Smoking cessation affects human platelet activation induced by collagen

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Received February 3, 2019; Accepted July 30, 2019

DOI: 10.3892/etm.2019.8025

Abstract. It is firmly established that smoking is a risk factor of cardiovascular disease, stroke and peripheral vascular disease. Although smoking alters the hemostatic process, the influence of smoking on human platelet activation remains controversial. For patients undergoing surgery, cessation of smoking prior to the procedure is recommended as it increases the risk of postoperative morbidity or mortality. The presented study investigated the effects of smoking cessation on human platelet activation induced via collagen (n=19 patients). Blood samples were taken on four occasions: Before smoking cessation, and at 4, 8 and 12 weeks after smoking cessation. Platelet aggregation using citrated platelet-rich plasma (PRP) was monitored using a PA-200 aggregometer, which determined the size of platelet aggregates using laser scattering methods. A low dose of collagen (1 μg/ml) accelerated platelet aggregation at 4 or 8 weeks after smoking cessation when compared with results before cessation. After 12 weeks, levels of platelet aggregation induced by collagen were almost equal to those recorded prior to smoking cessation. The secretion levels of collagen-induced platelet-derived growth factor (PDGF)-AB at 4 or 8 weeks after smoking cessation were significantly higher than those before smoking was stopped. Furthermore, smoking cessation markedly strengthened the collagen-induced phosphorylation of p38 mitogen-activated protein (MAP) kinase after 4 weeks. The results of the current study indicated that smoking cessation causes temporary short-term human platelet hyper-activation. The further suggest that the incidence of complications due to human platelet hyper-reactivity may be lowered by considering the period of smoking abstinence.

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Key words: platelet, platelet activation, smoking, smoking cessation, collagen

Introduction

Smoking has been recognized to cause serious health problems. It is established that smoking is a risk factor of diseases such as myocardial infarction (1,2), stroke (3) and peripheral vascular diseases (4). Smoking causes vascular thrombosis by altering the hemostatic process via changes in the function of endothelial cells and platelets affected by fibrinogen, or coagulation factors (2). Concerning the platelet function, the number of small aggregates of platelets in smokers' plasma is significantly higher than in non-smokers in the absence of chemical stimulants (5). Furthermore, acute smoking exposure in habitual smokers increases the platelet aggregation and induces greater resistance to thrombolysis than in non-smokers (5), and the mean platelet volume and platelet distribution width, which are indicators of platelet activation, are significantly higher in smokers than in non-smokers (6). However, there are no significant differences between smokers and non-smokers in platelet aggregation induced by ADP (7). Given these conflicting previous findings, the influence of smoking on platelet activation has not been fully clarified.

Quitting of smoking is recommended in patients undergoing surgery, because smoking increases the risk of postoperative morbidity or mortality (8,9). Smoking cessation reduces the rate of postoperative pulmonary complications such as pneumonia (10) or wound-healing complications such as surgical site infection (9). At least eight weeks are required for the disappearance of the cough-promoting effect of smoking and reduction of postoperative pulmonary complications (11) and more than four weeks are required to reduce wound-healing complications (10). In addition, surgical patients are at risk of developing venous thrombo-embolism (12). The main cellular components of thrombo-embolism are platelets, endothelial cells, monocytes and erythrocytes (12). However, the influence of smoking cessation for surgery on platelet activation is not fully known. Platelet aggregability in long-term smokers is not reversible in four weeks (13). However, only two weeks of smoking cessation reduced the ADP or collagen-induced platelet aggregations through suppression of oxidative stress (14).

Collagen initiates human platelet activation in the process of thrombus formation. The interactions of platelet glycoprotein VI with the collagen of the exposed vessel wall and the complex of platelet glycoprotein Ib/IX/V with von Willebrand factor (vWF) result in the adhesion of platelets to the site of injury. After this adhesion, thromboxane A_2 and ADP lead to the activation of glycoprotein IIb/IIIa (αIIbβ3) and thrombosis formation (15). Thrombus formation is also associated with the secretion of granule contents, such as platelet-derived growth factor (PDGF)-AB (16). In the plasma of smokers, platelet α-granule constituents are increased, leading to platelet aggregation (2). Smoking have higher P2Y12 receptor expression on platelets, which increases the ADP-induced platelet aggregation (2). However, the effects of smoking or smoking cessation on platelet activation and the intracellular mechanism are not fully clarified. Furthermore, the time-dependent changes in the platelet function associated with smoking cessation remain unclear.

