【 ORIGINAL ARTICLE 】

Cigarette Smoking Cessation Temporarily Enhances the Release of Phosphorylated-HSP27 from Human Platelets

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Abstract:

Objective Cigarette smoking is a risk factor for arteriopathy, including acute coronary syndrome, stroke and peripheral vascular disease. Thus, cessation is strongly recommended in order to reduce these risks. We recently demonstrated that smoking cessation causes temporary hyper-aggregability of human platelets. We previously showed that heat shock protein 27 (HSP27) is released from human platelets stimulated by collagen, accompanied by its phosphorylation. Accumulating evidence indicates potent roles of extracellular HSP27 as a modulator of inflammation. In the present study, using the stored samples obtained in the previous study, we investigated the effect of cigarette smoking cessation on the release of phosphorylated-HSP27 from collagen-activated human platelets (n=15 patients).

Methods We enrolled patients who visited smoking cessation outpatient services between January 2012 and November 2014. Platelet-rich plasma, chronologically obtained before and after the cessation, was stimulated by collagen using a PA-200 aggregometer in the previous study. The levels of phosphorylated-HSP27 released from platelets were determined by an enzyme-linked immunosorbent assay. The phosphorylation of HSP27 in platelets was evaluated by a Western blot analysis.

Results Cessation of cigarette smoking significantly upregulated the levels of collagen-stimulated release of phosphorylated-HSP27 at four and eight weeks after quitting smoking compared to before cessation. However, there was no significant difference between the levels before cessation and those at 12 weeks after cessation. The levels of phosphorylated-HSP27 stimulated by collagen in the platelets at four weeks after smoking cessation were remarkably enhanced compared to before cessation.

Conclusion Cigarette smoking cessation temporarily enhances the collagen-stimulated release of phosphorylated-HSP27 from human platelets in the short term.

Key words: smoking cessation, platelet, collagen, HSP27, phosphorylation

Introduction

Cigarette smoking is established as a crucial risk factor causing myocardial infarction, brain stroke and peripheral vascular diseases (1-4). It is recognized that smoking evokes vascular thrombosis by altering the functions of platelets and vascular endothelial cells (2). Smoking reportedly affects the dynamics of clot formation, leading to clots that are more resistant to thrombolysis than those in non-smokers (5, 6).

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Thus, smoking cessation is strongly recommended in order to reduce the risks of cardiovascular diseases and mortality (7-9).

Quitting smoking is also recommended for patients who are scheduled to undergo surgery because of the increased risk of the postoperative complications, such as pneumonia, wound-healing distress and even mortality (10-15). With regard to the relationship between cigarette smoking and human platelets, it has been reported that platelets from habitual smokers exhibit increased spontaneous aggregation compared with those of non-smokers (16), and the activity of platelets in smokers is said to be much higher than in non-smokers (17). Collagen is well recognized as a strong stimulator for human platelet activation, leading to the release of other stimulators, such as thromboxane A2 and ADP (18-20). Regarding the function of human platelets, it has been shown that a mere two weeks' cessation reduces the ADP- and collagen-induced platelet aggregability by decreasing the oxidative stress (21), whereas the elevated platelet aggregability in long-term smokers is reportedly prolonged at four weeks after cessation. (22). A recent study from our laboratories showed that smoking cessation temporarily deteriorates the aggregability of platelets stimulated by collagen in the short term (23). However, the details behind the influence of cigarette smoking cessation on the human platelet functions have not yet been fully clarified.

Heat shock proteins (HSPs), which are induced in cells in response to various environmental stresses, such as chemical, metabolic and pathophysiological stresses, facilitate the re-folding of nonnative or stress-accumulated unfolded intracellular proteins as molecular chaperons (24, 25). Based on the systematic gene symbols, HSPs are currently classified into seven major groups: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPD/E (HSP60/HSP10), CCT and HSPB (small HSP) (24). HSP27, which belongs to the HSPB group, is ubiquitously expressed in a variety of cells, including human platelets, and accepts post-translational modification, such as phosphorylation (25). Human HSP27 can be phosphorylated at three serine residues: Ser-15, Ser-78 and Ser-82 (26). p38 mitogen-activated protein (MAP) kinase is well known to regulate the phosphorylation of HSP27 (27). In addition to chaperon activity, it is generally established that HSP27 is involved in various other biological activities, including membrane stability, actin polymerization, proinflammatory gene expression and apoptosis (25, 28).

