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Mammalian Target of Rapamycin Pathway Assessment in Antiphospholipid Antibody–Positive Patients with Livedo

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ABSTRACT. ***Objective.*** In antiphospholipid antibody (aPL) nephropathy, activation of the mammalian target of rapamycin (mTOR) contributes to endothelial cell proliferation, a key finding of aPL microvascular disease. Here, we examined mTOR activation in the skin of aPL-positive patients with livedo.

Methods. Three patient groups with livedo were studied: (1) persistently aPL-positive with systemic lupus erythematosus (SLE); (2) persistently aPL-positive without SLE; and (3) aPL-negative SLE (control). After collecting aPL-related medical history, two 5-mm skin biopsies of livedo were performed on each patient: (1) peripheral (erythematous-violaceous lesion); and (2) central (nonviolaceous area). We stained specimens for phosphorylated protein kinase B (p-AKT) and phosphorylated S6 ribosomal protein (p-S6RP) as mTOR activity markers, CD31 to identify endothelial cells, and Ki-67 to show cellular proliferation. We counted cells in the epidermis and compared mTOR-positive cell counts between peripheral and central samples, and between patient groups, using Freidman test and Wilcoxon signed-rank test.

Results. Ten patients with livedo reticularis were enrolled: 4 aPL-positive without SLE (antiphospholipid syndrome [APS] classification met, n = 3), 4 aPL-positive SLE (APS classification met, n = 3), and 2 aPL-negative SLE (control). In all aPL-positive patients, epidermal p-AKT and p-S6RP staining were significantly increased in both peripheral and central skin samples when compared to aPL-negative SLE controls; both were more pronounced in the lower basal layers of epidermis.

Conclusion. Our study demonstrates increased mTOR activity in livedoid lesions of aPL-positive patients with or without SLE compared to aPL-negative patients with SLE, with more prominent activity in the lower basal layers of the epidermis. These findings may serve as a basis for further investigating the mTOR pathway in aPL-positive patients.

Key Indexing Terms: antiphospholipid syndrome, livedo reticularis, mammalian target of rapamycin

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by vascular thrombosis (arterial, venous, and small vessel) and/or pregnancy morbidity in patients with persistently positive antiphospholipid antibodies (aPL), namely, lupus anticoagulant (LAC), anticardiolipin antibodies (aCL), and anti–β_2 glycoprotein I (anti-β_2GPI) antibodies.[1] In addition to thrombotic and pregnancy complications, aPL-positive patients can develop microvascular disease such as livedo reticularis/racemosa, livedoid vasculopathy, nephropathy, or diffuse alveolar hemorrhage.[1,2]

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Livedo reticularis/racemosa is an erythematous-violaceous, net-like discoloration of the skin that can be seen in both pathological and physiological conditions. Impaired blood flow to the skin in the central arterial cones leads to reactive dilation of peripheral dermal venules and an increase in deoxygenated hemoglobin, which manifests as violaceous mottling with central clear cores. There are various systemic conditions associated with livedo, including APS.[3]

Endothelial dysfunction, often with a reactive proliferation, is one of the key pathological findings in aPL-positive patients with microvascular disease, and is well described in patients with kidney, skin, cardiac, and lung involvement.[4,5] The mammalian target of rapamycin (mTOR) pathway is involved in the development of endothelial dysfunction and proliferation in aPL-associated nephropathy.[6] However, these findings have not been investigated in aPL-positive patients with skin involvement. Thus, our primary objective was to investigate the mTOR pathway activation in the skin biopsies of persistently aPL-positive patients with livedo.

