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Angiogenesis in the skin of SHARPIN-deficient mice with chronic proliferative dermatitis

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Abstract

Angiogenesis is a common feature of pathological processes including wound healing, tumor formation, and chronic inflammation. Chronic inflammation can also be associated with dilation or proliferation of lymph vessels. We examined blood vessels and lymphatics and the expression of pro- and antiangiogenic genes in the skin of SHARPIN-deficient mice which spontaneously develop a chronic proliferative dermatitis (*cpdm*). The number of blood vessels in the dermis of *cpdm* mice increased with age as the inflammation progressed. Lymphatics identified by labeling for LYVE1 and podoplanin were moderately dilated, but they were not increased in number. The expression of proangiogenic *Vegfa*, *Flt1* and anti-angiogenic *Sema3a* mRNA was increased. VEGFA was primarily localized in keratinocytes of *cpdm* skin. There was also increased expression of *Ece1* and *Pdpn* mRNA. Podoplanin was restricted to lymphatic endothelial cells in normal skin, but fibroblasts in *cpdm* skin also reacted with anti-podoplanin antibodies indicating that they were activated. The expression of other angiogenic and lymphangiogenic factors was not altered or decreased. These results indicate that *cpdm* mice may be a useful model to study the pathogenesis of angiogenesis in chronic inflammation.

Keywords

Angiogenesis; lymphangiogenesis; VEGF; podoplanin; dermatitis

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Disclosure of conflicts of Interest

All other authors state no conflicts.

1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for organogenesis during embryonal and fetal development. Physiological angiogenesis rarely occurs in adults except during the ovarian and menstrual cycle and the hair follicle cycle (Gargett and Rogers, 2001; Robinson et al., 2009; Yano et al., 2001). Angiogenesis is an important component of pathological processes such as tumor growth, chronic inflammation, and wound healing (Carmeliet and Jain, 2011). Lymphangiogenesis refers to the formation of new lymphatics resulting in remodeling of the lymphatic network in tissues. Chronic inflammation can be accompanied by dilation of lymphatics and lymphangiogenesis (Liao and von der Weid, 2014). Angiogenesis and lymphangiogenesis are potential therapeutic targets for chronic inflammatory diseases, and it is therefore important to understand the role and processes underlying these processes in chronic inflammation (Tas et al., 2016).

Several acute and chronic skin diseases are associated with changes in the blood and lymph dermal vasculature (Varricchi et al., 2015). Psoriasis is a chronic inflammatory disease that is characterized by angiogenesis and dilation of dermal lymph vessels (Varricchi et al., 2015). Transgenic mice that overexpress *Vegfa* or *Stat3* driven by keratinocyte-specific promoters developed psoriasis-like skin lesions accompanied by an increased number of dermal blood vessels supporting the important role of angiogenesis in this disease (Sano et al., 2005; Xia et al., 2003). In atopic dermatitis, dermal blood vessels may be increased and more prominent, along with dilation of lymphatics (Mihm et al., 1976; Prose and Sedlis, 1960). Transgenic mice that overexpress *Il4* in keratinocytes, a model of atopic dermatitis, were reported to have a progressive increase of the number of blood vessels in the dermis in parallel with the inflammation (Chen et al., 2008). In addition, there was an increased number and dilation of lymphatics in the skin of these mice (Shi et al., 2012).

