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Brief Report

THROMBOSIS AND HEMOSTASIS

Targeting myeloid-cell specific integrin α9β1 inhibits arterial thrombosis in mice

Nirav Dhanesha,[1,*] Manasa K. Nayak,[1,*] Prakash Doddapattar,[1] Manish Jain,[1] Gagan D. Flora,[1] Shigeyuki Kon,[2] and Anil K. Chauhan[1]

[1]Division of Hematology/Oncology, Department of Internal Medicine, Iowa City, IA; and [2]Department of Molecular Immunology, Faculty of Pharmaceutical Sciences, Fukuyama University, Hiroshima, Japan

KEY POINTS

- Myeloid-specific integrin α9 modulates arterial thrombosis by enhancing NETosis.

Evidence suggests that neutrophils contribute to thrombosis via several mechanisms, including neutrophil extracellular traps (NETs) formation. Integrin α9β1 is highly expressed on neutrophils when compared with monocytes. It undergoes affinity upregulation on neutrophil activation, and stabilizes adhesion to the activated endothelium. The role of integrin α9 in arterial thrombosis remains unexplored. We generated novel myeloid cell-specific integrin $\alpha9^{-/-}$ mice ($\alpha9^{fl/fl}$LysMCre^{+}) to study the role of integrin α9 in arterial thrombosis. $\alpha9^{fl/fl}$ littermates were used as controls. We report that $\alpha9^{fl/fl}$LysMCre^{+} mice were less susceptible to arterial thrombosis in ferric chloride ($FeCl_3$) and laser injury-induced thrombosis models with unaltered hemostasis. Neutrophil elastase-positive cells were significantly reduced in $\alpha9^{fl/fl}$LysMCre^{+} mice concomitant with reduction in neutrophil count, myeloperoxidase levels, and red blood cells in the $FeCl_3$ injury-induced carotid thrombus. The percentage of cells releasing NETs was significantly reduced in $\alpha9^{fl/fl}$LysMCre^{+} mouse neutrophils stimulated with thrombin-activated platelets. Furthermore, we found a significant decrease in neutrophil-mediated platelet aggregation and cathepsin-G secretion in $\alpha9^{fl/fl}$LysMCre^{+} mice. Transfusion of $\alpha9^{fl/fl}$ neutrophils in $\alpha9^{fl/fl}$LysMCre^{+} mice restored thrombosis similar to $\alpha9^{fl/fl}$ mice. Treatment of wild-type mice with anti-integrin α9 antibody inhibited arterial thrombosis. This study identifies the potential role of integrin α9 in modulating arterial thrombosis. (*Blood*. 2020; 135(11):857-861)

Introduction

In addition to being the earliest cells to arrive at the site of vascular injury, leukocytes (predominantly neutrophils) influence multiple aspects of thrombosis by facilitating a coordinated interaction with endothelial cells and platelets.[1] Depletion of neutrophils has been shown to inhibit thrombosis, which suggests their role in modulating platelet adhesion, activation, and coagulation.[2,3] NETosis is characterized by the release of neutrophil extracellular traps (NETs), which are composed of chromatin and antimicrobial proteins (histones, myeloperoxidase, elastase, and cathepsin G). Activated platelets are known to promote NETosis[4] mediated by P-selectin and high-mobility group box 1.[5,6] NETs also bind to plasma proteins such as fibrinogen, von Willebrand factor, and fibronectin, all known to contribute toward the progression of arterial thrombosis.[7]

Integrin α9 (which is absent in platelets; supplemental Figure 1, available on the *Blood* Web site) is highly expressed on neutrophils, in contrast to monocytes.[8] On activation, neutrophils undergo affinity upregulation followed by transmigration to the plasma membrane. Although the role of integrin α9 in stabilizing neutrophil adhesion to the activated endothelium is known,[8] its role in the context of arterial thrombosis remains unexplored. We sought to determine whether myeloid-cell integrin α9 regulates arterial thrombosis by modulating the release of NETs. To investigate this, we generated novel myeloid cell-specific integrin $\alpha9^{-/-}$ ($\alpha9^{fl/fl}$LysMCre^{+}) mice and evaluated arterial thrombosis using $FeCl_3$ and laser injury-induced thrombosis models. To explore the underlying mechanisms, we determined the levels of myeloperoxidase (MPO) and cathepsin G (markers of neutrophil activation), along with the extent of NET formation by neutrophils. The results of our study suggest that myeloid-specific integrin α9 modulates arterial thrombosis, most likely by promoting NETosis.