METHODS

Study design. Ethical approval for this study was obtained from the Hospital for Special Surgery Institutional Review Board (IRB no. 2015-256). In this cross-sectional study, our goal was to recruit patients between the ages of 18 and 75 years, with active skin involvement (livedo reticularis, livedo racemosa, and/or livedoid vasculopathy) who fell into 1 of 3 groups: (1) persistently aPL-positive patients with no other systemic autoimmune diseases; (2) persistently aPL-positive patients with systemic lupus erythematosus (SLE); and (3) aPL-negative patients with SLE (control). aPL positivity was defined as persistent (at least 12 weeks apart) positive LAC tests based on the International Society on Thrombosis and Hemostasis recommendations,[1] aCL IgG/IgM ≥ 40 U, and/or anti-β_2GPI IgG/IgM ≥ 40 U. aPL negativity was defined as negative LAC, aCL IgG/IgM/IgA, and anti-β2GPI IgG/IgM/IgA within 1 year prior to study entry. The definition of SLE was based on the American College of Rheumatology SLE Classification Criteria.[7] Livedo reticularis was defined as the net-like, mottled, erythematous-violaceous discoloration of the skin that was uniform, symmetric, and nonfixed (variations usually seen with temperature changes). Livedo racemosa was differentiated from livedo reticularis by its nonuniform, asymmetric, and fixed appearance. Livedoid vasculopathy was characterized histologically by thrombotic occlusion of the cutaneous capillaries and endothelial proliferation, as well as the presence of superficial skin ulcers.[3,8]

Patients were excluded from the study if they had any other systemic autoimmune disease (eg, systemic sclerosis, rheumatoid arthritis), biopsy-proven cutaneous vasculitis, treatment with or exposure to mTOR inhibitors (eg, rapamycin), steroid use > 10 mg/day prednisone or equivalent < 30 days prior to enrollment, any immunosuppressive drug use within 3 months before screening (except hydroxychloroquine, mycophenolate mofetil, azathioprine, or methotrexate), acute or chronic skin infection or any other skin diseases around the skin biopsy area, acute infection receiving any antibiotics, acute thrombosis within 30 days prior to screening, or malignancy within 1 year prior to screening (except for nonmetastatic squamous or basal cell skin carcinomas and cervical carcinoma if received curative surgical treatment, or if they were pregnant).

Study procedures. Before the study visit, we obtained written informed consent from all subjects, including permission to publish the study data. Study visits followed these procedures: (1) clinical data (demographics, medical history including medications and APS-related medical history) were collected; (2) aPL/APS-specific physical examination including detailed skin examination was conducted; and (3) two 5-mm skin biopsies (epidermis) were performed on each patient: one peripheral (erythematous-violaceous lesion); and one central (nonviolaceous area).[9]

Immunohistochemistry studies in clinical specimens. Each biopsy specimen was fixed in formalin, then dehydrated and embedded in paraffin. Formalin-fixed, paraffin-embedded blocks were sectioned using a microtome (Leica), and the 7-μm thick sections were mounted on Superfrost Plus Microscope Slides (Cat# M6146-PLUS; Cardinal Health). All stainings were performed at the Molecular Cytology Core Facility of Memorial Sloan Kettering Cancer Center using Discovery XT processor (Roche Tissue Diagnostics).

Phosphorylated protein kinase B (p-AKT; Ser473) rabbit monoclonal (1 μg/mL, #4060; Cell Signaling Technology), phospholyrated S6 ribosomal protein (p-S6RP; p-Ser235/236) rabbit monoclonal (0.36 μg/ml, #4858; Cell Signaling Technology), CD31 mouse monoclonal (2.5 μg/ml, #M0823; Dako), and Ki-67 rabbit monoclonal (0.5 μg/ml, #9027; Cell Signaling Technology) antibodies were used for human immunohistochemistry (IHC) according to the manufacturer's instructions.