We previously demonstrated that ADP (17,18) and collagen (19,20) induce the secretion of PDGF-AB via the activation of p38 mitogen-activated protein (MAP) kinase in human platelets. In the present study, to investigate the effects of smoking and the cessation of smoking on human platelet activation, we examined the effects of collagen on platelet aggregation, the secretion of PDGF-AB and the underlying mechanism in patients who were quitting smoking for surgery over time for 12 weeks.

Materials and methods

Materials. Collagen was purchased from Takeda Austria GmbH (Linz, Austria). A PDGF-AB enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D System, Inc. (Minneapolis, MN, USA). Phospho-specific p38 MAP kinase antibodies and p38 MAP kinase antibodies were obtained from Cell Signaling, Inc. (Beverly, MA, USA). GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other materials and chemicals were obtained from commercial sources.

Subjects. This study was approved by the Ethics Committee of Gifu University Graduate School of Medicine and Gifu Prefectural General Medical Center. All participants signed an informed consent agreement after receiving a detailed explanation of the study.

We enrolled 19 patients who visited smoking cessation outpatient services at Gifu University Graduate School of Medicine or Gifu Prefectural General Medical Center between January 2012 and November 2014. However, four patients were excluded from the current study as they only visited outpatient services once. A self-assessment was administered, and we examined the concentration of carbon monoxide (CO) exhaled to confirm smoking cessation. Blood samples were donated 4 times as follows: before smoking cessation, and 4, 8 and 12 weeks after smoking cessation. We avoided drawing blood except for at these time points in order to avoid influencing the platelet function. Blood samples were drawn from the antecubital vein by careful venipuncture in a 21-G sterile syringe.

Human blood samples were combined with 1/10 volume of a 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained from blood samples by centrifugation at 155 x g for 12 min at room temperature. Platelet-poor plasma (PPP) was prepared from the residual blood by centrifugation at 2,500 x g for 5 min.

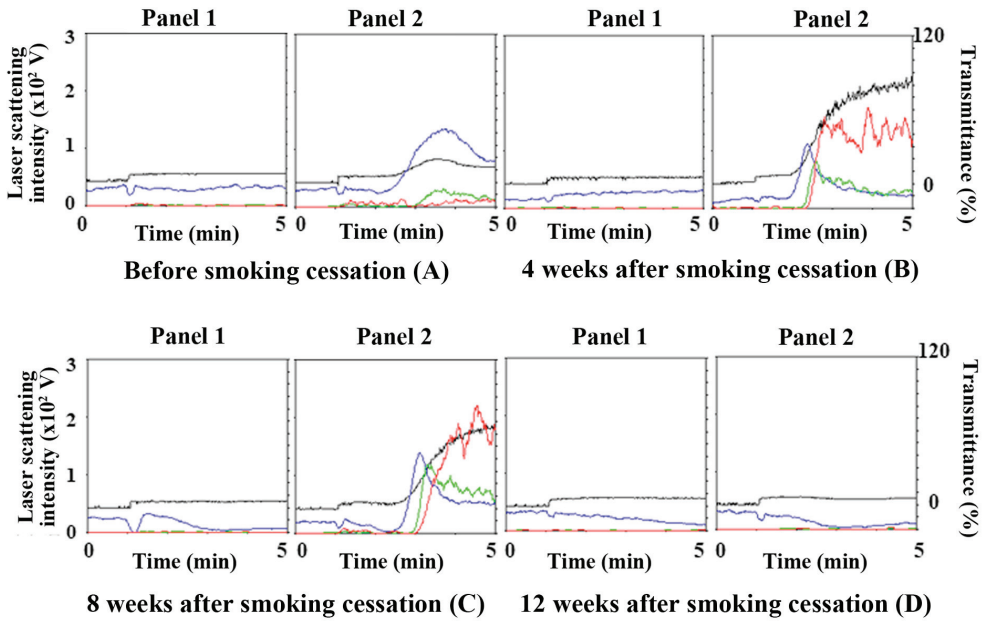
Two patients with severe hypertriglycemia (No. 10 and No. 12) were excluded because their PRP and PPP were highly turbid and the samples are inappropriate for an analysis of platelet function. Therefore, a total of 13 patients who ceased smoking were analyzed in the current study.

