Inflamm. res. 58 (2009) 306 – 311
1023-3830/09/060306-6
DOI 10.1007/s00011-008-8084-8

Inflammation Research

CD40/CD154 system and pro-inflammatory cytokines in young healthy male smokers without additional risk factors for atherosclerosis

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Received 1 October 2007; returned for revision 5 November 2007; received from final revision 28 April 2008; accepted by G. Wallace 9 June 2008

Published Online First 4 February 2009

Abstract. *Objective and design:* Atherosclerosis, as inflammatory disease, is characterized by pathologically altered levels of cytokines. We investigated whether smoking affects the CD40/CD154 system and pro-inflammatory cytokines in young males without other risk factors for atherosclerosis.
Subjects: Young male smokers (n=13) and 14 non-smoking controls were investigated.
Methods: The differences in CD40/CD154 system and serum cytokines between the groups were measured using flow cytometry and ELISA.
Results: In smokers, there was a strong trend ($P<0.06$) for increased CD40 expression on platelets as compared with non-smokers. However, there were no significant differences in CD40 expression on monocytes or in CD154 expression on platelets and T-cells between smokers and non-smokers. There was a strong trend for increased platelet-monocyte aggregates in smokers ($P<0.06$). Also, smokers had slightly but not significantly elevated hsCRP and IL-6 levels, and slightly decreased TNF-α and MCP-1. Interestingly, IL-18, a cytokine which has the ability to promote both Th1 and Th2 responses, was significantly decreased in smokers group ($P=0.03$ vs controls).
Conclusions: In young healthy males, smoking is not associated with dramatic changes in CD40/CD154 system. However, cigarette smoke alters the secreted cytokine profile, leading to significant decrease in systemic IL-18 levels.

Key words: Smoking – CD40/CD154 – Platelets – Cytokines – Interleukin-18

1. Introduction

Atherosclerosis, the leading cause of death in the Western world, is considered an inflammatory disease. Progressive stages of atherosclerosis are associated with enhanced activation of monocytes, T-cells, and platelets. Moreover, elevated levels of inflammatory cytokines and adhesion molecules have been shown in patients with coronary heart disease [1, 2].

One of the potent immune mediators involved in thrombosis and atherosclerosis is CD40, and its counterpart CD40 ligand (CD154). CD40, an important activation receptor, is over-expressed in human and experimental atherosclerotic lesions, especially in advanced plaques [3]. It had been originally identified on B lymphocytes, and was later found to be expressed constitutively on monocytes/macrophages, smooth muscle cells, and endothelial cells. Activation of CD40 by its ligand CD154 triggers the release of pro-inflammatory cytokines, adhesion receptors, tissue factor, metalloproteinases and prostaglandins [4, 5]. CD154, a transmembrane protein structurally related to TNF-α, is expressed on T lymphocytes, mast cells, basophils, and platelets. Rapid upregulation of CD154 on activated platelets leads to the release of soluble CD154 (sCD154) and inflammatory response in cells constitutively expressing CD40. Therefore, platelet activation generates signal for recruitment of leukocytes to the sites of injury and can rapidly activate the coagulation cascade and inflammatory response by interactions with cells of the vessel wall. Accordingly, increased platelet activation and elevated levels of CD154 were shown in patients with acute cerebral symptoms, myocardial infarction, and hypercholesterolemic subjects [6, 7, 8, 9].

As several cohort studies demonstrated, smoking is one of the main risk-factors for atherosclerosis [10, 11].

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Smoking has been associated with elevation of a number of inflammatory markers, such as C-reactive protein (CRP), fibrinogen, and homocysteine [12], and with progression of atherosclerosis [13]. Moreover, smoking has been shown to enhance platelet activation and aggregation [14, 15]. Previous study by Harding et al. showed a moderate upregulation of CD40/CD154 dyad in smokers [16]. However, the group of interest in that study comprised both male and female middle-aged smokers, with significantly decreased levels of HDL cholesterol as compared with non-smoking controls. Therefore, the aim of our study was to determine whether the up-regulation of CD40/CD154 system occurs already in a younger population (mean age 25.1 ± 3.5 years) of healthy male smokers without any additional risk factors for atherosclerosis. Furthermore, pro-thrombotic and pro-inflammatory parameters closely linked with CD40/CD154 system have been compared in young smokers and non-smoking controls.