For p-S6RP, CD31, and Ki-67 stainings, after 32 minutes of heat and Cell Conditioning Solution 1 (CC1, #950-500; Roche Tissue Diagnostics) retrieval, the tissue sections were blocked first for 30 minutes in background blocking reagent (Background Buster, #NB306; Innovex). The incubation with the primary antibody was done for 6 hours, followed by 60 minutes' incubation with biotinylated goat anti-rabbit IgG (5.75 μg/ml, #PK6101; Vector Laboratories). For p-AKT staining, the tissue sections were blocked for 30 minutes in 10% normal goat serum. The incubation with the primary antibody was done for 5 hours in phosphate-buffered saline with 2% bovine serum albumin, followed by 60 minutes' incubation with biotinylated goat anti-rabbit IgG (#PK6101; Vector Laboratories) at 1:200 dilution. Blocker D, Streptavidin–horseradish peroxidase, and diaminobenzidine detection kit (Roche Tissue Diagnostics) were used according to the manufacturer's instructions. Finally, the slides were counterstained with hematoxylin and coverslipped with Permount Mounting Medium (Fisher Scientific).

On nearby sections, IHC staining for p-AKT at Ser473 and p-S6RP were performed to evaluate mTOR activation. We used CD31 to identify endothelial cells, and Ki-67 to show cellular proliferation. We counted the cells in the epidermis using ImageJ[10] to measure mTOR activity and proliferation by calculating the number of cells stained positive for p-AKT and p-S6RP, and compared mTOR activity between different groups of peripheral and central samples in aPL-positive patients (with and without SLE) vs aPL-negative SLE controls. We subgrouped each skin sample analysis as upper superficial and lower basal layers of the epidermis. Investigators performing IHC and cell counting were blinded as to which samples were peripheral or central, as well as to the patient groups.

Statistical analysis. Descriptive analysis was performed on the demographic data collected. For skin biopsy sample analyses of the counted cells, we used Freidman test and Wilcoxon signed-rank test to compare the number of mTOR-positive cells in different groups of skin samples, followed by Bonferroni post hoc test for subgroup analysis.

RESULTS

Of 31 patients screened, 21 were excluded: no active lesions at the time of screening, n = 12; refused or unable to participate, n = 5; and immunosuppressive use within 3 months, n = 4. Ten patients were ultimately enrolled (9 female, all White, mean age 45 [SD 13.6] yrs): 4 aPL-positive without SLE (APS classification met, n = 3), 4 aPL-positive with SLE (APS classification met, n = 3), and 2 aPL-negative SLE (control).

All patients had active livedo reticularis (no livedo racemosa or livedoid vasculopathy). Of the 4 aPL-positive patients without SLE, 1 had positive LAC, 2 had positive aCL and anti-β_2GPI, and 1 had triple aPL positivity, whereas of the 4 aPL-positive

patients with SLE, 3 had positive LAC and 1 had triple aPL positivity. None of the patients had aPL-associated nephropathy (Supplementary Table S1, available with the online version of this article).

In both central and peripheral skin biopsy samples of aPL-positive patients with or without SLE, there was no consistent p-AKT, p-S6RP, or Ki-67 staining in the corresponding areas of endothelial lining identified by CD31 (at 10× magnification; Supplementary Figure, available with the online version of this article). The control group (aPL-negative SLE) samples also did not show any consistent p-AKT, p-S6RP, or Ki-67 activity.

Staining for p-AKT and p-S6RP was noticeable in the epithelium (at 40× magnification; Figure), and thus, we assessed the epithelium for the extent of staining for these markers. We assessed the number of p-AKT– and p-S6RP–positive cells in upper and basal layers separately. On average, aPL-positive patients had higher numbers of p-AKT and p-S6RP epidermal cells compared to aPL-negative patients, and this was true in both the upper and basal layers and in both peripheral and central samples (Table 1). Among aPL-positive patients (with or without SLE), p-AKT–positive cells were higher in the peripheral skin samples compared to central skin samples, whereas p-S6RP–positive cells were lower in the peripheral skin samples compared to the central skin samples; both were more prominent in the lower basal layers (Table 2). Further analysis of Ki-67 staining in epithelium suggested a trend toward the highest percentage of positive cells in aPL-negative patients with SLE, but sample numbers were inadequate at this time for a clear conclusion (Supplementary Table S2, available with the online version of this article).