Methods

Because of the word limit, detailed methods are provided in the supplemental Data.

Mice

To generate myeloid-specific $\alpha9^{-/-}$ mice, we crossed $\alpha9^{fl/fl}$ mice with LysMCre^{+} mice (supplemental Figure 2). $\alpha9^{fl/fl}$ littermates were used as controls.

$FeCl_3$ and laser injury-induced carotid thrombosis

The $FeCl_3$ (7.5%) injury-induced carotid artery thrombosis was assessed by intravital microscopy, as described previously.[9,10]

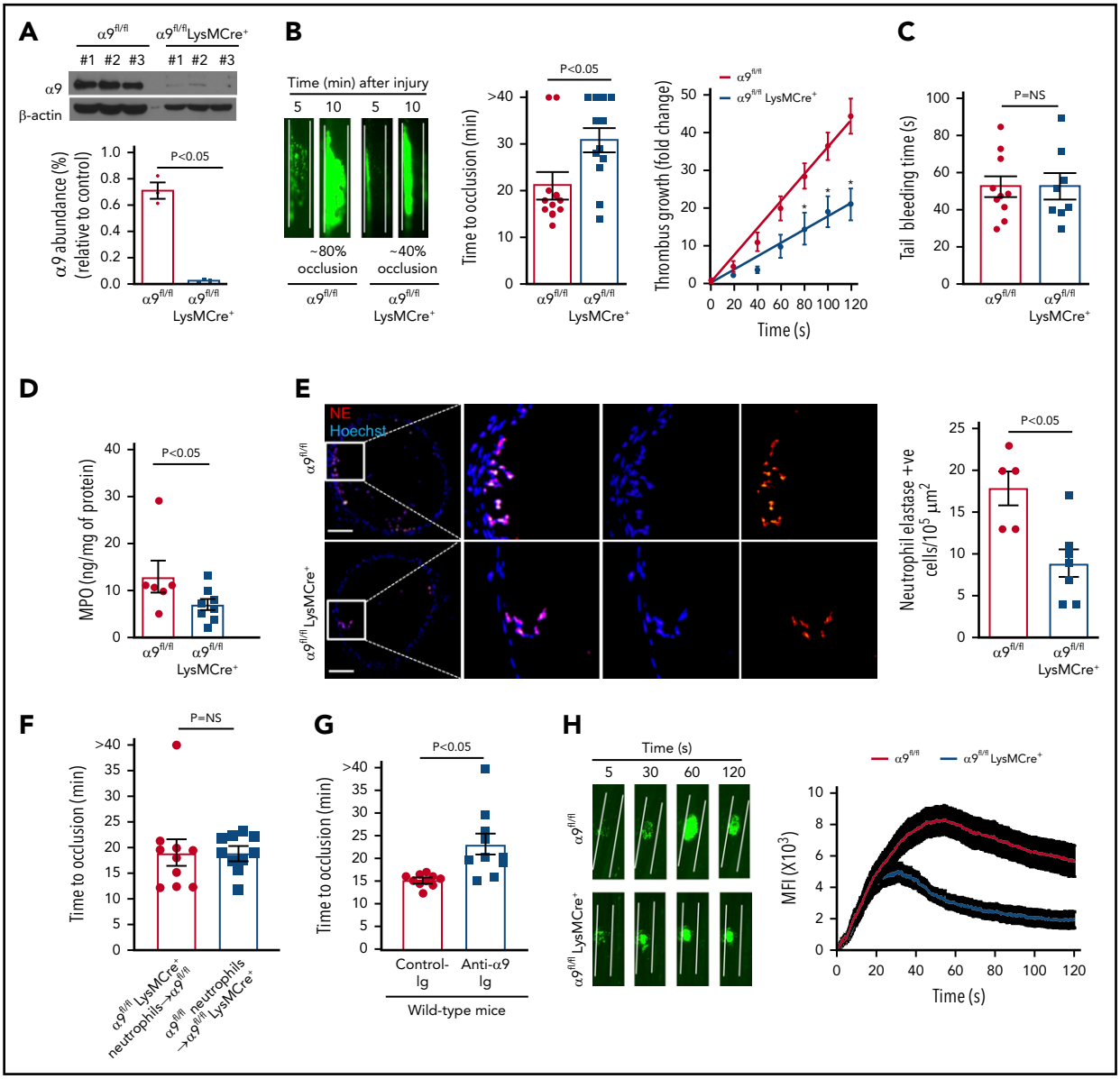

Figure 1. Myeloid specific $\alpha 9^{-/-}$ mice are less susceptible to experimental arterial thrombosis. (A) Western blot analysis of α9 integrin from bone marrow-derived neutrophils (top). Quantification (n = 3 mice/group) (bottom). (B) Representative microphotographs of carotid artery thrombus (7.5% $FeCl_3$ injury) (left). Platelets were labeled ex vivo with calcein green. Time to occlusion (n = 11-12 mice/group) (middle). Rate of thrombus growth (n = 11-12 mice/group) (right). The rate of thrombus growth over a period of 2 minutes was calculated by dividing the area of the thrombus at time (n) by the area of the same thrombus at time (0) (defined as the time point at which the thrombus diameter first reached 30 μm). Slopes over time showed that the rate of thrombus growth in the $\alpha 9^{fl/fl}$LysMCre$^+$ mice (slope, 0.1758) was decreased when compared with $\alpha 9^{fl/fl}$ mice (slope, 0.3575). *$P < .05$. (C) Tail bleeding assay (n = 8-10 mice/group). (D) MPO quantification by ELISA in carotid artery