2. Materials and Methods

2.1. Patients and controls

In this study, 13 young male smokers (mean age 25.1 ± 3.5 years, cigarettes ~ 20 per day) and 14 sex- and age-matched healthy controls were investigated between April 2002 and August 2003.

Smokers as well as non-smokers had no clinical evidence of a peripheral coronary or cerebral atherosclerotic or inflammatory disease, no atherosclerotic risk profile except the smoking, and no acute inflammatory process such as coughing, sore throat, or other complaints (by clinical history and physical examinations) within the preceding 2 weeks. None of the subjects was taking any drugs (including anti-inflammatory and anti-histaminic drugs). No drinks or foods containing alcohol were permitted within 3 days before blood samples were taken. Smokers had to restrain from cigarette consumption for at least 8 hours before the blood sampling.

Further exclusion criteria for both groups were: renal insufficiency, proteinuria, altered hepatic function, chronic alcohol abuse, and a body mass index (BMI) > 25. Subjects with diabetes mellitus, hypertension (systolic blood pressure > 140 mmHg, diastolic blood pressure > 90 mmHg and/or antihypertensive treatment), and hypercholesterolemia were also excluded.

After overnight fasting and a rest of at least 20 minutes, blood samples were taken for laboratory tests (between 8–10 a.m.). The study was approved by local ethics committee, and written informed consent was obtained from all subjects.

2.2. Blood sampling protocol

Peripheral venous blood was drawn into blood collection tubes containing sodium citrate. Citrated blood samples were either centrifuged (190 *g* for 10 minutes at room temperature) to obtain platelet-rich plasma (PRP) or immediately fixed with 1 % formaldehyde (1:1, v:v). A separate aliquot of blood without any additives was put into the refrigerator and allowed to clot for 1 hour before centrifugation (1500 *g* for 10 minutes). The serum supernatant was stored at –80°C until analysis. Samples were thawed only once. Serum albumin, total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL) were measured by standard laboratory techniques.

2.3. Flow cytometry

The following antibodies were used: fluorescein isothiocyanate (FITC)–conjugated anti-CD154 (CBL 543F, IgG_{2a}) and phycoerythrin (PE)-conjugated anti-CD4 (RFT-4, IgG_1) from Dianova, Germany, and anti-CD62P (FITC, mouse IgG_1), anti-CD40 (FITC, 5C3, IgG_1), anti-CD25 (FITC, M-A251, IgG_1), mouse IgG_{2a} (FITC, G155–178), mouse IgG_1 (FITC, MOPC-21), anti-CD41a (PE, HIP8, IgG_1), mouse IgG_1 (PE, MOPC-21), anti-CD18 (PE, 6.7, IgG_1) from PharMingen, Germany.

Platelet immunostaining was performed as previously described [9]. Fixed blood was diluted 1:50 with phosphate buffered saline (PBS, pH 7.4) for at least 15 minutes and incubated with antibodies (anti-CD154, anti-CD62P, anti-CD40 and anti-CD41a) for 30 minutes at room temperature. In PRP, the number of platelets was adjusted to 20 000/μL using PBS. Aliquots of PRP were mixed with appropriate antibodies and incubated for 30 minutes at room temperature. Before analysis, cells were diluted with 800 μL PBS and a total of 10 000 cells was measured by flow cytometry (FACS Calibur, Becton Dickinson) within 2 hours after sampling and analysed by CellQuest Software (Becton Dickinson). FITC and PE conjugated mouse IgG_1 and FITC conjugated IgG_{2a} served as isotype controls. Fluorescence calibration was achieved using calibration beads (Calibrite BEADS, Becton Dickinson). Data is expressed as net mean fluorescence (specific binding minus unspecific binding [isotype]) if not stated otherwise. Platelets were identified with anti-CD41a, which labelled all platelets, and gated according to staining for the platelet specific antigen and their characteristic light scatter. The platelet population evaluated was found to be ~ 98 % positive for CD41a. Before starting the study, preliminary experiments had excluded the alterations of the expression of any of the measured epitopes by the fixation procedure with 1 % formaldehyde.