DISCUSSION

Our study demonstrates significantly increased mTOR activity in both peripheral and central skin biopsies of aPL-positive patients with livedo (with or without SLE), compared to the aPL-negative SLE controls. Increased mTOR activity was more prominent in the lower basal layers of the epidermis compared to the upper surface layer; however, our study did not demonstrate any endothelial cell proliferation.

The signaling network of mTOR regulates cell growth, proliferation, and survival through various extracellular and intracellular pathway interactions.[11] A previous study identified the mTOR pathway as a potential plausible pathway involved in small vessel impairment of persistently aPL-positive patients with renal involvement.[6] Chronic vascular

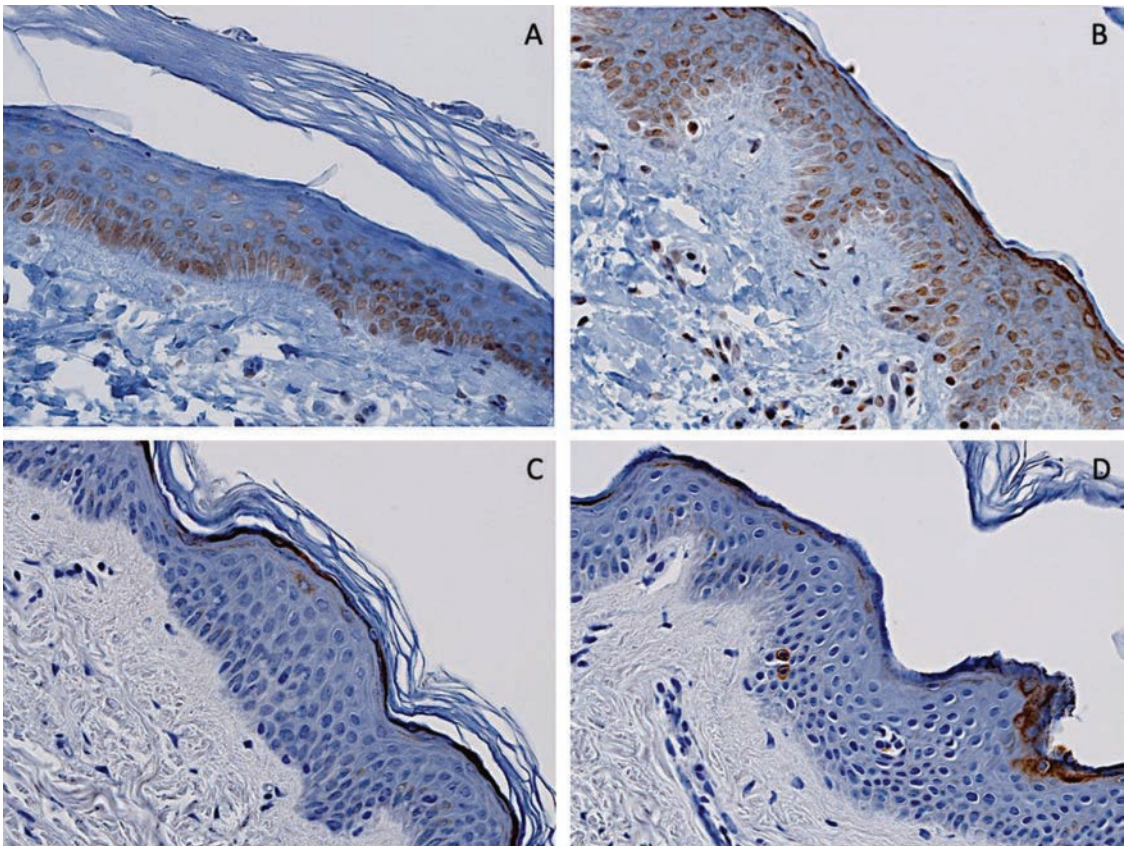

Figure. Epidermal mammalian target of rapamycin (mTOR) activity in the skin biopsies of antiphospholipid antibody (aPL)-positive and aPL-negative patients with SLE with livedo reticularis. (A) Peripheral and (B) central skin biopsies of an antiphospholipid antibody (aPL)-positive patient with SLE with livedo, compared to the (C) peripheral and (D) central skin biopsies of an aPL-negative patient with SLE. Increased mTOR activity is shown by the p-S6RP staining (brown) in A and B, but not in C and D. mTOR: mammalian target of rapamycin; p-S6RP: phosphorylated S6 ribosomal protein; SLE: systemic lupus erythematosus.