Mice with a null mutation in the *Sharpin* gene (C57BL/KaLawRij-*Sharpin*^{cpdm}/RijSunJ) develop a chronic proliferative dermatitis characterized by prominent ortho- and parakeratosis and accumulation of eosinophils, neutrophils, mast cells, and macrophages (HogenEsch et al., 1993; Seymour et al., 2007). The inflammation develops independently of B and T lymphocytes (Potter et al., 2014). Dermal blood vessels are prominent in these mice, but a detailed description and analysis of angiogenic factors is lacking. The SHARPIN protein forms a linear ubiquitination assembly complex (LUBAC) together with proteins HOIL1L (RBCK1) and HOIP (RNF31) that adds ubiquitin protomers to proteins in a linear Met1-linked fashion (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). This process plays an important role in NFkB signaling through cytokine and pattern recognition receptors (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011; Wang et al., 2012; Zak et al., 2011). Recent studies identified a Met1-specific deubiquitinase, OTULIN (ovarian tumor domain deubiquitinase with linear linkage specificity), that regulates signaling through these receptors (Fiil et al., 2013; Keusekotten et al., 2013; Mevissen et al., 2013; Rivkin et al., 2013). OTULIN-deficient mice die *in utero* with defects in angiogenesis (Rivkin et al., 2013). The role of linear ubiquitination in postnatal angiogenesis has not been described. Here, we report on a systematic analysis of blood and lymph vessels in the dermis of SHARPIN-deficient mice from before the onset of the dermatitis to severe chronic disease.

2. Materials and methods

2.1 Mice

C57BL/KaLawRij-*Sharnin*^{cpdm}/*Sharnin*^{cpdm} RijSunJ (JR# 7599; referred to hereafter as *cpdm*) mutant and wild type (WT, +/+) mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in specific pathogen-free conditions on a 12:12 hour light:dark cycle with constant temperature and humidity. Each double-pen polycarbonate cages (330 cm² floor area) housed a maximum of four per pen. Commercial autoclaved food (NIH 31, 6% fat; LabDiet 5K52, Purina Mills, St. Louis, MO) and acidified water (pH 2.8– 3.2) were fed *ad libitum*. Mice were genotyped by PCR as previously described to distinguish –/– or +/+ mice at day 10 after birth (Potter et al., 2014). All work was approved by The Jackson Laboratory and Purdue University Animal Care and Use Committees.

2.2. Histology and immunohistochemistry

Female *cpdm* mice and WT littermates were euthanized at 2, 4, 6, 8, and 10 weeks of age by CO₂ asphyxiation. Skin was fixed overnight by immersion in Fekete's acid–alcohol–formalin solution and then transferred to 70% ethanol. The skin was trimmed, processed routinely, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin (H&E). Serial sections were labeled with antibodies directed against CD31 (Abcam, Cambridge MA, cat# ab28364, 1:100), LYVE1 (Abcam, Cambridge MA, cat# ab14917, 1:100), smooth muscle actin isoform (SMA; Sigma, St Louis, MO, cat# A2547, 1:200), and vascular endothelial growth factor A (VEGFA; AbD Serotec, Raleigh NC, cat# AAM51, 1:400) using a Ventana autostainer (Tuscon, AZ). Diaminobenzidine (DAB; Sigma, St. Louis, MO) was used as the chromogen. Alternatively, skin was embedded in Optimal Cutting Temperature (OCT) medium, snap frozen in liquid nitrogen, and stored at –80 °C. Frozen sections were fixed in 100% cold acetone, labeled with Alexafluor 488 anti-podoplanin (Biolegend, San Diego, CA, cat# 127405) and counterstained with 4', 6-diamidino-2-phenylindole (DAPI; ThermoFisher Scientific, Waltham, MA).

2.3. Transcriptome analyses

Full thickness biopsies were collected from three female WT (four WT mice at 6 weeks of age) and three *cpdm* mice at 2, 4, 6, 8, and 10 weeks of age. Skin and tumor samples were stored in RNeasy Lysis Buffer (Qiagen, Crawley, UK) and homogenized in TRIzol (Life Technologies, Carlsbad, CA). Total RNA was isolated by standard TRIzol methods, and quality assessed using a 2100 Bioanalyzer instrument and RNA 6000 Nano LabChip assay (Agilent Technologies, Palo Alto, CA). Following reverse transcription with an oligo(dT)-T7 primer (Affymetrix, Santa Clara, CA), double-stranded cDNA was synthesized with the GeneChip Expression 3'–Amplification One-cycle kit (Affymetrix). In an *in vitro* transcription reaction with T7 RNA polymerase, the cDNA was amplified and labeled with biotinylated nucleotides (Affymetrix). Ten micrograms of biotin-labeled and fragmented cDNAs were hybridized onto GeneChip Mouse Genome 1.0 ST arrays (Affymetrix) for 16 hours at 45°C. Post-hybridization staining and washing was done according to the manufacturer's protocols using a Fluidics Station 450 instrument (Affymetrix). Finally, the arrays were scanned with a GeneChip Scanner 3000 laser confocal slide scanner. The images were quantified using GCOS 1.0 software (GeneChip Operating Software, Affymetrix).