Accumulating evidence indicate the potential roles of extracellular HSP27, which is recognized as a biomarker of various diseases, such as chronic obstructive pulmonary disease and diabetic neuropathy (29). Extracellular HSP27 reportedly acts as a potent modulator of inflammatory responses (29). We previously showed that the release of phosphorylated HSP27 from collagen-stimulated human platelets is observed in patients with type 2 diabetes mellitus, a major risk factor for atherosclerosis (30). In our recent study (23), we demonstrated that smoking cessation transiently up-regulates p38 MAP kinase, accompanied by hyper-aggregability of collagen-activated human platelets. These findings prompted us to speculate that quitting cigarette smoking might affect the release of phosphorylated HSP27 from the activated platelets.

In the present study, we investigated the effect of cigarette smoking cessation on the release of phosphorylated-HSP27 from human platelets stimulated by collagen, using the stored samples obtained in our previous study (23). We demonstrated that smoking cessation temporarily enhances the phosphorylation of HSP27 in human platelets stimulated by collagen, resulting in the short-term upregulation of the release of phosphorylated-HSP27.

Materials and Methods

Materials

Collagen was purchased from Takeda Austria (Linz, Austria). Anti-phospho-specific HSP27 (Ser-78) antibodies and a phosphorylated-HSP27 (Ser-78) enzyme-linked immunosorbent assay (ELISA) kit were obtained from Enzo Life Sciences, (Farmingdale, USA). Anti-HSP27 antibodies were purchased from Stressgen Biotechnologies (Victoria, Canada). Other materials and chemicals were obtained from commercial sources.

Subjects

Subjects were identical to those of our previous study, and the details were described in that report (23). In brief, we enrolled 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 (n=15 patients). We confirmed smoking cessation via patients' self-assessment and evaluated the concentration of exhaled carbon monoxide (CO). Blood samples drawn from the antecubital vein were sequentially donated 4 times as follows: before smoking cessation and 4, 8 and 12 weeks after cessation. Platelet-rich plasma (PRP) was obtained from blood samples immediately combined with 1/10 volume of 3.8% sodium citrate by centrifugation at $155 \times g$ for 12 minutes at room temperature.

This study was approved by the Ethics Committee of Gifu University Graduate School of Medicine and Gifu Prefectural General Medical Center. Written informed consent was obtained from all participants after receiving a detailed explanation of the study.

Platelet stimulation and protein preparation after stimulation

PRP was stimulated by 3 μg/mL of collagen for 5 minutes in an aggregometer (PA-200 apparatus; Kowa, Tokyo, Japan) at 37℃ with a stirring speed of 800 rpm, as described previously (23, 30). After the stimulation by collagen, platelet aggregation was terminated by the addition of an ice-cold ethylenediaminetetraacetic acid (EDTA) (10
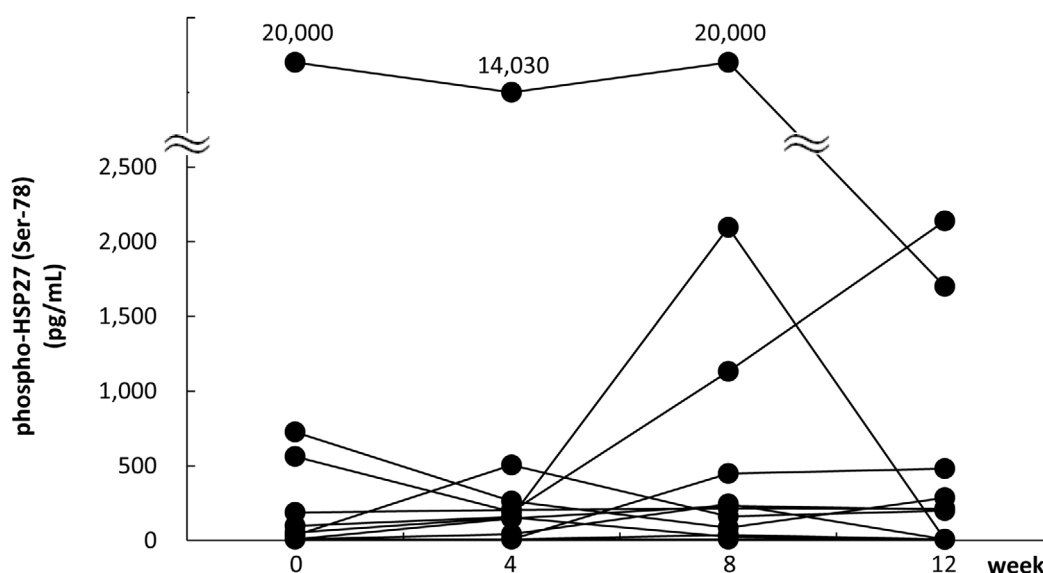
Figure 1.   **Effect of smoking cessation on the collagen-induced release of phosphorylated-HSP27 from human platelets. The net increase in phosphorylated-HSP27 released from collagen-stimulated (3 μg/mL) human platelets obtained from 12 patients at the indicated point - before smoking cessation (0) and 4, 8 and 12 weeks after smoking cessation - are shown. Values above the upper limit (20,000 pg/mL) are plotted as 20,000 pg/mL. For the statistical analysis, in values exceeded the upper limit, 20,000 pg/mL was adopted instead of the real value. The Friedman test revealed no statistical significance. HSP27: heat shock protein 27**

