Vitamins A and D are potent inhibitors of cutaneous lymphocyte-associated antigen expression

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Background: Cutaneous lymphocyte-associated antigen (CLA) is a surface glycoprotein expressed by skin-homing T cells. This carbohydrate moiety expressed on mucin-like surface glycoproteins, including P-selectin glycoprotein ligand 1 and CD43, confers binding activity to dermal endothelial E-selectin and is critical for T-cell recruitment to the skin. Vitamin A (retinoic acid [RA]) and the active form of vitamin D3 (1,25 dihydroxyvitamin D3 [1,25D(3)]) have been used to treat certain T cell–mediated inflammatory skin diseases, as well as cutaneous T-cell lymphomas; however, their effect on CLA expression has not been studied.

Objective: We analyzed the effects of RA and 1,25D(3) on expression of CLA and other lymphocyte-homing receptors on human T cells.

Methods: We cultured human T cells with 1,25D(3) and RA and analyzed the expression of CLA and other homing receptors. We also pretreated mice with either vitamin and then induced an antigen-dependent contact hypersensitivity response.

Results: Both RA and 1,25D(3) downregulated expression of the CLA and, in parallel, functional E-selectin ligand. Whereas RA increased expression of the gut-homing receptor α4β7 and reduced L-selectin expression, 1,25D(3) had no effect on other homing receptors. In an *in vivo* assay treatment with RA or 1,25D(3) downregulated the skin infiltration of effector $CD4^+$ T cells.

Conclusion: These findings suggest that 1,25D(3) can selectively downregulate CLA expression without influencing lymphocyte migration patterns to other tissues.

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Key words: *1, 25-Dihydroxyvitamin D(3), retinoic acid, cutaneous lymphocyte-associated antigen, chemokine receptor*

Abbreviations used

7-ADD:	7-Amino-actinomycin
AP:	Alkaline phosphatase
CCR:	Chemokine receptor
CLA:	Cutaneous lymphocyte-associated antigen
CTCL:	Cutaneous T-cell lymphoma
1,25D(3):	1, 25-Dihydroxyvitamin D(3)
FITC:	Fluorescein isothiocyanate
FucT-VII:	α1,3-Fucosyltransferase
β4GalT:	β1,4-galactosyltransferase
LN:	Lymph node
NP-40:	Nonidet P-40
PSGL-1:	P-selectin glycoprotein ligand 1
RA:	Retinoic acid
ST3Gal:	α2,3-sialyltransferase

Lymphocyte trafficking to the skin is dependent on the expression of cutaneous lymphocyte-associated antigen (CLA), a sialyl $Lewis^x$–related epitope expressed on P-selectin glycoprotein ligand 1 (PSGL-1) and CD43.[1-6] CLA is highly expressed on skin-infiltrating T cells in inflammatory skin diseases, including psoriasis, allergic contact dermatitis, and atopic dermatitis.[2-5,7-9] Malignant clonal T cells in cutaneous T-cell lymphoma (CTCL) also express CLA.[10] Because CLA is a major E-selectin ligand for dermal vascular E-selectin and promotes T-cell entry into the skin, this molecule might be a useful target for the treatment of T cell–mediated skin diseases.[3,4,9] Recent studies suggest that reduction of CLA or E-selectin ligand expression on effector T cells can ameliorate T-cell trafficking to inflamed skin and development of the T cell–dependent cutaneous inflammatory response.[4] CLA function is dependent on α1,3 fucosylation of PSGL-1 O-glycans by α1,3-fucosyltransferase (FucT) VII[1-4] and appears to be controlled in part by IL-12 and TGF-β expression[5,11,12]; however, other factors regulating CLA expression or fucosyltransferase expression have not been determined.

Vitamin D(3) (cholecalciferol) is produced in the skin after exposure to sunlight and is converted to its biologically active metabolite, 1, 25-dihydroxyvitamin D(3) (1,25D[3]). In the liver and kidney 1,25D(3) has been shown to influence the development of autoimmune diseases, including ulcerative colitis and Crohn's disease,[13,14] as well as skin diseases, such as psoriasis.[5] 1,25D(3) has been reported to decrease the production of IFN-γ and IL-2 in T cells, while increasing their production of IL-4.[14,15] These effects on cytokine production suggest that 1,25D(3) can skew T-cell development from a T_H1 to a T_H2 phenotype. 1,25D(3) is also known to inhibit the differentiation of human dendritic cells from monocytes, as well as their maturation into antigen-presenting cells.[16] Specific alterations of T cell–homing receptor expression by 1,25D(3) in the treatment of psoriasis, atopic dermatitis, or CTCL have not been reported. On the other hand, retinoic acid (RA) synthesis is accomplished by 2 sequential oxidation steps in which retinol is oxidized to retinal and retinal is oxidized to RA. Interestingly, the induction of

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lymphocyte-homing receptor expression can be triggered by vitamin A analogues, as evidenced by a recent report that showed RA induces murine T cells to express gut-homing molecular features.[17]