For the evaluation of CD40 on leukocytes, fixed blood was diluted 1:5 and incubated with anti-CD40 and anti-CD18 (for identification of granulocytes) for 30 minutes at room temperature. To determine T-cell activation, blood was incubated with anti-CD4 (for identification of $CD4^+$ T-cells), anti-CD25 (for identification of interleukin-2 receptor as a marker of T-cell activation) and anti-CD154. Erythrocytes were removed by adding 1 mL FACS lysis solution (PharMingen) for 30 minutes at room temperature. Leukocytes were washed twice with PBS and fixed in 1 % formaldehyde in PBS. T-cells were identified by gating for $CD4^+$-cells.

Adhesion of platelets to monocytes and granulocytes was measured as previously described [17]. Briefly, 100 μL citrate-anticoagulated blood was diluted with PBS (1:1). Samples were incubated with anti-CD41a. After 30 minutes of incubation at room temperature, 1 mL FACS lysis solution was added for 30 minutes at room temperature to lyse erythrocytes. The cells were washed twice with PBS and fixed in 1 % formaldehyde, following which a total of 5000 cells was analysed by flow cytometry. Platelet-monocyte aggregates were identified by gating on the monocyte population, platelet-granulocyte aggregates by gating on the granulocyte population.

2.4. Measurement of serum cytokines

The levels of serum interleukin (IL)-1β, IL-6, IL-18, tumor necrosis factor-alpha (TNF-α), and monocyte chemotactic protein-1 (MCP-1) were analyzed in duplicates using commercially available ELISAs (IL-1β: detection limit 0.31 pg/mL, Biosource, Belgium; IL-6: detection limit 0.016 pg/mL, R&D, Germany; IL-18: detection limit 12.5 pg/mL, MBL, Japan; TNF-α: detection limit 7 pg/mL, R&D, MCP-1: detection limit 5 pg/mL, R&D) according to the manufacturers' instructions.

Concentrations of sCD154 in plasma were determined using a commercially available enzyme immunoassay, according to the manufacturer's instructions (Bender MedSystem, Austria; detection limit 95 pg/mL). High-sensitivity CRP (hsCRP) was measured using a standard laboratory technique.

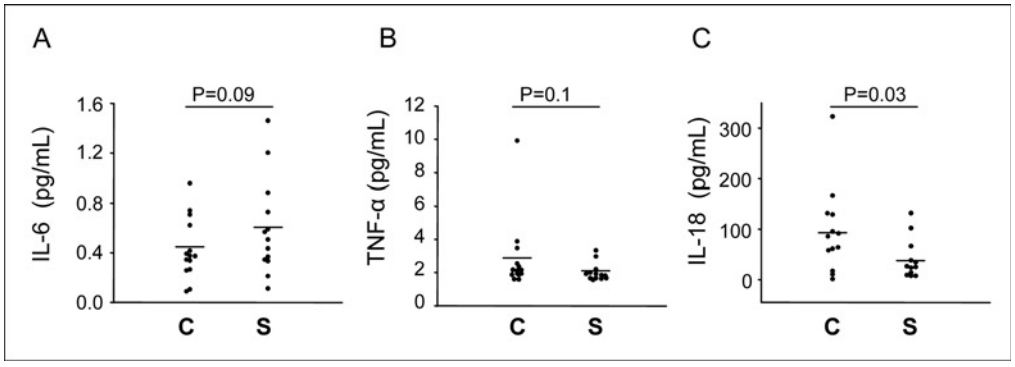

Fig. 1. Changes in cytokine profile in young smokers as compared with age- and gender- matched controls. (A) IL-6; (B) TNF-α; (C) IL-18. C, controls, n=14; S, smokers, n=13.