Average signal intensities for each probeset within arrays were calculated by and exported from Affymetrix's Expression Console (Version 1.2) software using the RMA method which incorporates convolution background correction, quantile-normalization, and summarization based on a multi-array model fit robustly using the median polish algorithm. Pairwise comparisons were used to statistically resolve gene expression differences between sample groups using the R/MAANOVA analysis package (Wu et al., 2003). Specifically, differentially expressed genes were detected by using F_s , a modified F-statistic incorporating shrinkage estimates of variance components from within the R/MAANOVA package (Cui et al., 2005; Wu et al., 2003). Statistical significance levels of the pairwise comparisons were calculated by permutation analysis (1000 permutations) and adjusted for multiple testing using the false discovery rate (FDR), q-value, method (Storey, 2002). Differentially expressed genes were declared at an FDR q-value threshold of 0.05.

The microarray data from this experiment have been deposited in the Gene Expression Omnibus under accession number GSE81683.

3. Results

3.1. Histology

The skin of *cpdm* mice was characterized by epidermal acanthosis with diffuse orthokeratosis and focal to locally extensive parakeratosis and occasional apoptotic keratinocytes, and a mixed population of inflammatory cells in the dermis as previously described (HogenEsch et al., 1993). There were frequent cross sections of thin-walled blood vessels, consistent with capillaries and venules, in the superficial dermis (Fig. 1). Lymphatic vessels are located along the hair follicle shaft in mouse skin, and these were moderately dilated.

3.2. Transcriptome analysis

The expression of several genes related to angiogenesis and lymphangiogenesis was analyzed by microarray (Table S1). There was significantly increased expression of *Vegfa*, as early as 4 weeks of age, and its expression increased as the lesions progressed (Fig. 2). *Flt1* encodes for VEGFR1, one of the receptors for VEGFA. The expression of *Flt1* was increased in mice of 6 weeks of age and older. In contrast, the expression of *Kdr*, which encodes for VEGFR2, another receptor for VEGFA, was decreased (Fig. 2). The expression of the related genes *Vegfc*, *Figf* (encodes for VEGFD), and *Flt4* (encodes for VEGFR3), was either decreased or not significantly changed. The expression of *Pdpn* (encodes for podoplanin expressed on lymphatic endothelial cells) was increased at 4, 6, and 8 weeks of age, but this only reached significance at 6 weeks of age (Fig. 2). There was no change in the expression of *Lyve1*, another marker of lymphatic endothelial cells. There was a significant increase of *Ece1* mRNA and *Sema3a* mRNA (Fig. 2). The expression of other endothelial cell-related genes was either unchanged (*Ecel1*, *Edn1*, *Edn3*, *Ednra*, *Ednrb*, *Nrp2*) or decreased (*Nrp1*, *Edn2*) at one or more time points.

3.3. Immunohistochemistry

Labeling with anti-CD31 antibodies identified few blood vessels in the dermis of WT mice, and an increase of blood vessels in the dermis of *cpdm* mice as the mice aged (Fig. 3A). Both blood vessels and arrector pili muscles in the skin were labeled with anti-SMA antibodies. An increased number of SMA-positive blood vessels occurred in the skin of *cpdm* mice similar to the labeling with anti-CD31 (Fig. 3A). The majority of cells in *cpdm* skin that reacted with anti-VEGFA antibodies were keratinocytes (Fig. 3A). The labeling became more pronounced as the disease progressed.

Lymphatics in skin of WT mice were labeled with anti-LYVE1 and anti-podoplanin antibodies. LYVE1 was restricted to lymphatics in *cpdm* skin, and the lymphatics were moderately dilated (Fig. 3A). The number of LYVE1-positive lymphatics was not increased. Podoplanin was expressed by lymphatic endothelial cells and by fibroblasts in the dermis of *cpdm* mice (Fig. 3B).