mM) solution. The reaction mixture was collected and centrifuged at 10,000×g at 4℃ for 2 minutes. The supernatant was collected for the ELISA and stored at -30℃. The pellet lysed by boiling in a lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol was collected for a Western blot analysis and stored at -30℃. The samples obtained in the series of the previous study (23) were used.

Western blotting

A Western blot analysis was performed as described previously (30). In brief, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed via the method described by Laemmli using a 10% polyacrylamide gel (31). The proteins in the gel were transferred onto a polyvinylidene fluoride membrane, which was then blocked with 5% fat-free dry milk in phosphate-buffered saline with 0.1% Tween 20 (PBS-T; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4, 137 mM NaCl, 2.7 mM KCL and 0.1% Tween 20) for 2 hours before incubation with anti-phospho-specific HSP27 (Ser-78) antibodies or anti-HSP27 antibodies as primary 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 optimal concentrations with 5% fat-free dry milk in PBS-T. The peroxidase activity on the membranes was visualized on X-ray film using an ECL Western blotting detection system (GE Healthcare, Buckinghamshire, UK) as described in the manufacturer's instructions.

Measurement of the release of phosphorylated-HSP 27

The levels of phosphorylated-HSP27 in the supernatant of the conditioned mixture after platelet aggregation were determined using an ELISA kit for phosphorylated-HSP27 (Ser-78) in accordance with the manufacturer's instructions.

Statistical analyses

The data are presented as box plots representing the median ±25th and 75th percentile values. The data were analyzed by Friedman's test followed by Wilcoxon's rank sum test for multiple comparisons using the Bonferroni method. Statistical analyses were performed using the SPSS software program, ver. 19.0 (IBM Japan, Tokyo, Japan). A probability of less than 5% was considered to be statistically significant.

Results

Effect of smoking cessation on the collagen-induced release of phosphorylated-HSP27 from human platelets

