–292.

Saari, H., Suomalainen, K., Lindy, O., Konttinen, Y. T. & Sorsa, T. (1990) Activation of latent human neutrophil collagenase by reactive oxygen species and serine proteases. *Biochemical and Biophysical Research Communications* **171**, 979–987.

Sugita, N., Kimura, A., Matsuki, Y., Yamamoto, T., Yoshie, H. & Hara, K. (1998) Activation of transcription factors and IL-8 expression in neutrophils stimulated with lipopolysaccharide from *Porphyromonas gingivalis*. *Inflammation* **22**, 253–267.

Swan, G. E., Hodgkin, J. E., Roby, T., Mittman, C., Jacobo, N. & Peters, J. (1992) Reversibility of airways injury over a 12-month period following smoking cessation. *Chest* **101**, 607–612.

Tappia, P. S., Troughton, K. L., Langley-Evans, S. C. & Grimble, R. F. (1995) Cigarette smoking influences cytokine production and antioxidant defences. *Clinical Science* **88**, 485–489.

US Department of Health and Human Services (1988) *The health benefits of smoking cessation: a report of surgeon general*. Washington DC: U. S. Department of Health and Human Services, DHHS Publication.

Address:
Hiromasa Yoshie
Division of Periodontology
Department of Oral Biological Science
Niigata University Graduate School of Medical and Dental Sciences
2-5274 Gakko-cho
Niigata 951-8514
Japan
Fax: +81 25 227 0808
E-mail: yoshie@dent.niigata-u.ac.jp