thrombus (n = 6-8 mice/group). (E) Representative immunofluorescence images of sections stained with anti-neutrophil elastase antibody. Scale bar, 100 μm. Boxed region is magnified to show colocalization of Hoechst/neutrophil elastase-positive cells (left). Quantification of neutrophil elastase-positive cells (n = 5-7 mice/group) (right). Value for each mouse represents a mean from 3 to 4 serial sections (each section separated by ~70 μm). (F) Time to occlusion in $\alpha 9^{fl/fl}$LysMCre$^+$ mice transfused with $\alpha 9^{fl/fl}$ mice neutrophils or $\alpha 9^{fl/fl}$ mice transfused with $\alpha 9^{fl/fl}$ LysMCre$^+$ mice neutrophils (n = 10 vessels from 5 mice/group). (G) Time to occlusion in the wild-type mice (C57BL/6J) treated with anti-integrin α9 antibody or vehicle control (n = 10 vessels from 5 mice/group). (H) Representative microphotographs of mesenteric artery thrombus in the laser-injury model (left). Mean fluorescence intensity (MFI) over time (n = 19-22 vessels from 4 mice/genotype) (right).

Laser injury-induced mesenteric artery thrombosis was evaluated using the Micropoint laser ablation system (Andor Technology), as described previously.[10,11] Assays on both the models were performed by the investigator blinded to the genotypes.

Statistical analysis

Data represent mean ± SEM and are analyzed by Student *t* test and repeated measures ANOVA. Shapiro-Wilk test was used to check normality, and Bartlett's test was used to check equal variance. The results were considered significant at $P < .05$.

