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ENHANCED mRNA EXPRESSION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 IN LIVEDOID VASCULOPATHY LESIONS

Running head: Plasminogen activator inhibitor-1 in livedoid vasculopathy

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Abstract

Aim: Thrombosis and inflammation play an important role in pathophysiology of livedoid vasculopathy (LV). Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of fibrinolysis and is a pivotal modulator in a broad range of biological processes.

Method: The study specimens were retrospectively selected from archives of pathology department. We investigated PAI-1 mRNA expression in the paraffin blocks of patients with biopsy proven LV and controls. We analyzed the presence of thrombus, fibrinoid necrosis, ulcer and epidermal atrophy in study samples. The correlation between histologic findings and PAI-1 expression was investigated.

Results: Analyses were performed in 14 LV patients (mean age 31±20, 79 % female) and 4 controls (mean age 64±19, 50 % female). PAI-1 gene expression was significantly higher in LV compared to the control group (median 7.74 (Iqr:13.94) versus 1.0 (0.31)), p=0.011. Histological analysis displayed that fibrinoid necrosis was present on all patients with LV, 61.5% displayed thrombus, 46.2 % displayed ulcer, and 15.4 % displayed epidermal atrophy. Overall, we did not observe any discerning difference in PAI-1 expression between the LV blocks with or without thrombus, fibrinoid necrosis, or epidermal atrophy, yet the LV specimens that displayed ulcer histologically, had higher PAI-1 mRNA expression compared to those without ulcer (median 13.98 (Iqr:19.21) versus 2.86 (5.59)), (p=0.046).

Conclusion: PAI-1 mRNA expression is significantly increased in cutaneous lesions of patients with LV. Histological finding of ulcer is associated with increased PAI-1 expression in LV specimen. In the current era of PAI-1 inhibitors, enhanced local PAI-1 expression can form a novel and local therapeutic target in LV.

Keywords: Fibrinolysis, Plasminogen inactivators**,** Plasminogen activator inhibitor 1
Vascular diseases, Thrombosis, Topical administration

Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of both tissue type (t-PA) and urokinase-type (uPA) plasminogen activators.[1,2] PAI-1 prevents the generation of plasmin, the active fibrin degrading protease of fibrinolysis, from the inactive precursor plasminogen. In addition to fibrinolysis, PAI-1 is a pivotal modulator in a broad range of biological processes including thrombosis, fibrosis, tumorigenesis, cancer metastasis, and atherosclerosis.[1,2] Livedoid vasculopathy (LV) is a chronic skin disease with uncertain etiology. Thrombophilic conditions occur frequently in patients with LV.[3-11] Livedoid vasculopaty is clinically characterized by the triad consisting of livedo rasemosa, episodic painful cutaneous ulcerations located on the distal part of the lower extremities and small porcelain-white scars called atrophie blanche that occurs after the healing of the lesions.[3-12] Thrombosis, fibrinoid necrosis, ulceration and epidermal atrophy are the main histological features of LV. Histopathologically LV is characterized by occluded capillary vessels by fibrin thrombi in the upper and middle dermis, fibrinoid degeneration of the vessel walls. Painful ulcers occur at the advanced stages. Unlike to primary vasculitis, perivascular inflammatory infiltration is not present. Erythrocyte extravasation and dermal hyalinization are also observed in the histopathology of the disease.[12] Although the pathogenesis of LV

remains to be elucidated, thrombosis and inflammation play a role in the disease mechanism. Cutaneous injury in LV pathophysiology can stimulate PAI-1 activation which inhibits fibrinolysis and causes thrombus. The etiology seems to be multifactorial and a combination of genetic and environmental triggering factors can result in LV.[3-12] Pro-inflammatory vascular cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6, are important in the pathophysiology of LV.[3-12] We previously reported that LV and vitiligo patients display enhanced stability of circulating PAI-1 levels which can contribute to the pathophysiology of these complex diseases.[13,14] Since, disease phenotype and symptoms are cutaneous, the local expression of PAI-1 in LV lesions is clinically important. A strategy to selectively inhibit cutaneous PAI-1 might therefore be therapeutically useful in preventing the progression LV lesions. In this study, we investigate PAI-1 mRNA expression in LV lesions from patients with biopsy proven LV.