Table 1. Comparison of mammalian target of rapamycin (mTOR)-positive cell counts between antiphospholipid antibody (aPL)-positive (with and without SLE) and aPL-negative patients with SLE.

	aPL-Positive Without SLE, n = 4	aPL-Positive With SLE, n = 4	aPL-Negative SLE, n = 2	*P*
p-AKT				
Peripheral				
Upper surface layer	247/1215 (20)	201/1411 (14)	21/261 (8)	**< 0.001***
Lower basal layer	982/1710 (57)	1185/1967 (60)	151/571 (26)	**< 0.001****
Total	1229/2925 (42)	1386/3378 (41)	172/832 (21)	**< 0.01*****
Central				
Upper surface layer	468/2852 (16)	456/4117 (11)	38/1773 (2)	**< 0.001***
Lower basal layer	2544/4048 (63)	1784/5656 (32)	301/1916 (16)	**0.001***
Total	3012/6900 (44)	2240/9773 (23)	339/3689 (9)	**< 0.001***
p-S6RP				
Peripheral				
Upper surface layer	457/1241 (37)	1128/1567 (72)	452/1484 (30)	**< 0.001***
Lower basal layer	1547/2346 (66)	3039/3530 (86)	693/3247 (21)	**< 0.001***
Total	2004/3587 (56)	4167/5097 (82)	1145/4731 (24)	**< 0.001***
Central				
Upper surface layer	833/1836 (45)	1712/2439 (70)	942/3570 (26)	**< 0.001***
Lower basal layer	2195/2872 (76)	3917/4400 (89)	1889/5450 (35)	**< 0.001***
Total	3028/4708 (64)	5629/6839 (82)	2831/9020 (31)	**< 0.001***

Values are expressed as positive cells/total cells (%). Values in bold are statistically significant. * Statistical significance was preserved among all subgroup analyses. ** Subgroup analysis for aPL without SLE vs aPL with SLE: $P = 0.90$; aPL with and without SLE vs aPL-negative SLE: $P < 0.001$. *** Subgroup analysis for aPL without SLE vs aPL with SLE: $P = 0.11$; aPL with and without SLE vs aPL-negative SLE: $P < 0.001$. aPL: antiphospholipid antibody; p-AKT: phosphorylated protein kinase B; p-S6RP: phosphorylated S6 ribosomal protein; SLE: systemic lupus erythematosus.

Table 2. Comparison of mammalian target of rapamycin (mTOR)-positive cell counts between peripheral vs central skin samples in aPL-positive patients (n = 8).

	Peripheral Skin Samples	Central Skin Samples	*P*
p-AKT			
Upper surface layer	448/2626 (17)	924/6969 (13)	**< 0.001**
Lower basal layer	2167/3677 (59)	4328/9704 (45)	**< 0.001**
Total	2615/6303 (41)	5252/16673 (32)	**< 0.001**
p-S6RP			
Upper surface layer	1585/2805 (57)	2545/4275 (60)	**0.003**
Lower basal layer	4586/5876 (78)	6112/7272 (84)	**0.003**
Total	6171/8684 (71)	8657/11547 (75)	**0.003**

Values are expressed as positive cells/total cells (%). Values in bold are statistically significant. aPL: antiphospholipid antibody; p-AKT: phosphorylated protein kinase B; p-S6RP: phosphorylated S6 ribosomal protein.