Results and discussion

Given the early lethality associated with the development of α9 global knockout mice,[12] we generated a cohort of $\alpha 9^{fl/fl}$LysMCre$^+$ and littermate control $\alpha 9^{fl/fl}$ mice on a C57BL/6J background

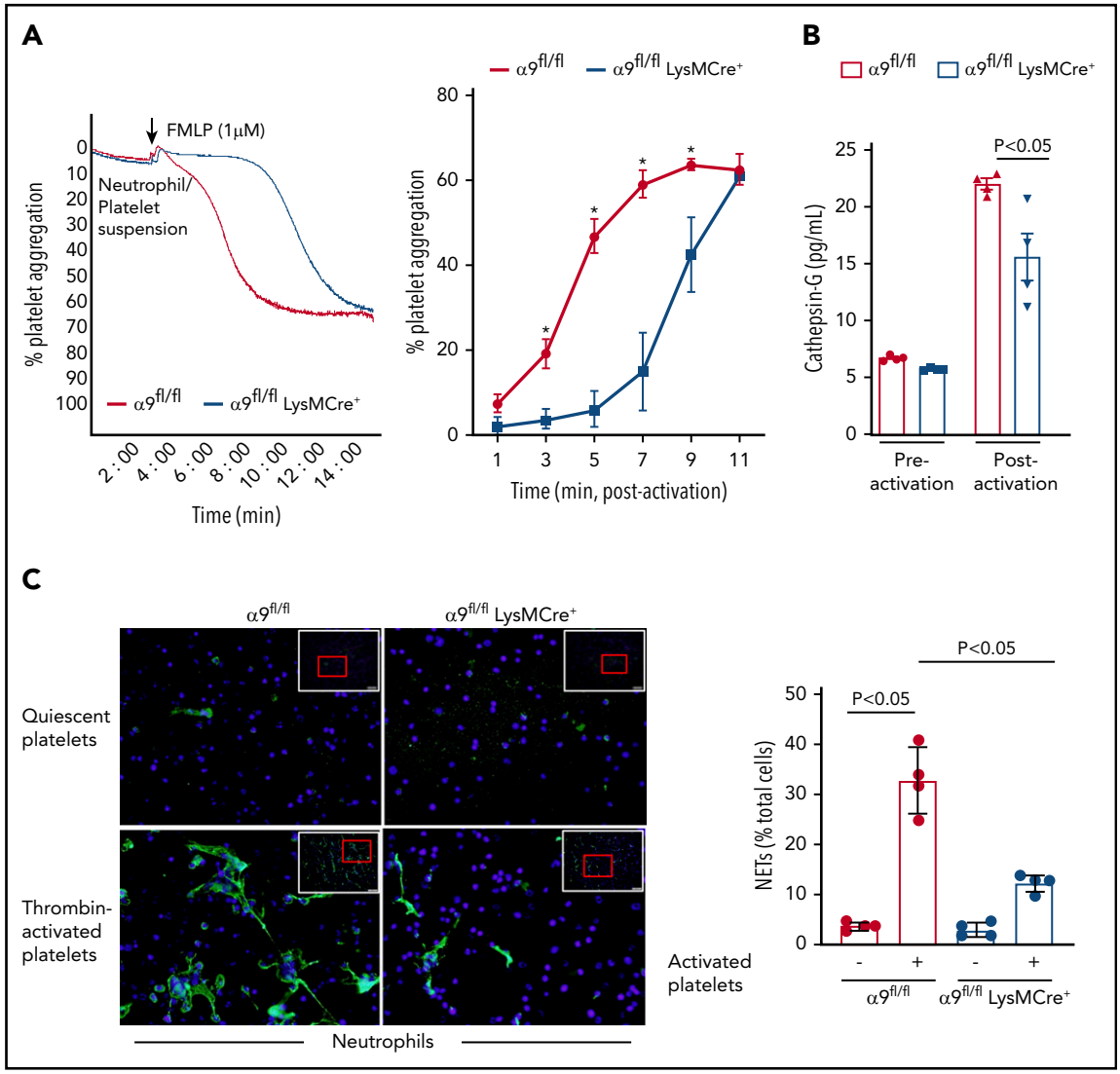

Figure 2. $\alpha 9^{fl/fl}$ LysMCre^{+} mice display reduced neutrophil mediated platelet aggregation and decreased NETosis. (A) Neutrophils from each genotype and wild-type platelets were stirred in a Chrono-log Whole Blood/Optical Lumi-Aggregometer (model 700-2) before the addition of fMLP. Representative aggregation curve for the fMLP stimulated neutrophils-induced platelet aggregation (left). Quantification (n = 4-5/group) (right). (B) Cathepsin G release as quantified by ELISA from the fMLP-stimulated neutrophils (n = 4 mice/group). (C) NETs assay was performed by stimulating neutrophils with thrombin-activated platelets. Representative microphotographs of NETs stained with PlaNET green (stains extracellular DNA, green) and counterstained with Hoechst (stains nuclei, blue) (left). Boxed region (lower magnification). Red insert in the boxed region is magnified and shown in the microphotographs. Quantification of the percentage of cells releasing NETs (n = 4 mice/group) (right). Value for each mouse represents a mean from 2 fields.

(supplemental Figure 2). As expected, the expression of the integrin α9 receptor was nearly undetectable in the neutrophils isolated from the bone marrow of $\alpha 9^{fl/fl}$LysMCre^{+} mice (Figure 1A). Previously, it was shown that the global deletion of α9 in mice results in defective granulopoiesis.[13] In contrast, complete blood counts were comparable in the $\alpha 9^{fl/fl}$ and $\alpha 9^{fl/fl}$LysMCre^{+} mice (supplemental Table 1), suggesting that myeloid-cell specific integrin α9-deficient mice could be used as a genetic model to define the role of integrin α9 in arterial thrombosis. Eight- to 10-week-old male $\alpha 9^{fl/fl}$ and $\alpha 9^{fl/fl}$LysMCre^{+} mice were subjected to 7.5% $FeCl_3$ induced-carotid artery thrombosis. $\alpha 