Methods

The study specimens were retrospectively selected from archives of Pathology Department. PAI-1 mRNA expression was analyzed in the paraffin blocks of the patients with biopsy proven LV and controls (Table 1). The study protocol was approved by the Institutional Review Board. We identified paraffin blocks of 14 patients with biopsy proven LV. The blocks were reviewed and the diagnosis was confirmed by 2 pathologists (PG, FVA). Histopatological features of some of the study patients with LV were presented in Figure 1. Four paraffin embedded normal skin tissue samples were selected as controls (Figure 2). Specifically, the control specimens were taken from surgical border normal skin tissue in 2 patients who underwent squamous cell carcinoma removal, after cholecystectomy in 1 patient, and at the surgical border of fibroepithelial polyp removal in 1 patient.

We analyzed the presence of thrombus, fibrinoid necrosis, ulcer and epidermal atrophy in the samples of 13 LV patients and all controls. The block was not available for histological assessment in 1 LV patient.

RNA isolation and cDNA synthesis from formalin fixed paraffin embedded (FFPE) sections

Total RNA isolation of the FFPE sections were done by Roche High Pure RNA Paraffin kit (Roche, Germany), according to the manufacturer's instructions. Two 10 µm FFPE sections of the tissue samples were deparaffinized prior to RNA isolation. The concentration and purity of RNA were determined by measuring absorption at 260 to 320 nm spectrophotometrically. DEPC treated water used in all spectrophotometric analysis. 200 ng of total RNA was used for cDNA synthesis in a reaction volume of 20 µl, using Transcriptor High Fidelity cDNA synthesis kit (Roche, Germany), according to the manufacturer's instructions. cDNA samples were stored at -20^{o}C until use.

Real-Time Q-PCR for SERPINE-1 (PAI-1) and Beta-actin

Real time quantitative PCR for SERPINE-1 (PAI-1) and Beta-actin was performed using LightCycler 480 Probes Master (Roche, Germany) and 2.5 µl of cDNA was used in a reaction volume of 10. All reactions were performed in triplicate for housekeeping gene, beta actin, and SERPINE-1. The primer probe mix for each gene was purchased as RealTime ready Custom Single Assay (Roche, Germany) and used according to the manufacturer's instructions. Analysis and quantification was performed using LightCycler 480 software. Relative quantification was calculated by delta Ct method, SERPINE-1 expressions were normalized with respect to the expression of an endogenous control, Beta-actin.

Statistical Analysis

Statistical analysis was done using IBM, SPSS Statistics 21.0 software (IBM corp) and GraphPad. Nonparametric test (Mann-Whitney U) was used to compare the groups for quantitative variables. Fisher's Exact test was used to compare groups for categorical variables. Median (interquartile range (IQR)) values were displayed for variables. P values of less than 0.05 were considered as statistically significant for all tests.

Results

We identified 20 paraffin blocks of LV patients in the archives of Pathology Department, 6 samples were not included in the analysis as both PAI-1 and the house keeping gene expressions were absent. Final analysis was performed in 14 patients and 4 control subjects (Table 1). Characteristics of study participants were displayed in Table 1. Control group patients were older compared to LV patients. Median duration of disease was 36.93 months. PAI-1 gene expression was significantly higher in paraffin blocks of LV patients compared to the control samples: median (IQR) level was median 7.74 (Iqr:13.94) versus 1.0 (0.31) (p=0.011, Mann-Whitney U) (Figure 3), (Table 2).

Histological analysis displayed that fibrinoid necrosis was present on all patients with LV, 61.5 % displayed thrombus, 46.2 % displayed ulcer, and 15.4 % displayed epidermal atrophy. The control samples did not display thrombus, fibrinoid necrosis, ulcer or epidermal atrophy. Overall, we did not observe any discerning difference in PAI-1 expression between LV blocks with or without thrombus, fibrinoid necrosis, or epidermal atrophy, yet the LV specimens that displayed ulcer histologically, had higher PAI-1 mRNA expression compared to those without ulcer (median 13.98 (Iqr:19.21) versus 2.86 (5.59)), (p=0.046) Table 3.