Platelet aggregation. Platelet aggregation using citrated PRP was monitored using a PA-200 aggregometer (Kowa Co., Ltd., Tokyo, Japan), which can determine the size of platelet aggregates via particle counting using laser scattering methods (small, 9-25 μm; medium, 25-50 μm; large, 50-70 μm) (21) at 37˚C with a stirring speed of 800 rpm. Platelet aggregation was monitored for 4 min after stimulation with collagen (n=13 patients). The percentage of transmittance of the isolated platelets was recorded as 0%, and that of the appropriate PPP (blank) was recorded as 100%.

Determination of the ED_{50} value. We calculated the ED_{50} of collagen for the platelet aggregation using an aggregometer with the laser scattering system. The percentage of aggregation in each subject was analyzed at a dose of 0, 1, 3 and 10 μg/ml collagen. Using the ALOKA curve software program included in the ALOKA RIA programs (ALOKA, Tokyo, Japan), a dose-response curve was plotted. From the regression equation, the collagen dose corresponding to 50% aggregation was calculated as the individual ED_{50} value (22).

Protein preparation after stimulation. After the stimulation with collagen, platelet aggregation was terminated by the addition of an ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4˚C for 2 min. To measure the secreted PDGF-AB as described below, the supernatant was isolated and stored at -30˚C for a subsequent enzyme-linked immunosorbent assay (ELISA). For the Western blot analysis, the pellet was washed twice with phosphate-buffered saline, lysed, and immediately boiled in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol.

Western blotting. Western blot analysis was performed as described previously (23). Briefly, SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method described by Laemmli (24) using a 10% polyacrylamide gel. The proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane, which was then blocked with 5% fat-free dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20) for 2 h before incubation with the indicated primary antibodies. The primary antibodies used in this study were phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies or GAPDH antibodies. Peroxidase-labeled anti-rabbit IgG antibodies or anti-goat IgG antibodies were used as secondary antibodies. The primary and secondary antibodies were diluted to the optimum concentration with 5% fat-free dry milk in TBS-T. The peroxidase activity on the PVDF membranes was visualized on X-ray film using an



Panel	(A)-1	(A)-2	(B)-1	(B)-2	(C)-1	(C)-2	(D)-1	(D)-2
Collagen (1 μg/ml)	−	+	−	+	−	+	−	+
Transmittance (%)	5	22	6	99	5	73	6	9
Ratio (%)								
small	98	83	98	26	95	41	98	98
medium	1	10	1	18	2	27	1	1
large	1	7	1	56	3	32	1	1
Total	100	100	100	100	100	100	100	100

Figure 1. Effect of smoking cessation on platelet aggregation induced by collagen. PRP plasma was stimulated by 1 μg/ml of collagen or vehicle for 5 min. Results were obtained (A) before cessation, at (B) 4 weeks, at (C) 8 weeks and at (D) 12 weeks. The reaction was terminated by adding ice-cold EDTA (10 mM) solution. (A) Representative pattern of platelet aggregation induced by collagen (high dose, 10 μg/ml; low dose, 1 μg/ml) as detected using an aggregometer with a laser scattering system. The black curve indicates the percentage of transmittance in each sample (isolated platelets were recorded as 0%; platelet-free plasma was recorded as 100%). The blue line indicates small aggregates (9-25 μm) the green line indicates medium aggregates (25-50 μm) and the red line indicates large aggregates (50-70 μm). The distribution (%) of aggregated particle size was measured using laser scattering methods. PRP, platelet-rich plasma.

ECL Western blotting detection system as described in the manufacturer's instructions.

Measurement of PDGF-AB. The PDGF-AB levels in the samples were determined using a PDGF-AB ELISA kit in accordance with the manufacturer's instructions.

Statistical analyses. The data are presented as box-and-whisker plots representing the median ± 25 and 75 percentile values. Statistical analyses were performed using the SPSS software program, ver. 23.0 (IBM Japan Ltd., Tokyo, Japan). The data were analyzed by Friedman test followed by Wilcoxon signed-rank test for multiple comparisons using the Bonferroni method. A P-value<0.05 was considered to indicate statistical significance.

