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Dermal lymphatic dilation in a mouse model of alopecia areata

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ABSTRACT

Mouse models of various types of inflammatory skin disease are often accompanied by increased dermal angiogenesis. The C3H/HeJ inbred strain spontaneously develops alopecia areata (AA), a cell mediated autoimmune disorder that can be controllably expanded using full thickness skin grafts to young unaffected mice. This provides a reproducible and progressive model for AA in which the vascularization of the skin can be examined. Mice receiving skin grafts from AA or normal mice were evaluated at 5, 10, 15, and 20 weeks after engraftment. Lymphatics are often overlooked as they are small slit-like structures above the hair follicle that resemble artifact-like separation of collagen bundles with some fixatives. Lymphatics are easily detected using lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) by immunohistochemistry to label their endothelial cells. Using LYVE1, there were no changes in distribution or numbers of lymphatics although they were more prominent (dilated) in the mice with AA. *Lyve1* transcripts were not significantly upregulated except at 10 weeks after skin grafting when clinical signs of AA first become apparent. Other genes involved with vascular growth and dilation or movement of immune cells were dysregulated, mostly upregulated. These findings emphasize aspects of AA not commonly considered and provide potential targets for therapeutic intervention.

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1. Introduction

Angiogenesis is a hallmark of inflammation, wound healing, and neoplasia (Patan, 2004). Angiogenesis is also the initial gross and histological finding in two stage cutaneous chemical carcinogenesis or UVB induced skin cancers long before there is any evidence of epidermal hyperplasia or neoplasia (Binder et al., 1998; Konger et al., 2013). Angiogenesis is present in the dermis of various mouse models commonly used to study genetically based inflammatory skin diseases. For example, the chronic proliferative dermatitis mouse (C57BL/KaLawRij-*Sharpin*^{cpdm}/RijSunJ) has an autoinflammatory skin disease where the mice develop progressively worsening inflammation consisting primarily of eosinophils, neutrophils, macrophages, and mast cells that are independent of B and T lymphocytes. These mice develop progressive and prominent neovascularization (HogenEsch et al., 1993). This phenotype is due to a null mutation in the mouse *Sharpin* gene (Seymour et al., 2007) which interacts with a series of pathways, one of which activates the NFKB pathway which regulates inflammation (Ikeda et al., 2011; Liang et al., 2011; Wang et al., 2012a, 2012b). Similarly, the flaky skin mutant mouse (CByJ.A-*Ttc7*^{fsn}/J) also has marked dermal neovascularization but less is known about the molecular pathogenesis of this mouse skin disease (Helms et al., 2005; Sundberg et al., 1997).

Although described in detail in various anatomic locations in the skin of mice (Karlsen et al., 2006), lymphatics are largely ignored in the evaluation of mouse models with various types of skin disease, probably because they appear as thin slit-like structures, not unlike separation in the dense irregular collagenous connective tissue that makes up the dermis. LYVE1 is a widely used marker for lymphatic endothelial cells (Jackson, 2004) making it very useful for evaluating these structures in mice and other species. Lymphatics are an essential part of the body's immune defense in which lymph circulates back to the blood circulation by being filtered through lymph nodes where foreign material is taken up by antigen presenting cells which can initiate specific immune responses (Alitalo et al., 2005). Lymphatics, like blood vessels, can proliferate during inflammation (Alitalo et al., 2005; Pullinger and FLorey, 1937).

Alopecia areata (AA) is a lymphocyte-dependent autoimmune disease (Carroll et al., 2002). Human AA patients have increased lesional

Abbreviations: AA, alopecia areata; *Ccr7*, chemokine (C-C motif) receptor 7; *Cxcl11*, chemokine (C-X-C motif) ligand 12; *Cxcr4*, chemokine (X-X-C motif) receptor 4; *Ece1* and *2*, endothelin converting enzyme 1 and 2; *End1*, endothelin 1; *Flt1*, FMS-like tyrosine kinase 1; H&E, hematoxylin and eosin stain; *Kdr*, Kinase insert domain receptor, also known as vascular endothelial growth factor 2, *VEGF2*; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1; PECAM1, platelet/endothelial cell adhesion molecule 1, formerly CD31; *Pdpn*, podoplanin; *Sharpin*^{cpdm}, SHANK-associated RH domain interacting protein, chronic proliferative dermatitis allelic mutation; SMA, smooth muscle actin isoform; *Ttc7*^{fsn}, tetratricopeptide repeat domain 7, flaky skin allelic mutation.

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skin temperature that was proposed to be the result of the degree of vascularization (Mijailović et al., 1997). Conversely, other early studies suggested that angiogenic activity was defective in monocytes from alopecia universalis (the most severe form of AA) patients (Skoutelis et al., 1990). Several inbred strains, most notably C3H/HeJ (McElwee et al., 1999; Sundberg et al., 1994), develop a complex genetic based cellmediated autoimmune disease, AA (Carroll et al., 2002; Sundberg et al., 2003). These mice develop a mixed but primarily lymphocytic inflammation in and around hair follicles without obvious changes in the dermal vasculature. This is a well-established mouse model for AA. C3H/HeJ mice naturally develop AA at a frequency of around 20–25% by one year of age (Sundberg et al., 1994). These mice can be used in experimental studies, although lesions tend to wax and wane. A more consistent model was developed using full thickness skin grafts from old mice with AA onto young mice, 10 weeks of age, who developed disease that repeatedly and consistently progressed from patchy alopecia at 8–10 weeks after grafting to diffuse alopecia by 20 weeks after grafting (McElwee et al., 1998; Silva and Sundberg, 2013; Sundberg et al., 2004). Using the skin graft model, changes in the dermal vasculature were evaluated at multiple time points from initial development through progression of diffuse AA. Mild to moderate changes in the lymphatics were found but not in blood vessels using histology, immunohistochemistry, and transcriptome analyses.