Among the 15 patients who were also included in the previous study (23), 2 were excluded (Pt. 8 and 11) because their samples were unsuitable for usage in the platelet function analysis (because of highly turbid PRP due to lipidemia) In addition, one patient (Pt. 5) whose sample volume was insufficient for an ELISA was excluded. After the exclusion, the samples from 12 patients were ultimately sub-
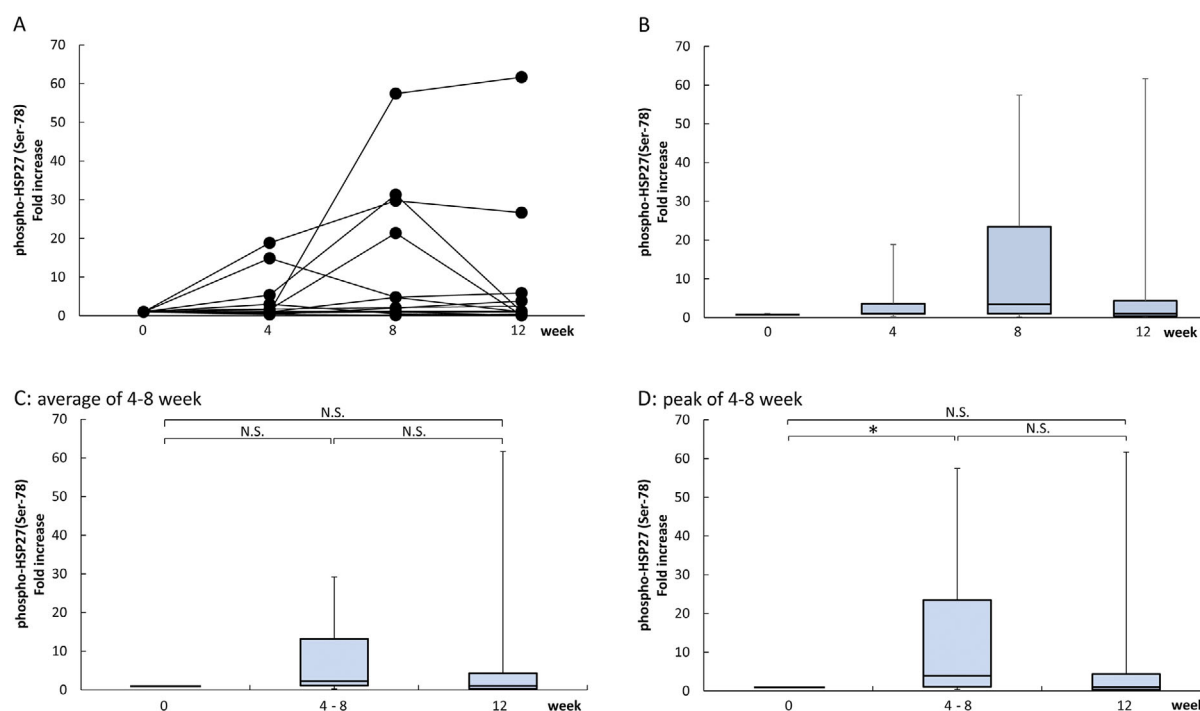
Figure 2. **Effect of smoking cessation on the collagen-induced release of phosphorylated-HSP27 from human platelets: change from the baseline. The collagen-induced net increase in the levels pre-smoking cessation is represented as 1, and the levels at 4, 8 and 12 weeks after smoking cessation are expressed as the fold increase compared to the pre-smoking cessation value. (A) The line graph shows the values before smoking cessation and at 4, 8 and 12 weeks after smoking cessation in each case (n=12). (B) Each boxplot shows the values before smoking cessation and the values at 4, 8 and 12 weeks after smoking cessation (n=12). The Friedman test revealed no statistical significance. (C) Each boxplot shows the pre-smoking cessation value, the average of 4 and 8 weeks after smoking cessation, and the values at 12 weeks after smoking cessation (n=12). The Friedman test revealed no statistical significance. N.S. indicates no significant difference between the indicated pairs. (D) Each boxplot shows the pre-smoking cessation value, the peak level at 4 or 8 weeks after smoking cessation, and the values at 12 weeks after smoking cessation (n=12). *p<0.05, significant difference between the indicated pairs. N.S. indicates no significant difference between the indicated pairs. HSP27: heat shock protein 27**

jected to an ELISA.

We previously showed that HSP27 is released from human platelets, after its phosphorylation is stimulated by collagen (30). Therefore, we first examined the effect of smoking cessation on the collagen-induced release of phosphorylated-HSP27 from human platelets. All data from the 12 patients were plotted and shown in Fig. 1. The results of a statistical analysis by the Friedman test showed no significant difference in the data among these patients. The levels of phosphorylated-HSP27 released from collagen-stimulated human platelets were widely distributed, even at the baseline.

We next analyzed the collagen-induced levels of phosphorylated-HSP27 released from human platelets compared to the baseline levels. The levels of collagen-induced phosphorylated-HSP27 released at four and eight weeks after smoking cessation seemed to be higher than those pre-smoking cessation (Fig. 2A), although there was no statistical significance (Fig. 2B). We presumed that the level of collagen-induced phosphorylated-HSP27 release peaked dur-

ing this period. Therefore, for further analyses, we decided to combine the data from four and eight weeks after smoking cessation.