9^{fl/fl}$LysMCre^{+} mice exhibited smaller thrombi, prolonged occlusion time, and a significant decrease in the rate of thrombus growth ($P < .05$ vs $\alpha 9^{fl/fl}$; Figure 1B). Despite being less susceptible to arterial thrombosis, the tail bleeding time was comparable between $\alpha 9^{fl/fl}$ and $\alpha 9^{fl/fl}$LysMCre^{+} mice, suggesting that myeloid cell-specific deficiency of integrin α9 does not alter hemostasis (Figure 1C). The time to complete occlusion was comparable between $\alpha 9^{fl/fl}$ and $\alpha 9^{+/+}$LysMCre^{+} mice, thus ruling out the nonspecific effects of Cre-recombinase expression (supplemental Figure 3). The MPO levels (Figure 1D), neutrophil elastase-positive cells (Figure 1E), and neutrophil count (supplemental Figure 4) in the carotid artery thrombus of $\alpha 9^{fl/fl}$LysMCre^{+} mice were substantially decreased, suggesting fewer neutrophils getting recruited to the site of injury ($P < .05$ vs $\alpha 9^{fl/fl}$). Transfusion of $\alpha 9^{fl/fl}$ neutrophils in $\alpha 9^{fl/fl}$LysMCre^{+} mice restored thrombosis similar to $\alpha 9^{fl/fl}$ mice (Figure 1F). Treatment of wild-type (C57BL/6J) mice with anti-α9 immunoglobulin blocking antibody (55A2C) inhibited arterial thrombosis (Figure 1G).

Next, to ensure that the observed effects are applicable to a broader context, susceptibility to arterial thrombosis was evaluated

using another model: laser injury-induced mesenteric artery thrombosis. Consistent with $FeCl_3$ injury-induced carotid artery thrombosis, $\alpha9^{fl/fl}$LysMCre$^+$ mice displayed significantly reduced thrombus growth ($P < .05$ vs $\alpha9^{fl/fl}$ mice; Figure 1H). Susceptibility to $FeCl_3$-induced carotid artery thrombosis was unaltered in monocyte-depleted (using clodronate liposomes) wild-type mice, demonstrating the minimal role of monocytes in the progression of arterial thrombosis in the conditions tested here (supplemental Figure 5). Together, these results suggest the potential role of integrin α9 expressed on neutrophils, but not on monocytes, toward the progression of arterial thrombosis.