4. Discussion

The chronic dermatitis in *cpdm* mice is associated with prominent angiogenesis and moderate dilation of lymphatics, but no evidence of lymphangiogenesis. The absence of lymphangiogenesis was evident in the lack of an increased number of LYVE1-positive vessels and the absence of changes in the expression of *Lyve1* mRNA at any of the examined ages. Consistent with this, there was no increased expression of *Flt4* mRNA which encodes for VEGFR3 expressed on lymphatic endothelial cells, or its ligands, VEGFC and VEGFD. The interaction of VEGFC or VEGFD with VEGFR3 mediates lymphangiogenesis (Veikkola et al., 2001). The role of lymphangiogenesis in chronic inflammation is poorly understood (Liao and von der Weid, 2014). Stimulation of lymphangiogenesis via transgenic overexpression of VEGFC or VEGFD reduced the severity of inflammation in a contact hypersensitivity dermatitis model (Huggenberger et al., 2010) raising the possibility that the lack of lymphangiogenesis in *cpdm* mice contributes to the chronic inflammation.

VEGFA is the major angiogenic factor in tissues and a target of successful therapies aimed at inhibiting neovascularization (Carmeliet and Jain, 2011; Varricchi et al., 2015). The expression of *Vegfa* mRNA was significantly increased in *cpdm* mice at four weeks and older suggesting an important role in the development of the skin lesions as this is the age at which the mutant mice first develop clearly visible skin lesions. VEGFA binds to both VEGFR1 and VEGFR2 with NRP1 as a co-receptor which facilitates signaling. VEGFA-induced angiogenesis is thought to be principally mediated by activation of VEGFR2 and the role of VEGFR1 in angiogenesis is controversial (Carmeliet and Jain, 2011). However, the increased expression of *Flt1* mRNA which encodes for VEGFR1 and decreased expression of *Kdr* which encodes for VEGFR2 suggests that the VEGFA-VEGFR1 interaction is an important mechanism underlying the prominent angiogenesis in *cpdm* dermatitis. VEGFA can be produced by various skin residential and inflammatory cells (Varricchi et al., 2015). Immunohistochemical labeling indicated that VEGFA was primarily expressed by keratinocytes in the *cpdm* skin. The dermatitis in *cpdm* mice has similarities to atopic dermatitis including the accumulation of mast cells and eosinophils and the increased expression of type 2 cytokines (HogenEsch et al., 2001). An increase of VEGFA has been

reported in the skin and blood of patients with atopic dermatitis although the role of this angiogenic factor in the pathogenesis of the disease has not been established (Amarbayasgalan et al., 2012; Koczy-Baron et al., 2012; Zhang et al., 2006). There was also increased expression of *Vegfa* mRNA and a progressive increase of the concentration of VEGFA protein in skin lesions of K14-*II4* transgenic mice, a model for atopic dermatitis (Chen et al., 2008). In contrast to the *cpdm* dermatitis, the expression of mRNA encoding for VEGFR2, but not VEGFR1, was increased in these mice.

Semaphorins are a family of transmembrane and secreted proteins that bind to plexin receptors. Semaphorin 3A binds type A or D1 plexin receptors and has an anti-angiogenic function (Carmeliet and Jain, 2011). The overexpression of *Sema3a* observed in the skin of *cpdm* mice may serve as an attempt to limit the degree of neovascularization in the skin during chronic inflammation.

Endothelin-converting enzyme (ECE) 1 and 2 catalyze the proteolytic processing of the precursor big endothelin into biologically active endothelin peptides (Davenport et al., 2016). In addition, they play a role in degradation of various neuropeptides (Roosterman et al., 2007). Endothelins are best known for their role in vasoconstriction, but they can also cause itching and pain (Davenport et al., 2016; Kido-Nakahara et al., 2014). In addition, endothelins can contribute to tumor angiogenesis (Kandalaf et al., 2011). Increased expression of *Ece1* mRNA in *cpdm* skin may result in increased production of biologically active endothelin which can contribute to the neovascularization and the pruritus which is a prominent feature of the clinical phenotype of these mice.