Regarding the average values of released phosphorylated-HSP27 of four and eight weeks after smoking cessation, the fold-increase values were not significantly different from those pre-smoking cessation (Fig. 2C). In addition, there was no significant difference between the average values and the values at 12 weeks after cessation (Fig. 2C). Based on the changes in the phosphorylated-HSP27 release between 4 and 8 weeks after smoking cessation among individual patients, the value of phosphorylated-HSP27 released between 4 and 8 weeks increased in 9 cases but decreased in 3 cases among the 12 total patients. Therefore, we decided to adopt the higher value of released phosphorylated-HSP27 observed at 4 or 8 weeks after smoking cessation as the peak value and compared it with the values pre-smoking cessation or at 12 weeks after smoking cessation. The peak values at four and eight weeks were significantly higher than that before smoking cessation (Fig. 2D). However, there was no signifi-
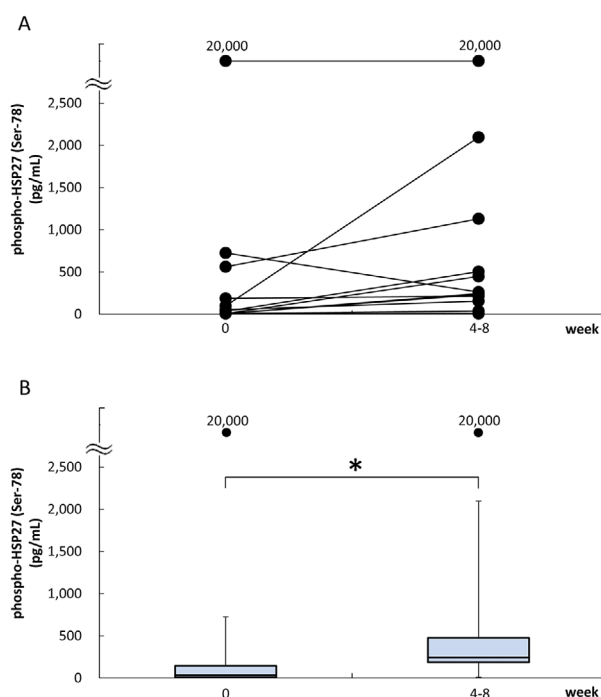
Figure 3. Effect of smoking cessation on the collagen-induced release of phosphorylated-HSP27 from human platelets: A comparison between the pre-smoking cessation value and the peak level at 4 or 8 weeks after smoking cessation. The net increase in phosphorylated-HSP27 released from collagen-stimulated (3 μg/mL) human platelets obtained from 12 patients before smoking cessation and the peak value of phosphorylated-HSP27 at 4 or 8 weeks after smoking cessation are shown in a line graph (A) and box plots (B). Values above the upper limit (20,000 pg/mL) are plotted as 20,000 pg/mL. For the statistical analysis, in values exceeded the upper limit, 20,000 pg/mL was adopted instead of the real value. All 12 cases are included in the statistical analysis. *p<0.05, significant difference between the indicated pairs. HSP27: heat shock protein 27

cant difference between these peak values at four and eight weeks and the value at 12 weeks after cessation (Fig. 2D).

We further compared the peak levels of released phosphorylated-HSP27 at four and eight weeks after smoking cessation with those before smoking cessation. The peak levels of released phosphorylated-HSP27 at four and eight weeks were significantly higher than those pre-smoking cessation (Fig. 3).

Effect of smoking cessation on the collagen-induced phosphorylation of HSP27 in human platelets

We previously showed that collagen induces the phosphorylation of HSP27 in human platelets, leading to the subsequent release of the phosphorylated-HSP27 into the plasma (30). We therefore examined the effect of smoking cessation on the collagen-stimulated phosphorylation of HSP 27 (Ser-78) in human platelets. Regarding the baseline values of phosphorylated HSP27, the levels observed at 4 and 12 weeks after smoking cessation were lower than those pre-smoking cessation (Fig. 4, Lane 1). In contrast, the levels of phosphorylated HSP27 from the platelets stimulated by 3 μg/mL of collagen observed at 4 weeks after smoking cessation were markedly amplified compared to those pre-smoking cessation, whereas the levels were gradually decreased by 12 weeks (Fig. 4, Lane 2)

Discussion

In the present study, we investigated the effect of smoking cessation on the release of phosphorylated-HSP27 from human platelets stimulated by collagen. We showed that the cessation of smoking for 4 and 8 weeks significantly upregulated the release of phosphorylated-HSP27 from activated human platelets compared to pre-cessation, while the
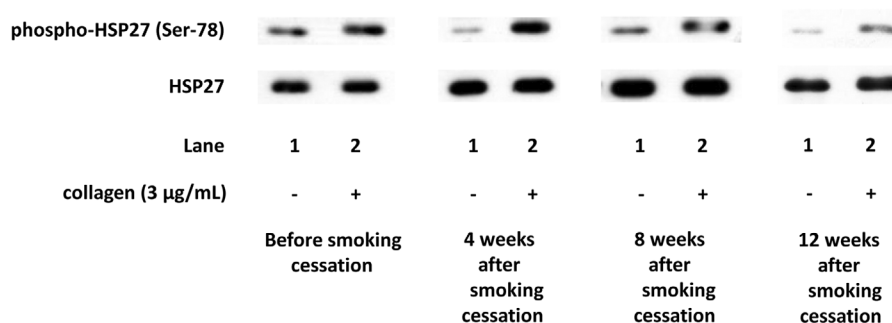

