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Endogenous Retinoids in the Pathogenesis of Alopecia Areata

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Alopecia areata (AA) is an autoimmune disease that attacks anagen hair follicles. Gene array in graft-induced C3H/HeJ mice revealed that genes involved in retinoic acid (RA) synthesis were increased, whereas RA degradation genes were decreased in AA compared with sham controls. This was confirmed by immunohistochemistry in biopsies from patients with AA and both mouse and rat AA models. RA levels were also increased in C3H/HeJ mice with AA. C3H/HeJ mice were fed a purified diet containing one of the four levels of dietary vitamin A or an unpurified diet 2 weeks before grafting and disease progression followed. High vitamin A accelerated AA, whereas mice that were not fed vitamin A had more severe disease by the end of the study. More hair follicles were in anagen in mice fed high vitamin A. Both the number and localization of granzyme B–positive cells were altered by vitamin A. IFNγ was also the lowest and IL13 highest in mice fed high vitamin A. Other cytokines were reduced and chemokines increased as the disease progressed, but no additional effects of vitamin A were seen. Combined, these results suggest that vitamin A regulates both the hair cycle and immune response to alter the progression of AA.

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INTRODUCTION

Alopecia areata (AA) is an autoimmune, nonscarring, hair loss disease affecting up to 1.7% of humans (Safavi *et al.*, 1995). Current treatments are often ineffective in inducing prolonged remission (Tosti and Duque-Estrada, 2009; Harries *et al.*, 2010). AA is characterized by a loss of hair follicle immune privilege, increased IFN gamma (IFNγ) and other T helper 1 (Th1) cytokines, and an increase in $CD8^+$ T cells (McElwee *et al.*, 1996; Gilhar *et al.*, 2005; King *et al.*, 2008). NK or NKT cells and T regulatory (Tregs) cells may also be involved (McElwee *et al.*, 2005; Ito *et al.*, 2008; Petukhova *et al.*, 2010). Studies suggest that AA is a complex polygenetic disease (Sundberg *et al.*, 2004; Petukhova *et al.*, 2010). Little is known about the environmental factors, such as diet, that impact the course of AA.

Several interactions between retinoids and immunity exist (Duriancik *et al.*, 2010). Vitamin A deficiency impairs the development of cell-mediated immunity (Smith *et al.*, 1987) and promotes Th1 responses while delaying Th2 development (Cantorna *et al.*, 1994). Recently, additional T-cell subtypes were appreciated as important in autoimmunity, including Th17 and T Tregs cells, which are both regulated by vitamin A to maintain gut immune tolerance (Stockinger *et al.*, 2007; Sojka *et al.*, 2008). Retinoic acid (RA) synthesis occurs in dendritic cells (DCs) in the gut (Iwata *et al.*, 2004), which increases FOXP3-positive Tregs (Coombes *et al.*, 2007) and inhibits Th17 cell development (Mucida *et al.*, 2007) via RA receptor alpha (RARA) *in vitro* (Schambach *et al.*, 2007). Inducing *in vivo* endogenous RA synthesis through activation of toll-like receptor 2 (Manicassamy *et al.*, 2009) or PPARG agonist (Housley *et al.*, 2009) inhibited Th17 cells and increased FOXP3. Exogenous RA inhibited Th17 cells but had no effect on FOXP3 (Xiao *et al.*, 2008), suggesting that RA needs to be induced in a precise location endogenously to maintain gut immune tolerance. Similar to the gut, skin has a major barrier function. DCs in the dermis have similar characteristics to those in the gut, including langerin expression (Bursch *et al.*, 2007). Mouse ear dermal DCs had aldehyde dehydrogenase activity and induced FOXP3-positive

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Abbreviations: AA, alopecia areata; ALDH1A1, 2, 3, retinal dehydrogenase1, 2, 3; CRABP2, cellular retinoic acid–binding protein II; CRABP2, cellular retinoic acid–binding protein II; DGAT1, diacylglycerol acyltransferase 1; DHRS9, dehydrogenase reductase SDR family member 9; GZMB, granzyme B; LRAT, lecithin:retinol acyltransferase; NKG2D, natural killer group 2D; RA, retinoic acid; Raldhs, retinal dehydrogenases; RARA, B, G, retinoic acid receptor alpha, beta, gamma; RBP1 (formerly CRBP), cellular retinol–binding protein 1; Roldhs, retinol dehydrogenases; STRA6, stimulated by retinoic acid 6; Th1, T helper 1; VAA, vitamin A adequate; VAD, vitamin A deficient; VAE, vitamin A excess; VAH, vitamin A high. Genes and RNA message expression are italicized, whereas proteins are not. Proteins from mice, rats, and humans are all capital letters.

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Tregs in an RAR-dependent manner, although it was not confirmed that these cells also expressed langerin or retinal dehydrogenase 2 (ALDH1A2, (Guilliams *et al.*, 2010). Collectively, the results from these studies suggest that RA synthesis within the gut and skin DCs regulates immune tolerance.

Excess RA leads to alopecia (Ruzicka *et al.*, 1992; Ries and Hess, 1999; Shih *et al.*, 2009), which may result from many factors including dysregulated immune function. Vitamin A deficiency leads to follicular hyperkeratosis and rupture in humans and rodents (Wolbach and Howe, 1925; Girard *et al.*, 2006). In rodents, vitamin A deficiency also leads to a thin hair coat that is frequently seen but rarely reported (Anzano *et al.*, 1979, unpublished observation; Everts and Berdanier, 2002). The results from these studies suggest that precise RA levels are needed for optimal hair follicle function.