Table 1. Baseline characteristics of non-smokers and current smokers

	Non-smokers (n=14)	Current smokers (n=13)	P value
Age, y / [range]	24.8 ± 2.0 / [22–29]	25.1 ± 3.5 / [20–33]	ns
Male sex, %	100	100	ns
Body mass index, kg/m^2	23.2 ± 1.4	22.4 ± 2.0	ns
Cigarettes/day	-	18.9 ± 5.7	<0.0001
Smoking period, y	-	7.8 ± 2.3	<0.0001
Systolic blood pressure, mmHg	120.7 ± 10.9	122.3 ± 10.9	ns
Diastolic blood pressure, mmHg	80.0 ± 6.8	78.9 ± 11.2	ns
Albumin, mg/dL	46.3 ± 1.3	46.7 ± 2.6	ns
Triglycerides, mg/dL	114.9 ± 60.5	116.1 ± 57.3	ns
Total cholesterol, mg/dL	187.6 ± 31.5	194.4 ± 29.3	ns
LDL cholesterol, mg/dL	124.0 ± 28.8	123.3 ± 22.1	ns
HDL cholesterol, mg/dL	45.9 ± 11.0	51.9 ± 14.1	ns
LDL/HDL ratio	2.9 ± 1.1	2.6 ± 1.9	ns

Data are mean ± SD; ns, not significant.

2.5. Statistical analyses

The data were analysed by non-parametric methods to avoid assumptions about the distribution of the measured variables. Comparisons between groups were done using the Mann-Whitney *U* test. The association of measurements with other biochemical parameters was assessed by the Spearman rank correlation test. All values are reported as mean ± SD. Statistical significance was indicated by the value of $P < 0.05$.

3. Results

The baseline characteristics of non-smokers and current smokers are given in Table 1. Both groups were comparable with respect to age, sex, BMI, blood pressure, risk factor profile, serum albumin, and lipid profile. Importantly, there were no significant differences between the two groups in total cholesterol, LDL cholesterol or LDL/HDL ratio, which were previously described to affect CD40/CD154 system [9]. There were no significant associations between age, BMI, or lipid levels and CD40/CD154 expression.

In the serum analyses, young smokers were found to have slightly but not significantly elevated IL-6 (0.60 ± 0.4 vs 0.43 ± 0.2 pg/mL in controls, P=0.09 Fig. 1A) and hsCRP levels as compared with non-smoking subjects (Table 2). Among other measured inflammatory markers, lower levels of MCP-1 (Table 2) and a trend for lower TNF-α was observed in smokers (2.0 ± 0.5 vs 2.8 ± 2.1 pg/mL in controls, P=0.1; Fig. 1B). Moreover, there was a significant decrease in IL-18 levels in smokers group (40.7 ± 39.9 vs 95.4 ± 84.3 pg/mL in controls; P=0.03, see Fig.1C).

As membrane-bound CD154 from T-cells and platelets can be released into circulation, we analysed sCD154 levels in both study groups. Contrary to our expectation, there were no significant differences in sCD154 levels between smokers and non-smokers (Table 2).

Expression of activation markers on platelets, monocytes, and T-cells was analysed. There were no differences between the groups in P-selectin expression on platelets (Table 3). When the percentages of platelets positive for CD154 were compared, smokers had slightly but not significantly lower CD154 expression than controls. However, there was a strong trend for increased CD40 in platelets from smokers (2.8 ± 1.3 vs 2.3 ± 0.9 in non-smokers; P<0.06). Similar results were obtained with platelets isolated from PRP (not shown). The analyses of CD40 expression on monocytes showed very similar levels of this receptor in smokers and non-smokers. Smokers had slightly increased levels of T-cell activation marker CD25 as compared with non-smoking subjects, but their CD154 expression was lower (Table 3).

To assess the effect of smoking on platelet adhesion to monocytes and granulocytes, we used flow cytometry to identify platelet-monocyte and platelet-granulocyte aggregates. In blood samples from smokers, platelet-granulocyte aggregates were slightly less frequent than in non-smokers (35.0 ± 22.0 vs 43.9 ± 38.3; P=ns). On the contrary, there was a strong trend for increased platelet-monocyte aggregation in smokers (96.8 ± 58.4 vs 69.2 ±

27.7 in non-smokers; P=0.06; see Fig. 2). However, this trend showed no correlation with platelet activation markers or with CD40 expression on monocytes.

Table 2. Plasma levels of systemic inflammatory markers among smokers and non-smokers.

	Non-smokers (n=14)	Current smokers (n=13)	P value
hsCRP, mg/L	0.91 ± 0.5	1.1 ± 1.3	ns
IL-1β, pg/mL	0.59 ± 0.5	0.61 ± 0.6	ns
MCP-1, pg/mL	75.5 ± 51.3	65.3 ± 40.2	ns
sCD154, pg/ml	14.3 ± 4.5	12.7 ± 5.1	ns

Data are mean ± SD; ns, not significant.