Discussion

In this study, we demonstrate that PAI-1 mRNA expression is significantly increased in cutaneous lesions of patients with LV. We previously reported that enhanced stability of PAI-1 may contribute to the pathophysiology of LV.[13,14] Enhanced local expression of PAI-1 in LV lesions is clinically important to explain the disease process and expand therapeutic options. A number of potential mechanisms may be responsible for the increased expression of PAI-1 in LV lesions including presence of thrombus, inflammation, point mutation(s) in the coding domain sequences of PAI-1 gene, alterations in mRNA degradation, and binding stabilizing co-factors. Histological feature such as ulcer formation is associated with increased PAI-1 expression. Ulcer formation can be a histological marker of disease severity in LV.[3,12] Ulcer indicates that vascular obliteration and cutaneous thrombus load are severe enough to block the dermal circulation and cause cutaneous infarction. The pathogenesis of LV remains unclear. LV is considered an occlusive thrombotic process due to an abnormalities in the hemostasis cascade resulting in cutaneous ischemia and infarction.[12] Therefore, the finding of higher PAI-1 expression in blocks with ulcer implicates the role of PAI-1 in the pathophysiology of LV.

Disease mainly affects dermal layers yet, there have been no reports of peripheral venous insufficiency and collagen vascular diseases associated with LV.[15] The common hypothesis is that LV is a primary thrombotic disturbance at the microcirculatory level, determining a local state of hypercoagulability, instead of being a vasculitic process.[3-12] Urokinase plasminogen activator is an extracellular matrix-degrading protease involved in cancer invasion and metastasis, interacting with PAI-1, which was originally identified as a blood-derived endogenous fast-acting inhibitor of u-PA .

Plasminogen activator inhibitor-1 is a serine protease inhibitor that inhibits fibrinolysis and promotes thrombosis by specifically inactivating t-PA and u-PA.[2] High plasma concentrations of PAI-1 play a role in fibrosis, vascular and extracellular matrix remodeling.[2,3] Thus, elevated PAI-1 levels have been reported in association with coronary artery disease, ischemic stroke and venous thrombosis.[2,16] However, local effects of PAI-1 in the dermis remain to be elucidated.

Several polymorphisms of the PAI-1 gene have been described, including a sequence length dimorphism 4G/5G.[16] Subjects who are homozygous for the 4G allele (4G/4G genotype) have a higher frequency of high PAI-1 levels.[17] In fact, there are case reports indicate that LV is associated with PAI-1 promoter homozygosity (4G/4G), and systemic administration of t-PA has been reported as a treatment alternative in such patients.[11] However, systemic treatment options for LV come with significant risk. Furthermore, individual mutations or polymorphisms are associated with only a mild increase in the probability of a thrombotic event and more common opinion is that alterations in mRNA degradation, and binding stabilizing co-factors can enhance the local effects of PAI-1.[8]

Plasminogen activator inhibitor-1 plays an important role in a wide variety of physiologic and pathologic processes.[18] The biological actions exerted by PAI-1 are complex. There is widespread expression of PAI-1 in many different tissues, including liver, adipose stromal tissue, platelets, and endothelial cells, and increased expression is a feature of inflammation and tissue damage.[19] Mediators of increased PAI-1 expression are likely to be locally determined. The effects of PAI-1 in the local milieu appear to reflect a local rather than systemic effect. In the case of the stomach, gastrin and of H. pylori directly induce PAI-1 expression in epithelial cells.[20] Increased PAI-1 expression as a result of transforming growth factor-β is another example.[18]

Plasminogen activator inhibitor-1 expression is part of a defense response to damage that follows exposure inflammatory injury. Experimental data from animal models indicate the actions of PAI-1 to stabilize fibrin clots by inhibition of u-PA or t-PA and so promote hemostasis and minimize the formation of hemorrhagic lesions.[21] For instance, gastric PAI-1 mRNA expression is part of a defense response to mucosal damage that follows aspirin or non-steroidal anti-inflammatory drug exposure.[21]

In normal skin, PAI-1 is present at low levels, whereas in pathological conditions such as keloid tissue PAI-1 is significantly increased.[22] The relationship between PAI-1 and collagen accumulation fueled interest in exploring the role of PAI-1 in skin lesions. As a result, PAI-1 has been tested as a therapeutic target in dermal pathologies. For instance, silencing of PAI-1 induces keloid shrinkage.[23,24]