RA synthesis occurs locally in or near the cells where it will ultimately be used. Precise spatial and temporal levels of RA in the skin are achieved by regulating several key steps in cellular vitamin A metabolism: storage as retinyl esters, RA synthesis, and RA degradation (Everts, 2012). In brief, vitamin A circulates as retinol bound to retinol-binding protein (RBP4). Retinol is transported into the cell via stimulation by RA 6 (STRA6) and binds cellular retinol binding protein 1 (RBP1, aka CRBP). This bound retinol can either be esterified by lecithin:retinol acyltransferase (LRAT) for storage or reversibly oxidized to retinal via retinol dehydrogenases, such as dehydrogenase reductase SDR family member 9 (DHRS9) or RDH10. Retinal is further oxidized to RA by retinal dehydrogenases 1–3 (ALDH1A1–3). RA is then sent to the nucleus with the assistance of cellular RA binding protein 2 (CRABP2) to bind its RA receptors alpha, beta, and gamma (RARA, B, C) and activate the transcription of 500+ genes (Balmer and Blomhoff, 2002). When RBP1/CRBP is saturated or absent, retinol can be esterified by acyl CoA: diacylglycerol acyltransferase 1 (DGAT1) or cleared by conversion to retinal by alcohol dehydrogenases 1–4. Retinal is then oxidized to RA via ALDH1A1 and further metabolized by cytochrome P450 26 family members (CYP26A1, B1, C1) with the assistance of cellular RA binding protein 1 (CRABP1).

To better understand AA, transcriptome analysis of C3H/HeJ mice with AA was performed. As retinoid metabolism was not part of the network analysis software, the expression of retinoid metabolism genes was examined with the hypothesis that RA metabolism was not altered in AA. Transcripts coding for proteins metabolizing retinoids were altered, which was confirmed in biopsies from AA patients and rodent AA models. High dietary vitamin A accelerated disease progression and numbers of hair follicles in anagen. Lack of vitamin A resulted in a more severe disease. A few immune factors were also altered by diet, suggesting that retinoids alter AA by regulating both the hair cycle and immune response.

RESULTS

The capacity for RA synthesis was increased in AA

Analysis of graft-induced AA transcripts revealed that the expression of most genes involved in RA synthesis was significantly increased (Figure 1a, Supplementary Tables S3–S6 online), whereas the expression of Rbp4 and RA degradation genes was significantly decreased (Figure 1b) in AA compared with sham controls at 10, 15, and sometimes 20 weeks after grafting. Only Rbp1 (Crbp1), Crabp2, and Aldh1a3 transcripts were significantly increased and Stra6 significantly decreased in mice with spontaneous AA compared with wild-type C3H/HeJ mice (Figure 1c).

Because differences in the hair cycle between the AA mice and controls were found (Supplementary Figure S2 online) and RA synthesis components changed during the hair cycle (Everts *et al.*, 2007), these gene array results were confirmed using immunohistochemistry (IHC) on human, DEBR rat, and C3H/HeJ mouse skin, with or without AA, using antibodies against specific RA synthesis and degradation proteins to better control for hair cycle changes. RBP1/CRBP had the greatest increase in immunoreactivity in biopsies of patients with AA and both rodent models (Figure 2a–d and Supplementary Figure S3a and b online). RBP1/CRBP was also high in CBA/CaHN-*Btk*^{xnl}/J, SWR/J, and A/J mice with AA, but not in C3H/HeOuJ mice with AA (Supplementary Figure S4 online). DHRS9 and CRABP2 were also increased in mice but only slightly increased in AA patients and DEBR rats (Figure 2e–h, q–t and Supplementary Figure S3c, d, g, and h online). ALDH1A1 and ALDH1A2 were increased in biopsies from humans, but were not different in rodent models (Figure 2i–l and Supplementary Figure S6a–d online). ALDH1A3 was greatly increased in mice, absent from the pre-medulla in DEBR rats, and not different in biopsies from humans, although no pre-medulla was present in human samples (Figure 2m–p and Supplementary Figure S3e, f online). Immunoreactivity of CYP26B1 was not different between C3H/HeJ mice with AA or controls, but localization changed during the hair cycle (data not shown). DHRS9, ALDH1A1, and ALDH1A2 also localized to infiltrating immune cells in biopsies from human patients with AA and C3H/HeJ mice (Figure 2f, h, j, l and Supplementary Figure S6a–d arrow online). In DEBR rats, immune cells expressed DHRS9 but not ALDH1A1 or ALDH1A2 (Supplementary Figure S3d online, data not shown). ALDH1A2 colocalized with DC markers langerin and natural killer group 2D (NKG2D; Supplementary Figure S6e, f arrow online).

Retinoid levels were measured by liquid chromatography/mass spectometry (LC/MS/MS) and HPLC to confirm this expression pattern. RA levels were significantly greater ($P<0.05$), whereas retinol levels were lower ($P=0.061$), in AA mice compared with controls (Supplementary Figure S7a and b online). There was no difference in retinyl ester levels (Supplementary Figure S7c online).

Dietary vitamin A altered the progression of AA

To determine whether dietary vitamin A altered AA, C3H/HeJ mice were fed one of the five diets, starting 2 weeks before receiving a graft from a mouse with AA, and the progression of the disease was analyzed. Ventral hair loss was significantly increased at 13, 14, and 18 weeks post grafting in mice fed high vitamin A (12 IU, VAH) compared with mice fed unpurified chow (control, Figure 3a). There was also a trend at 14 weeks with mice fed VAH diet having more hair loss than mice fed the vitamin A–deficient diet