renal lesions in aPL-nephropathy were associated with the activation of the mTOR pathway. Further, investigators reported decreased vascular proliferation after treatment with sirolimus, which inhibits mTOR.[6] In cultured vascular endothelial cells, IgG antibodies from patients with APS stimulated the mTOR pathway, suggesting that aPL can cause endothelial proliferation through the mTOR pathway. Although assessment of endothelial cell proliferation and mTOR activation in endothelial cells was inconclusive (the epidermis, in contrast to the vessels in the dermis, is easy to appreciate in tissue sections by the location and morphology of the cells), we did find increased mTOR activity in aPL-positive patients with or without SLE compared to aPL-negative SLE controls.

In livedo, there is either partial narrowing or vasospasm (reticularis), or significant narrowing or frank occlusion (racemosa) in cutaneous arterioles. The purplish discoloration is a consequence of reduced blood flow through central arterioles. Therefore, it is recommended that skin biopsies be performed from both the peripheral and center of the livedoid segment in an effort to best capture the pathogenic process.[8,9] We found differences in these distinct regions, with more pronounced activity of p-AKT in the erythematous-violaceous peripheral and p-S6RP in the nonviolaceous central areas. Thus, our findings support the recommendation to obtain skin biopsies from both areas when evaluating livedoid lesions.

The significance of the apparent higher mTOR activity in the epidermis of aPL-positive patients is currently unknown. The higher activity in the basal layers than in the upper layers

could reflect the greater level of proliferation that occurs in the basal epidermis, an area that is physiologically enriched in epidermal stem cells.[12] However, we were not able to show higher cellular proliferation in aPL-positive patients to correlate with mTOR activation markers, possibly due to our small sample size. Whether there are alterations in the basal keratinocytes in aPL-positive patients, and their functional significance, will need to be addressed in future, larger-scale studies.

Livedo is one of the microvascular, noncriteria manifestations seen in aPL-positive patients.[1] Despite being on the milder end of the microthrombotic disease spectrum of aPL-positive patients,[2] livedo reticularis and more often livedo racemosa are associated with other manifestations (arterial thrombosis, neurological, or cardiovascular) and predictors of morbidity in APS.[5,13,14] The mechanisms that cause livedo potentially reflect the similar underlying pathophysiology of more severe complications such as stroke, cognitive impairment, or pregnancy morbidity. Thus, skin as a proxy to other systemic presentations of APS may allow us to better understand aPL-related manifestations and serve as a guide for possible treatment options.

There is increased knowledge and ongoing research for the role of mTOR in SLE pathogenesis. To date, no study has investigated mTOR activation in skin samples of patients with SLE. Researchers studied mTOR activity in T-cell lineage development in patients with SLE and found increased p-S6RP along with decreased p-AKT activation in T cells of patients with SLE, both reversed by sirolimus.[15] Our results showed highest p-S6RP activity in aPL-positive patients with SLE, but not p-AKT. An explanation could be the synergetic effect of both aPL positivity and SLE, since aPL-positive patients without SLE had higher activity than aPL-negative patients with SLE.

The limitations of our study include relatively homogeneous demographics, a clinically stable patient population, and potential human error. Even though we performed analysis on cell counts, we acknowledge that our sample size is small, especially for the control group. Additionally, we did not have any patients with livedo racemosa or livedoid vasculopathy and/or skin ulcers that could have been helpful to assess more severe disease activity and may have strengthened our findings. Further, aPL-negative patients with livedo were not on any antiplatelet or anticoagulant treatment, suggesting a milder disease activity overall. Last, double staining for mTOR activation and endothelial cell markers may have enhanced our findings.

In conclusion, our results showed increased mTOR activity in livedoid lesions of aPL-positive patients with or without SLE, compared to aPL-negative patients with SLE. We also found more profound mTOR activity in the lower basal layers of epidermis compared to the upper surface layer. The findings of our study may serve as a basis for further investigating the effects of the mTOR pathway in aPL-positive patients.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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