Results

Effects of smoking cessation on the human platelet aggregation induced by collagen. Nineteen patients who intended to abstain from smoking participated in this study, but four who stopped visiting the hospital during the treatment period were excluded. The characteristics of the patients are detailed in Table I. The concentration of exhaled CO decreased after smoking cessation in all patients (Table I). We first examined the effect of smoking cessation on the platelet aggregation stimulated by collagen. Before smoking cessation, collagen dose-dependently elicited platelet aggregation between 1 and 10 μg/ml. For platelet aggregation induced by a high dose of collagen (10 μg/ml), the transmittance of aggregation was over 100% throughout the observation periods. There were no significant differences between the findings before and after smoking cessation (data not shown). In contrast, a low dose of collagen (1 μg/ml) accelerated platelet aggregation at 4 or 8 weeks after smoking cessation compared with that before cessation (Fig. 1). In addition, 12 weeks after smoking cessation, the levels of platelet aggregation induced by collagen (1 μg/ml) had nearly returned to the levels before smoking cessation. Representative patterns of low-dose collagen (1 μg/ml)-induced platelet aggregation are shown in Fig. 1.

Table I. Characteristics of 15 patients who ceased smoking and participated in the present study.

Pt	Age (years)	Sex	Height/Weight (cm/kg)	HTN	DM	HL	Platelet ($x10^4/\mu l$)	Concentration of CO (ppm)		Brinkman Index	Anti-platelet medication
								(before)	(12 weeks after)		
1	44	F	170/58	-	-	-	20.5	20	0	480	-
2	71	M	160/76	+	+	+	20.3	11	2	1375	+
3	49	M	167/58	-	-	-	27.7	66	2	2040	-
4	35	F	162/44	-	-	-	35.1	38	6	300	-
5	63	M	168/59	-	+	-	21.6	25	1	1350	+
6	62	M	156/64	-	-	-	27.5	30	2	840	-
7	53	M	170/65	-	-	-	22.2	24	3	600	-
8	46	M	163/80	-	-	+	25.4	13	1	780	-
9	75	M	165/54	-	-	-	16.6	2	0	900	-
10	72	M	169/52	-	-	+	22.5	23	0	1040	-
11	49	M	168/61	-	-	-	21.0	27	2	1240	-
12	47	F	149/40	-	-	-	28.0	23	8	580	-
13	75	M	151/61	-	-	-	18.6	22	3	500	-
14	47	M	178/77	-	-	-	20.3	14	0	600	-
15	46	M	176/90	-	-	-	24.9	8	2	405	-

Brinkman index indicates the number of cigarettes smoked per day multiplied by the number of years of smoking PT, patient; HTN, hypertension; DM, diabetes mellitus; HL, hyperlipidemia; CO, carbon monoxide; M, male; F, female.