2. Materials and methods

2.1. Mice

Graft recipient C3H/HeJ (JR# 659) female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 10 weeks of age. Donor C3H/HeJ mice were obtained from production colonies as retired breeders, maintained for 6 months, and those that developed spontaneous AA were used as donors. Surgical methods for full thickness skin grafts have been described in detail elsewhere (McElwee et al., 1998; Silva and Sundberg, 2013). All work was approved by The Jackson Laboratory Animal Care and Use Committee (Approval number 07005).

2.2. Histology and immunohistochemistry

Mice were euthanized by CO_2 asphyxiation. The dorsal skin was surgically removed at the time of necropsy, laid flat on aluminum foil in a cranio-caudal orientation, and fixed overnight by immersion in Fekete's acid–alcohol–formalin solution and then transferred to 70% ethanol (Silva and Sundberg, 2012). The skin was trimmed, processed routinely, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E). Serial sections were immunolabeled using antibodies directed against PECAM1/CD31 (for endothelial cells; Abcam, Cambridge MA, cat# ab14917, 1:100), LYVE1 (for lymphatics, Abcam, Cambridge MA, cat# ab14917, 1:100), and smooth muscle actin isoform (SMA, for vascular smooth muscle; Sigma, St Louis, MO, cat# A2547, 1:200) (http://tumor.informatics.jax.org/html/antibodies.html) using a Ventana autostainer (Tuscon, AZ). Diaminobenzidine (DAB; Sigma, St. Louis, MO) was used as the chromogen.

2.3. Transcriptome analyses

RNA samples from 3 AA graft and 3 normal graft-recipient mice were used at each time point and processed using the MOE430v2.0 GeneChip™ (Affymetrix) as described and reported previously (Duncan et al., 2013; McPhee et al., 2012). Briefly, skin and spleen samples were harvested in an RNAse free manner and stored in RNAlater (Ambion, Austin, TX) per the manufacturer's instructions and homogenized in TRIzol (Invitrogen, 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 Superscript double-stranded cDNA synthesis custom kit (Invitrogen). In an *in vitro* transcription (IVT) reaction with T7 RNA polymerase, the cDNA was amplified and labeled with biotinylated nucleotides (Enzo Diagnostics, Farmingdale, NY). Fifteen micrograms of biotin-labeled and fragmented cRNA was hybridized onto MOE430v2.0 GeneChip™ arrays (Affymetrix) for 16 h at 45 °C. Post-hybridization staining and washing was done according to the manufacturer's protocols using a Fluidics Station 450 (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). Data were imported into the R software environment and analyzed using the R/MAANOVA package. After graphical diagnostics and appropriate transformations, an analysis of variance (ANOVA) model was applied to the data, and F1, F2, F3, and Fs test statistics constructed along with their permutation p-values.

3. Results

3.1. Lymphatics

Lymphatics in the mouse skin are localized in the dermis and hypodermal fat layer. The lymphatics are uniformly located above the hair bulb and parallel to the hair follicle shaft. As the dense irregular collagenous connective tissue fragments and separates in routine histological sections stained with H&E and fixed in formalin based fixatives, these are usually overlooked. Some of the slit-like structures that resemble collagen bundle separations are lined by endothelial cells. LYVE1 clearly labeled the endothelial cells lining the lymphatics (Fig. 1). Lymphatic distribution and numbers did not change between age and gender matched sham grafted and AA grafted C3H/HeJ female mice as alopecia areata developed and progressed. However, lymphatics in the dermis above the hair follicles became progressively larger and more dilated as the disease progressed from normal to patchy to generalized alopecia.

3.2. Blood Vessels

Smooth muscle actin (SMA) labeled vascular smooth muscle and arrector pili muscles in skin sections. Platelet/endothelial cell adhesion molecule 1 (PECAM1) labeled only vascular endothelial cells but not those lining lymphatics. In both cases, blood vessels, not normally prominent in the dermis of the mouse, remained similar in size and distribution between the normal sham-grafted mice and those developing progressive AA (data not shown, available on http://pathbase.net/). This is in contrast to changes in the dermis seen in primarily neutrophil (flaky skin mutant mice, *Ttc7*^{fsn}) or a mixture of neutrophil and eosinophil driven (chronic proliferative dermatitis, *Sharpin*^{cpdm}) inflammatory scaly skin disease models in which lymphatics are unchanged, but blood vessels become prominent and tortuous in the dermis and hypodermal fat layer (data not shown).

3.3. Transcriptome results

While there were histologically evident changes in lymphatics in the dermis of mice with developing AA, there were no significant changes in *Lyve1* transcripts over time (Table 1). This is consistent with dilation of lymphatics rather than proliferation. Blood vessels were of normal size and distribution between controls and those mice developing progressive AA, although some heavily affected areas had focal areas where the blood vessels were more prominent and larger. Transcripts' coding for proteins that regulate blood vessels, lymphatics, endothelial cells, and the immune cells that are carried in these vessels (*End1*, *Flt1*, *Pdpn*, *Ece1* and *2*, *Ccr7*, *Cxcl12*, and *Cxcr4*) were significantly upregulated

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