It is known that *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)–mediated stimulation and activation of neutrophils results in platelet aggregation.[14] To further define the role of neutrophil integrin α9 on platelet activation, fMLP-stimulated neutrophil-induced platelet aggregation was determined in $\alpha9^{fl/fl}$ and $\alpha9^{fl/fl}$LysMCre$^+$ mice. fMLP-activated neutrophils from $\alpha9^{fl/fl}$LysMCre$^+$ significantly impaired the onset and extent of platelet aggregation ($P < .05$ vs $\alpha9^{fl/fl}$ mice; Figure 2A). Previous studies have suggested that the prothrombotic effects of neutrophils are significantly reduced on pharmacological inhibition or genetic deletion of cathepsin G (secreted from activated neutrophils and an important constituent of NETs).[15,16] To determine whether cathepsin G release was affected by neutrophil integrin α9 deficiency, we measured and compared its level in the supernatant of fMLP-stimulated neutrophils from both $\alpha9^{fl/fl}$ and $\alpha9^{fl/fl}$LysMCre$^+$ mice. Indeed, cathepsin G levels in the supernatant of activated neutrophils from $\alpha9^{fl/fl}$LysMCre$^+$ mice were found to be substantially reduced when compared with those of $\alpha9^{fl/fl}$ mice ($P < .05$; Figure 2B). The underlying mechanism by which integrin α9 deficiency in neutrophils reduces the release of cathepsin G remains unclear and requires further investigation.

Next, we performed NET assay in vitro, using stimuli such as thrombin-activated platelets or TNF-α to determine whether integrin α9 modulates NETosis and, thereby, the progression of arterial thrombus. The percentage of cells releasing NETs was significantly reduced in $\alpha9^{fl/fl}$LysMCre$^+$ mice ($P < .05$ vs $\alpha9^{fl/fl}$ mice; Figure 2C; supplemental Figure 6), suggesting that α9 may promote arterial thrombosis by enhancing NETosis. Previously, NETs were shown to promote red blood cell adhesion.[7] Indeed, we found that RBC content (supplemental Figure 7) was decreased in the carotid thrombus of the $\alpha9^{fl/fl}$LysMCre$^+$ mice ($P < .05$ vs $\alpha9^{fl/fl}$). Collectively, a marked reduction in NETosis, MPO levels, neutrophil count, and red blood cells in the carotid artery thrombus concomitant with reduced neutrophil-mediated platelet aggregation, and cathepsin G levels from activated neutrophils in the $\alpha9^{fl/fl}$LysMCre$^{+/-}$ mice provide potential mechanistic insights by which targeting integrin α9 may inhibit arterial thrombosis. In summary, these findings identify a novel role for integrin α9 in the modulation of arterial thrombosis.

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Authorship

Contribution: N.D. and M.K.N. performed experiments, analyzed results, and cowrote the manuscript; P.D. and M.J. performed experiments; G.D.F. performed experiments and edited the manuscript; S.K. provided the anti-integrin α9 antibody; and A.K.C. directed the project, interpreted results, and edited the manuscript.

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ORCID profiles: N.D., 0000-0001-9268-4363; M.K.N., 0000-0001-6461-0543; P.D., 0000-0003-4219-5240; M.J., 0000-0002-7263-9829; G.D.F., 0000-0002-4784-3094; S.K., 0000-0001-5410-8429; A.K.C., 0000-0001-8554-0898.

Correspondence: Anil K. Chauhan, Department of Internal Medicine, University of Iowa, 3120 Medical Labs, Iowa City, IA 52242; e-mail: anil-chauhan@uiowa.edu.

Footnotes

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*N.D. and M.K.N. contributed equally to this study.

For original data, please contact the corresponding author.

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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