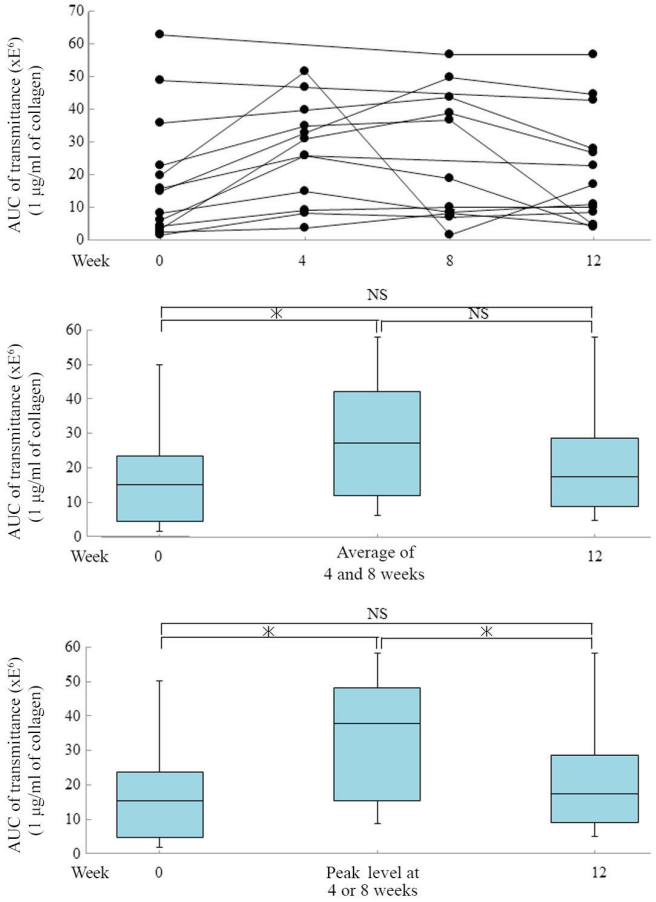


Figure 2. Effect of smoking cessation on the AUC of transmittance for platelet aggregation induced by collagen. PRP was stimulated by 1 μg/ml of collagen or vehicle for 5 min. The reaction was terminated by adding ice-cold EDTA (10 mM) solution. The values of AUC of transmittance were detected using an aggregometer with a laser scattering system (n=13 patients). Values before smoking cessation and at 4-8 and 12 weeks after smoking cessation in each case are presented in the upper line graph. Each upper boxplot presents the pre-smoking cessation values and the average value of 4-8 and 12 weeks after smoking cessation. Each lower boxplot presents the pre-smoking cessation value and the peak level at 4-8 and 12 weeks after smoking cessation. *P<0.05 as indicated. AUC, area under the curve; PRP, platelet-rich plasma; NS, not significant.

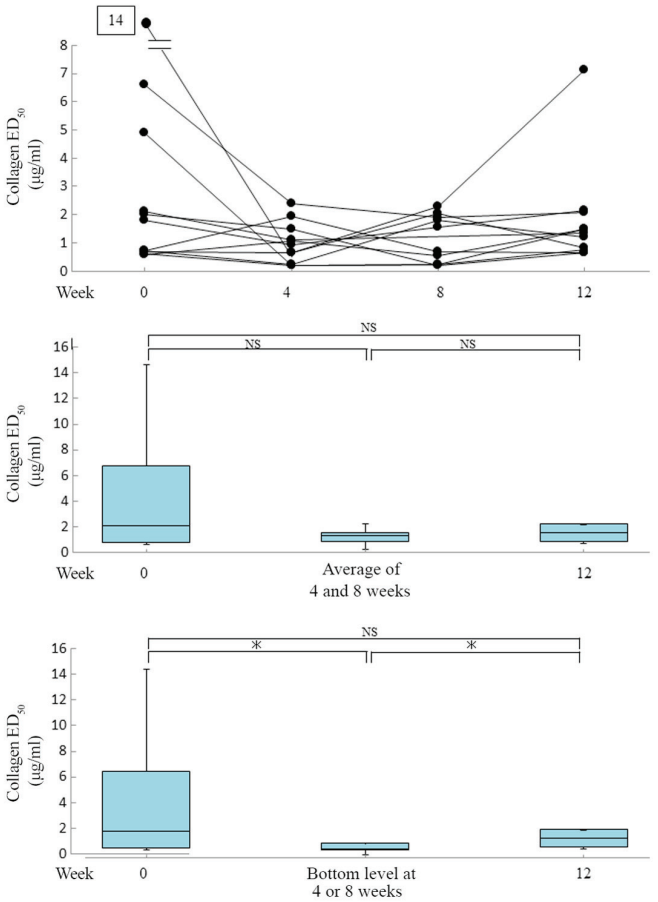


Figure 3. Effect of smoking cessation on the parameter (ED_{50}) for platelet aggregation induced by collagen. PRP was stimulated by 1 μg/ml of collagen or vehicle for 5 min. The reaction was terminated by the addition of ice-cold EDTA (10 mM) solution. The values of ED_{50} were determined using an aggregometer with a laser scattering system (n=13 patients). The values obtained before smoking cessation, and at 4-8 and 12 weeks after smoking cessation in each case are presented in the upper panel. Each upper boxplot presents pre-smoking cessation values and the average values obtained at 4-8 and 12 weeks after smoking cessation. Each lower boxplot presents the pre-smoking cessation value and the 0, 4-8 and 12 weeks after smoking cessation. *P<0.05 as indicated. PRP, platelet-rich plasma; NS, not significant.