Table 3. CD40/CD154 and activation markers expression in smokers and non-smokers.

	Non-smokers (n=14)	Current smokers (n=13)	P value
Platelet CD154	22.4 ± 4.7	19.1 ± 6.7	ns
Platelet CD40	2.3 ± 0.9	2.8 ± 1.3	0.06
Platelet P-selectin	1.29 ± 0.7	1.22 ± 0.9	ns
Monocyte CD40	5.9 ± 3.2	5.7 ± 3.4	ns
T-cell CD25	3.7 ± 1.0	4.0 ± 0.7	ns
T-cell CD154	0.69 ± 0.5	0.44 ± 0.3	ns

Data are averages of mean fluorescence ± SD; ns, not significant

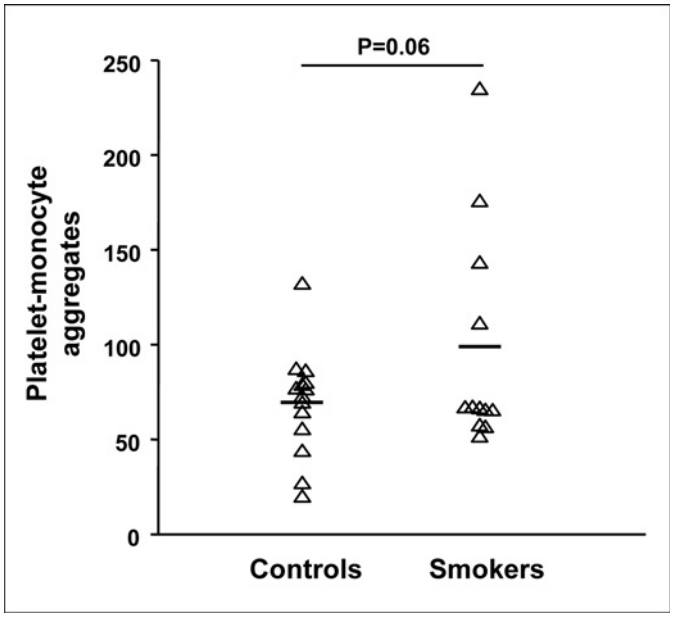

Fig. 2. Platelet-monocyte aggregates in smokers (n=12) and age- and gender-matched controls (n=14).

4. Discussion

This study was done to determine whether smoking affects the CD40/CD154 system and pro-inflammatory parameters in young male smokers without any additional risk factors for atherosclerosis. In smokers, we observed a strong trend for upregulation of CD40 expression on platelets. However, contrary to our expectations, there were no significant differences between smokers and non-smokers in CD40 expression on monocytes, or in CD154 expression on platelets and T-cells. In a recent study by Harding et al., upregulation of CD40/CD154 system was shown in older smokers (mean age 35 ± 8 y) as compared with non-smoking controls [16]. Apart from older age of the volunteers (presumably resulting in a longer smoking history), there are two main differences between that and our study. In the study of Harding et al., both controls and smokers had markedly higher BMI than our subjects, moreover, their smokers group had a significantly lower level of HDL cholesterol than controls. Therefore, the lack of CD40/CD154 up-regulation reported here may be due to relatively short smoking history of our volunteers, younger age, and the fact that their lipid profile was carefully matched with non-smoking subjects. It is not known whether age or BMI may constitute independent factors influencing CD40/CD154 system. However, although HDL cholesterol was not directly associated with CD154 up-regulation, LDL/HDL ratio was shown to correlate positively with CD40/CD154 increase in patients with moderate hypercholesterolemia [9]. Decreased HDL cholesterol in smokers group may thus partly account for the increase in CD40/CD154 reported by Harding et al., as opposed to the results of our study. The slight decrease we observed in platelet CD154 expression in the smokers group may be related to the upregulation of CD40 receptor on these cells, as the binding of CD154 to co-expressed CD40 leads to rapid cleavage and inactivation of this molecule [18].