Podoplanin is a sialylated membrane glycoprotein that is expressed by fibroblastic reticular cells in lymphoid organs, lymphatic endothelial cells, selected epithelial cells and podocytes. It binds to CLEC1B (previously known as CLEC2) on platelets, but its function is not well understood (Astarita et al., 2012). The expression of *Pdpm* mRNA was significantly increased in *cpdm* dermatitis, and this was confirmed by immunofluorescence at the protein level. However, whereas podoplanin was primarily present on lymphatic endothelial cells in normal skin, it was widely expressed on fibroblasts in *cpdm* skin. This contrasts with the expression of LYVE1 which remained restricted to lymphatic endothelial cells in normal and *cpdm* skin. Previous studies have shown that the expression of podoplanin is increased on activated fibroblasts in mouse skin following application of 12-O-tetradecanoylphorbol-13-acetate and during wound healing (Gandarillas et al., 1997), in human fibroblasts associated with tumors (Pula et al., 2013), and fibroblast-like synovial cells in patients with rheumatoid arthritis (Ekwall et al., 2011). Incubation of normal fibroblast-like synoviocytes with cytokines IL1B, TNF, or TGFB induced increased expression of podoplanin (Ekwall et al., 2011). There is an increase of IL1B in the skin of *cpdm* mice (Wang and HogenEsch, unpublished) suggesting that this could be one of the cytokines underlying the increased expression of podoplanin in the *cpdm* dermis.

In summary, chronic proliferative dermatitis in SHARPIN-deficient *cpdm* mice is associated with angiogenesis and moderate dilation of lymphatics, but not lymphangiogenesis. The angiogenesis is associated with upregulation of several proangiogenic factors, most importantly VEGFA. Increased expression of the anti-angiogenic factor semaphorin 3A may

represent an attempt to curtail angiogenesis. These data suggest that *cpdm* mice may be a useful model to study the pathogenesis of angiogenesis in chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>Cpdm</i>	chronic proliferative dermatitis allelic mutation
ECE	endothelin-converting enzyme
FDR	false discovery rate
HOIL1L	heme-oxidised IRP2 ubiquitin ligase 1L
HOIP	HOIL1L-interacting protein
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1
OTULIN	ovarian tumor domain deubiquitinase with linear linkage specificity
SHARPIN	SHANK-associated RH domain interactor
SMA	smooth muscle actin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WT	wild type

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Highlights

- SHARPIN-deficient mice develop a chronic proliferative dermatitis (*cpdm*)
- Angiogenesis and lymphatic dilatation are present in *cpdm* dermatitis
- Increase of *Vegfa* and *Flt1* mRNA in *cpdm* skin
- Podoplanin is increased on activated dermal fibroblasts in *cpdm* skin

Fig. 1. Increased number of blood vessels in the superficial dermis of *cpdm* mice. Eight week old female *cpdm* mouse. Hematoxylin and eosin. Arrows identify blood vessels that contain red blood cells; * lymphatic vessel.

Fig. 2.

Changes in expression of genes involved in angiogenesis with age in *cpdm* mice. The data represent the mean change in gene expression of three *cpdm* mice compared with control female littermates for each age. *significant different at $P < 0.05$.

Fig. 3.

(A) Immunohistochemistry for CD31, smooth muscle actin (SMA), LYVE1 and vascular endothelial growth factor A (VEGFA) in skin from 8 week old *cpdm* and WT mice. The number of blood vessels (labeled for CD31 and SMA) in the skin of *cpdm* mice is increased (arrows). Lymphatics identified with LYVE1 are moderately dilated. There is increased expression of VEGFA in keratinocytes of *cpdm* mice. (B) Increased expression of podoplanin in the skin of *cpdm* mice. Immunofluorescent labeling of frozen skin sections with Alexafluor 488-labeled anti-podoplanin antibodies. White arrows identify lymphatics.