Based on the analysis of the size of platelet aggregates using laser scattering methods, after 4 or 8 weeks smoking cessation, the ratio of large aggregates (50-70 μm) stimulated by 1 μg/ml collagen was significantly increased while the ratio of small aggregates (9-25 μm) was significantly decreased (data not shown). The peak value of the areas under the curve (AUCs) of transmittance stimulated by low-dose collagen (1 μg/ml) at 4 or 8 weeks after smoking cessation were significantly higher than the respective AUCs before smoking cessation (Fig. 2). In addition, the AUC of transmittance stimulated by 1 μg/ml collagen at 12 weeks after smoking cessation returned to levels similar to those before the cessation of smoking.

The bottom ED_{50} values of collagen (1 μg/ml) at 4 or 8 weeks after smoking cessation were significantly lower the respective ED_{50} values before smoking cessation, and at 12 weeks after the cessation, these values returned to levels similar to those observed before the cessation (Fig. 3).

Effect of smoking cessation on the collagen-induced PDGF-AB secretion in human platelets. We previously reported that collagen induces PDGF-AB secretion from human platelets (25). We further examined whether smoking cessation affects the collagen-induced PDGF-AB secretion from human platelets or not. The peak values of PDGF-AB at 4 or 8 weeks after the cessation of smoking were significantly higher than before the cessation (Fig. 4).

Considering the measured individual data, the peak or bottom value seems to be at 4 or 8 weeks. However, there is a trend but no statistically significant difference between 0, 4, 8 and 12 weeks (data not shown). We'd like to clarify the tendency of short-term hyperactivation of human platelets at 4 or 8 weeks. Therefore, we presented some plots weeks 4 and 8 together (Figs. 2-4).

Effect of smoking cessation on the collagen-induced phosphorylation of p38 MAP kinase in human platelets. In our previous studies (17-20), we demonstrated that collagen-induced PDGF-AB secretion is associated with the activation of p38 MAP kinase in human platelets. Therefore, we examined

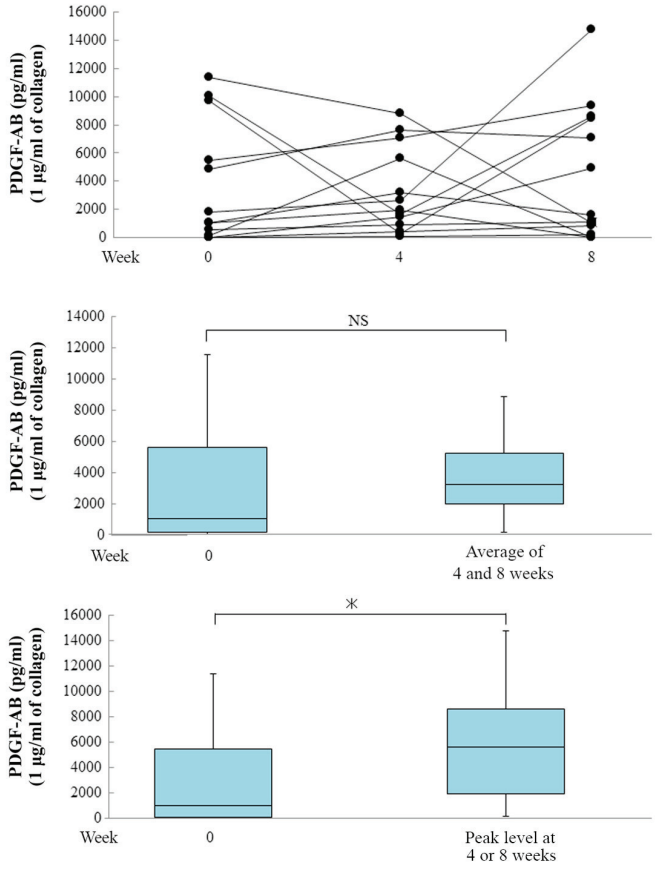


Figure 4. Effect of smoking cessation on collagen-induced PDGF-AB secretion in human platelets. PRP was stimulated by 1 μg/ml of collagen for 5 min. The reaction was terminated by adding ice-cold EDTA (10 mM) solution. The mixture was centrifuged at 10,000 x g at 4˚C for 2 min and the supernatants were subjected to ELISA. The values before smoking cessation and at 4-8 and 12 weeks after smoking cessation in each case are presented in the upper line graph. Each upper boxplot presents the pre-smoking cessation value and the average of 4-8 weeks after smoking cessation. Each lower boxplot indicates the pre-smoking cessation value and the peak level at 4-8 and 12 weeks after smoking cessation. *P<0.05 as indicated. PDGF, platelet-derived growth factor; PRP, platelet-rich plasma; NS, not significant.