With regard to sCD154 levels in circulation, the study of Harding et al. reported a slight, but not significant upregulation of this parameter in smokers group. In our study, there were no significant differences in sCD154 between smokers and non-smokers. However, in accordance with findings reported by Harding et al., we observed a strong trend for higher platelet-monocyte aggregation in smokers. This trend was not associated with general over-activation of platelets, as there was no increase in P-selectin or CD154 on platelets from smokers. Also, it did not depend on monocyte CD40 expression.

In our study, certain inflammation parameters such as hsCRP, IL-6, and the activation marker of T-cells were slightly increased in smokers while others (TNF-α, MCP-1) were lowered, suggesting that the compounds of the cigarette smoke are capable of modifying the profile of secreted cytokines. In the airways, smoking is known to increase IL-6 [19] and suppress the ability of macrophages to secrete pro-inflammatory cytokines such as TNF-α in response to inflammatory stimuli [20]. The results of this

study imply that overall systemic effect of smoking on pro-inflammatory cytokines may be similar to that observed in the lung. To our knowledge, this is the first report demonstrating a systemic decrease in IL-18 levels in young smokers. IL-18 is a pro-inflammatory cytokine secreted by activated macrophages. Functionally, IL-18 in synergy with IL-12 strongly induces the production of interferon-γ (IFN-γ) from T-cells and natural killer cells [21]. However, it was shown that IL-18 plays an important role in the development of both Th1 and Th2 responses, depending on a surrounding cytokine environment [22, 23], and it is able to induce IL-4, IL-13, and histamine production in basophils and mast cells [24]. In the airways, smoking has been shown to reduce the levels of secreted IL-18 [25]. It is thus possible, that similar suppressive mechanisms may be acting on systemic IL-18 production in smokers. IL-18 mRNA is expressed constitutively by peripheral blood mononuclear cells and can be upregulated by cytokines. Cigarette smoke may contain reactive compounds with ability to alter IL-18 gene expression. Furthermore, it is possible that neutrophil activation described in smokers [26, 27] leads to increased caspase-3 activity resulting in faster degradation of IL-18 [28].

With regard to the link between IL-18 and CD40/CD154 system, Takahashi et al. reported that IL-18 upregulated the expression of CD40 on monocytes [29], and this effect was inhibited by nicotine [30]. In mice, administration of IL-18 induced IgE production via upregulation of CD154 expression on T-cells [31]. Thus, our observations concerning the lack of CD40 upregulation in monocytes, and a slight decrease in CD154 levels on T-cells in young smokers may reflect the functional effect of systemic decrease in their IL-18 levels. In the light of many recent reports, it becomes clear that the differential regulation of inflammatory response greatly depends on the cytokine balance, i.e. between IL-18 and IFN-α, or between IL-18 and its dominant partner IL-12. It was recently demonstrated, that uterine inflammation in mice is enhanced by neutralization of IL-18, which leads to exaggerated Th1 response because of increased IL-12:IL-18 ratio [32]. Moreover, Gutzmer et al. have shown that preincubation of monocyte-derived dendritic cells with IFN-γ and IL-18 resulted in a significant decrease of their capacity to induce IFN-γ production in T-cells [33]. Therefore, the systemic reduction in IL-18 levels associated with smoking may, in the presence of normal IFN-γ and IL-12 levels, alter the balance of Th1/Th2 cytokine secretion towards Th1 responses and contribute to increased inflammation over the longer time period.

The limitation of this study was a relatively small size of the study groups due to very strict inclusion criteria. Further work on a larger number of subjects and longitudinal studies on subjects with longer history of smoking will be essential to determine the factors influencing cytokine alterations in smokers and the time scale of these changes. Moreover, additional studies will be necessary to analyse the likely molecular mechanisms and mediators responsible for modulating the cytokine secretion in these subjects.

In conclusion, in young male smokers without additional risk factors for atherosclerosis, smoking is not associated with an increase in CD40/CD154 system. However, even at this early stage, cigarette smoke is capable of altering the secreted cytokine profile, and leads to significant decrease in systemic IL-18 levels. Thus, it is possible that with growing age, BMI, and cigarette consumption in smokers the progressive CD40/CD154 upregulation takes place, and is accompanied by misbalanced cytokine expression, which may in long term lead to enhancement of pro-inflammatory and atherothrombotic conditions.

Acknowledgments. The authors thank Sabine Kesting for excellent technical assistance.

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