Figure 4. Effect of smoking cessation on the collagen-stimulated phosphorylation of HSP27 in human platelets. PRP obtained at indicated points was stimulated by 3 μg/mL of collagen or vehicle for 5 min, and the reaction was terminated by the addition of ice-cold EDTA solution in the series of the previous study. The lysate of platelets was harvested and subjected to SDS-PAGE with a Western blot analysis using antibodies against phospho-specific HSP27 (Ser-78) or HSP27. The representative results obtained from patient No. 6 are presented. HSP27: heat shock protein 27, PRP: platelet-rich plasma, EDTA: ethylenediamine tetra acetic acid, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

release at 12 weeks after cessation was not markedly affected. To our knowledge, this is the first report showing the temporary amplification of the release of phosphorylated-HSP27 from activated human platelets by smoking cessation.

Regarding protein kinases involved in the phosphorylation of HSP27, it is generally known that p38 MAP kinase positively regulates the phosphorylation of HSP27 (27). In our previous study (23), we showed that smoking cessation enhances the p38 MAP kinase activation in collagen-stimulated platelets observed at four and eight weeks after cessation in association with the temporary deterioration of hyper-aggregability.

In the present study, we showed that smoking cessation markedly strengthened the levels of phosphorylated HSP27 in the collagen-stimulated platelets compared to those observed pre-smoking cessation. Therefore, our findings suggest that the enhancement of the phosphorylated-HSP27 release from human platelets after smoking cessation is closely related to the temporary hyper-aggregability and that p38 MAP kinase regulates the enhancement. However, regarding the levels of phosphorylated HSP27 in resting platelets without stimulation, the levels observed at 4 and 12 weeks after smoking cessation were lower than those pre-smoking cessation. These changes likely reflect the favorable effect of abstinence, which results in an improvement from the upregulated platelet function caused by smoking.

Accumulating evidence indicates that HSP27 acts not only intracellularly as a molecular chaperon but also extracellularly as a modulator of inflammation (29). HSP27 reportedly stimulates the activation of nuclear factor kB in macrophages, leading to the secretion of anti-inflammatory cytokines, including IL-10 (32). HSP27 is therefore suspected to function as an atheroprotective agent by reducing the uptake of atherogenic lipids and attenuating inflammation (33). In contrast, extracellular HSP27 reportedly acts as a proinflammatory agent through the secretion of toll-like receptor (TLR)-2 and TLR-4 in mouse coronary vascular endothelial cells (34). Therefore, it is currently recognized that extracellular HSP27 is a potent modulator of inflammation (29).

Regarding the relationship between HSP27 and smoking, it has recently been shown that the plasma levels of HSP27 are higher in habitual smokers than in non-smokers and that the levels of plasma HSP27 in former smokers are almost within the normal range (35). Based on these present and previous findings, it seems that the temporary enhancement of phosphorylated-HSP27 release from human platelets after smoking cessation might be implicated in the deterioration of atherosclerotic pathophysiology through modulating inflammation progress. In addition, elevated levels of phosphorylated-HSP27 in plasma might be a useful indicator of platelet hyper-activation in the context of smoking cessation that can alert physicians to a potential risk of thrombus formation due to temporarily deteriorated platelet hyper-aggregability after smoking cessation. While the cessation of smoking is generally recommended in order to reduce post-operative complications (10-15), to our knowledge, there have been no reports showing that the incidence of complications, such as mortality and cardiovascular events, is affected by short-term smoking cessation (36, 37).

Although the relationship between the levels of extracellular HSP27 and the outcome of surgery remains unclear, the cessation of smoking as soon as possible should be encouraged for patients scheduled to undergo surgery in light of the platelet function after quitting cigarette shown in the present study. Careful follow-up during the abstinence period will likely help reduce the incidence of complications after smoking cessation. Further investigations are required to clarify the exact roles of extracellular HSP27 released from human platelets after smoking cessation.

In addition, we combined the data from four and eight weeks after smoking cessation as the peak value, leading to statistical significance despite the limited sample numbers. Although the large number of examinations may provide supporting evidence regarding the exact period of an elevated release of phosphorylated-HSP27, the analytical process used in the present study should be mentioned as a limitation.

In conclusion, our results strongly suggest that smoking cessation temporarily enhances the collagen-stimulated release of phosphorylated-HSP27 from human platelets in the short term. We should give patients clear and strong advice to quit smoking, regardless of timing, and pay attention to the abstinence period as well.

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