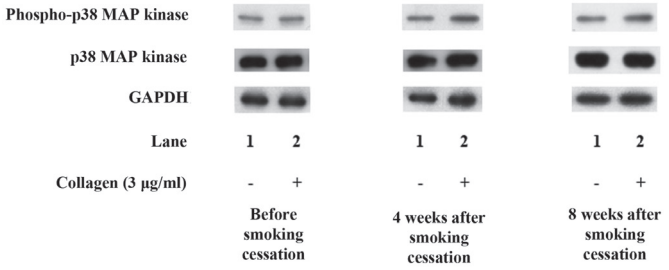


Figure 5. Effect of smoking cessation on the phosphorylation of p38 MAP kinase induced by collagen in human platelets. PRP was stimulated by 1 μg/ml of collagen for 5 min. The reaction was terminated by adding ice-cold EDTA (10 mM) solution. The lysate of platelets was harvested and subjected to SDS-PAGE using antibodies against phospho-specific p38 MAP kinase, p38 MAP kinase or GAPDH. Representative western blotting data before smoking cessation and at 4 and 8 weeks after smoking cessation are presented. MAP, mitogen activated protein; PRP, platelet-rich plasma.

whether smoking cessation affects the collagen-induced phosphorylation of p38 MAP kinase or not. Smoking cessation markedly enhanced the levels of collagen-induced phosphorylation of p38 MAP kinase at 4 weeks compared to before smoking cessation, whereas the total p38 MAP kinase levels were not affected. Though, there is no significant difference of the levels of the collagen-induced phosphorylation of p38 MAP kinase between 4 and 8 weeks. Representative results are shown in Fig. 5.

Discussion

In the present study, we investigated the effect of smoking cessation on human platelet activation. We showed that cessation for 4 and 8 weeks induced a significantly increase in the AUC (transmittance) of collagen (1 μg/ml)-induced platelet aggregation and a decrease in the ED_{50} of collagen compared with the values before smoking cessation. In addition, smoking cessation affected the distribution of aggregated particle sizes of human platelets as evaluated using laser scattering methods. These results suggest that the platelet aggregation induced by low-dose collagen (1 μg/ml) was temporarily up-regulated by the smoking cessation. We also found that the collagen-induced PDGF-AB secretion after cessation was temporarily amplified. In our previous studies (18-20), we demonstrated that collagen or ADP induces the secretion of PDGF-AB from human platelets associated with the activation of p38 MAP kinase. From this viewpoint, we focused on the effect of smoking cessation on the p38 MAP kinase activation. We found that smoking cessation remarkably enhanced the collagen-induced phosphorylation of p38 MAP kinase. Therefore, smoking cessation may up-regulate the secretion of PDGF-AB by collagen from human platelets associated with the enhancement of p38 MAP kinase activation. These results newly suggest that smoking cessation temporary causes hyper-reactivity of human platelets, which is observed relatively short-term from 4 to 8 weeks after cessation.

Platelet activation and aggregability are more augmented in smokers than in non-smokers (5,6,26-29). A previous report found that chronic exposure to cigarette smoke sustains the activation of the endothelial-coagulative system, such as by increasing vWF antigen, D-dimer, prothrombin fragment F1+2, platelet factor-4 and beta-thromboglobulin, and smoking abstinence may result in the improvement of several endothelial-coagulative system abnormalities (30).

vWF activity decreases as early as 2 months after starting smoking abstinence, and other circulating endothelial-coagulative activation markers are substantially modified at 6 and 12 months after smoking abstinence in regular smokers (30). The mean platelet volume, which is known to be a simple and convenient indicator for platelet activation, is significantly higher in regular smokers than in non-smokers (6,28) and decreases significantly at three months after smoking cessation (28). In contrast, Morita *et al* (14) reported that only two weeks of smoking cessation in long-term smokers was able to ameliorate the enhanced platelet aggregability and intraplatelet redox imbalance. In present study, we examined the time-course changes in the platelet function including the mechanism over 12-week at 4-week intervals. Our present findings showing that the hyper-reactivity in human platelet was transient, occurring relatively short-term after 4 to 8 weeks of smoking cessation, is inconsistent with these previous reports. While we did not gather data at very early

timing (e.g. 1 and 2 weeks) of the smoking cessation, smoking cessation might cause extremely complicated changes in the platelet function. At the very least, our present findings suggest that the time-dependent changes in the platelet function through the persistence of smoking cessation require physicians to monitor the temporary augmentation of the platelet aggregability.