To our knowledge, this is the first report of increased PAI-1 expression in LV lesions. Given the marked improvement in the skin lesions after anti-thrombotic therapy, local treatment options can favorably influence the course of the disease. As an important mechanism in the development of LV lesions, in the present study, we demonstrate that LV lesions express significantly higher PAI-1 than controls. Increased expression of PAI-1 induces local thrombosis, inflammation and can be the crucial target in the development of LV lesions. Lack of clinical evidence for systemic thrombosis in LV patients, suggest that the increase in PAI-1 expression is confined to skin in LV. Thus, current systemic treatment options remain limited for LV. For instance, systemic with novel PAI-1 inhibitors offer potential treatment alternatives in several disease states[25]. Recently, PAI-1 aptamers have been developed to bind reactive center loop and vitronectin binding[26]. They can potentially inhibit intracellular and cutaneous effects of PAI-1. In patients with LV, the emerging PAI-1 inhibitors by targeting the enhanced local PAI-1 expression can form a novel and local therapeutic option.

Our study comes with several limitations. First of all, study specimens were retrospectively selected from archives of pathology department. Due to retrospective nature of the study we could not retrieve entire clinical treatment regimen at the time of biopsy or after. Simultaneous measurement the amount of plasminogen, fibrin, fibrinogen, platelet, leukocytes, tPA and correlation with mRNA expression of PAI-1 were not possible due to the retrospective nature of the study. We previously reported that both Ag and specific activity levels of PAI-1 were moderately elevated in LV patients compared to the controls, and more importantly, PAI-1 kinetic studies demonstrated markedly enhanced stability of PAI-1 activity in plasma from patients with LV [14]. Although the above findings provide evidence that PAI-1 is involved in LV, future studies with biomarkers are needed to be recommended for routine clinical use. In order for emerging biomarkers to progress to the clinic, in addition to analytical and clinical validation, demonstration of clinical utility is necessary.

Considering the enhanced PAI-1 expression in LV lesions, evaluating the PAI-1 expression in skin biopsy samples can identify subjects who are likely to benefit from local application of novel PAI-1 inhibitors. Since systemic administration of PAI-1 inhibitors is limited due to side effects[25, 26], future studies can then test the efficacy of locally applied PAI-1 inhibitors in LV lesions in patients with enhanced local expression of PAI-1.

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Author contributions -

MA

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Table 1. Characteristics of study participants

Variables	Patients	Control Group	P Value
Mean Age ± SD	30.92±20.05	63.5±18.77	
Median Age (IQR)	27 (18.50)	60.50 (35)	0.017*
Female (%)	78,6	50	0.219**
Median duration of disease	36.93 months	-	

*Mann-Whitney U, **Fisher's Exact test

Table 2. PAI-1 gene expression was significantly higher in paraffin blocks of LV patients compared to the control samples.

Case Number	PAI-1 expression	Case Number	PAI-1 expression	Case Number	PAI-1 expression	Control Number	PAI-1 expression
1	,30	7	2,86	13	26,08	1	1.02
2	1,58	8	3,59	14	30,98	2	0.98
3	2,90	9	7,02			3	0.84
4	6,61	10	8,45			4	1.24
5	16,00	11	11,89				
6	19,15	12	16,06				

Table 3. Comparisons of the levels of PAI-1 mRNA expression according to histopathological findings

Histopathological findings	Present Median PAI-1 (IQR)	Absent Median PAI-1 (IQR)	P value
Thrombus	10.17 (19.13)	6.61 (15.34)	0.380*
Fibrinoid necrosis	8.45(14.36)	-	-
Ulcer	13.97(19.21)	3.59 (13.14)	0.046*
Epidermal atrophy	13.8 (-)	7.02 (14.16)	0.430*

*Mann-Whitney U

Figure legends.

Figure 1. a, b, c, d, e. The histopathological characteristics of livedoid vasculopathy in the study specimen. Hyaline thrombi are present in capillaries (circles), venules (circles) and an arteriol (arrow) of papillary and mid dermis. Fibrinoid material (x) is present in the vessel wall. Extravasated red blood cells are noted (arrowheads) in dermis. Perivascular slight lymphocytes (#), epidermal atrophy (*) and full thickness epidermal necrosis (§) are displayed.

Figure 2. Histological appearance of a control sample.

Figure 3. The box plots represents the distribution of PAI-1 mRNA expression for the LV (median 7.74 (Iqr:13.94) and control groups (median 1.0 (Iqr:0.31)). The horizontal line in the box represents median. The lower and upper margins of the boxes show the 25^{th} and 75^{th} percentile, respectively. Whiskers display the greatest and the least values.

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