Smoking cessation is recommended before surgery. A systematic review showed that the incidence of postoperative complications, such as general morbidity, wound complications, general infections and pulmonary or neurological complications, was significantly higher in smokers than in non-smokers (31). It has been reported that current smokers undergoing coronary artery bypass surgery have higher rates of pulmonary complications, such as postoperative pneumonia, pleural effusion and adult respiratory distress syndrome, than non-smokers, with the risk declining in patients after more than four weeks smoking cessation (32). In addition, four weeks of preoperative smoking cessation reduced the incidence of pulmonary complications after pulmonary surgery (33). Turan *et al* (34) demonstrated that in non-cardiac surgical patients, smoking was associated with a statistically significant increase in cardiovascular complications: In their large data set, there was a 57% increased chance of experiencing 30-day postoperative cardiac arrest, an 80% increased chance of experiencing a myocardial infarction, and a 73% increase chance of experiencing stroke.

However, conflicting findings have also been reported; for example, a meta-analysis shows that the preoperative smoking status was not associated with cardiovascular complications (31). Surgical patients are at risk of developing thrombo-embolism during the post-operative period with and without smoking (12). However, to our knowledge, there are no clinical reports describing an increase in perioperative thrombus formation due to smoking cessation. In the present study, we found that more than 4 to 8 weeks are needed to restore the human platelet function; however, the precise duration required remains unclear. Although the incidence of cardiovascular complications tended to decrease, no statistically significant decrease (P=0.08) was observed after 6 to 8 weeks of smoking cessation in those who had undergone hip or knee arthroplasty (35). In addition, we could not elucidate the benefits of smoking cessation regarding the prevention of cardiovascular complications (10,36). Our present findings, which show the non-linear recovery of the human platelet function according to smoking cessation, might provide a possible explanation for the phenomenon in which postoperative cardiovascular complications do not obviously decrease immediately after smoking cessation. Physicians should take measures to avoid thrombus formation in the perioperative period because smoking cessation can induce temporary hyper-activation of platelets in the short term. A longer duration of preoperative smoking cessation is likely to be desirable, and anesthesiologists should recommend the cessation of smoking as soon as possible before surgery. In addition, the incidence of complications such as mortality or pulmonary complications does not increase with short-term smoking cessation (36,37), so it is not necessary to postpone an operation even if patients have only stopped smoking for a short while.

Several limitations associated with the present study warrant mention. Both the self-assessment of patients and the CO concentration of breathing were used for the confirmation of smoking cessation. However, the expired CO concentration in smokers who have refrained from smoking for >8 h is almost as low as that in non-smokers. Therefore, the confirmation of complete quitting smoking during the 12-week period depended largely on the self-report of patients. Some patients had remarkable medical histories, such as hypertension, diabetes and hyperlipidemia or anti-platelet medication, which may have affected the platelet aggregation.

In conclusion, our results strongly suggest that smoking cessation causes the temporary hyper-activation of human platelets in the short term (4 to 8 weeks). Our present findings suggest that the incidence of complications due to hyper-reactivity of human platelets can be reduced by paying attention to the smoking abstinence period. We should give preoperative patients clear and strong advice to quit smoking as soon as possible and provide educational messages about the impact of smoking on surgery.

Acknowledgments

The authors would like to thank Yumiko Kurokawa (Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu, Japan) for her technical assistance.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YK and TO performed *in vitro* experiments and collected the data. MI, KT and TI drafted the manuscript and acquired the data. KN and MT performed statistical analysis. HT constructed the *in vivo* model and the associated experiments. OK and HI designed the present study, and drafted and revised the manuscript. All authors critically reviewed the manuscript and approved the final version for publication.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Gifu University Graduate School of Medicine (approval no. 23-209) and Gifu Prefectural General Medical Center (approval no. 30) All participants signed an informed consent agreement after receiving a detailed explanation of the study protocol.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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