In the present study we analyzed the effect of 1,25D(3) and RA on the expression of CLA, as well as other gut- and lymph node (LN)–homing receptors and chemokine receptors (CCRs), on human $CD3^+$ T cells. Using flow cytometry and Western blotting/immunoprecipitation approaches, we found that 1,25D(3) at concentrations as low as 1 nM decreased the decoration of PSGL-1 with the CLA epitope, whereas RA at similar pharmacologically attainable concentrations also ablated CLA expression. RA also decreased the expression of the skin-homing receptor CCR10 and the LN-homing receptor L-selectin (CD62L) and increased the expression of the gut-homing receptors $\alpha4\beta7$ integrin and CCR9. 1,25D(3), on the other hand, did not alter the expression of gut- or LN-homing receptors or of CCRs. Quantitative PCR of glycosyltransferase mRNAs showed that reduction of CLA expression mediated by both 1,25D(3) and RA was associated with reduced FucT-VII mRNA levels. Finally, using a murine model of contact hypersensitivity, we found that *in vivo* treatment with 1,25D(3) or RA resulted in the inhibition of skin infiltration of $CD4^+$ T cells. These findings suggest that 1,25D(3) might selectively downregulate CLA expression without influencing lymphocyte migration patterns to other tissues.

METHODS

Cell purification and culture

Fresh whole blood was collected from healthy donors in compliance with institutional review board policies, and PBMCs were prepared by means of density gradient centrifugation over Ficoll-Histopaque (Sigma Chemical Co, St Louis, Mo). $CD3^+$ T cells were purified with the pan-T cell isolation kit II (Miltenyi Biotec, Auburn, Calif). For analysis of CLA and PSGL-1 expression, $CD3^+$ T cells were cultured in XVIVO15 medium (BioWhittaker, Walkersville, Md) supplemented with 2 mM L-glutamine, 0.5 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 80 U/mL recombinant human IL-2.[11] For analysis of other tissue-homing receptor and CCR expression, $CD3^+$ T cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, Calif) supplemented with 2 mM L-glutamine, 0.5 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FCS. Cells were plated initially at 2×10^6 cells/mL in 24-well, plastic, tissue-culture plates (Costar, Cambridge, Mass) coated with azide-free murine anti-human CD3 mAb on goat anti-mouse IgG. To these cultures, 1,25D(3) (active vitamin D3), cholecalciferol (the inactive precursor of vitamin D3), RA (all-trans-RA, the active form of vitamin A), retinol (an inactive precursor of vitamin A), and retinal (all-trans-retinal, an active intermediate form of vitamin A) were added at final concentrations of 1 pM to 1 μM. After 2 days, cells were harvested, suspended in the same medium, and added to non–antibody-coated plates. Half of the medium was replaced with fresh medium every other day, and cells were collected on day 7. Cells were washed twice with PBS and then analyzed by means of flow cytometry, or cell lysates were prepared for Western blotting/immunoprecipitation experiments.

Flow cytometric studies

Flow cytometric analysis was performed by using directly conjugated mAbs to human CD3, CLA, CD162, CCR4, CCR6, CCR9, CD62L, α4 integrin, β7 integrin, CD25, and CD69, and isotype controls for these antibodies were purchased from BD Biosciences (San Diego, Calif). Fluorescein isothiocyanate (FITC)–annexin-V and 7-amino-actinomycin (7-AAD) were also purchased from BD Biosciences. Monoclonal antibodies to human CCR7 and CCR10 were purchased from R&D Systems (Minneapolis, Minn). Immunophenotypic analysis of cells was performed on a Becton Dickinson FACScan instrument with CellQuest software (Becton Dickinson, Mansfield, Mass).

Detection of E- and P-selectin ligands

Cultured T cells were first stained with FITC-conjugated anti-CLA antibody. The cells were washed and subsequently incubated with 10 μg/mL E- or P-selectin human IgG Fc chimera (R&D systems) in HBSS supplemented with 2 mM calcium, 5% FCS, and 1 mM HEPES. After incubation for 30 minutes at 4°C, the cells were gently washed and incubated with phycoerythrin-conjugated goat $F(ab')_2$ anti-human IgG (Beckman Coulter) for 30 minutes at 4°C.

Quantification of mRNA expression levels

We analyzed mRNA levels of 7 principal glycosyltransferases involved in the synthesis of CLA[18] in $CD3^+$ T cells cultured in the presence or absence of 1,25D(3), RA, or diluent control (ethanol). For quantitative RT-PCR analysis, total RNA was extracted with the Clontech RNA purification kit (Clontech, Palo Alto, Calif), according to the manufacturer's instructions. Total RNA (2.5 μg; A260/A280 = 1.7-2.0) was reverse transcribed with oligo-dT primers and Powerscript Reverse Transcriptase (Clontech, Palo Alto, Calif) in a final volume of 20 μL. One microliter of the cDNA was amplified by means of PCR to a final volume in 50 μL with SYBR Green PCR Core reagents (Biosystems, Warrington, United Kingdom) and 200 nM of glycosyltransferase primers. cDNAs were also probed for the expression of β-actin to control for fidelity and efficiency of cDNA synthesis from each cell preparation. The following glycosyltransferase mRNAs were probed: core 2 β1, 6 N-acetylglucosaminyltransferase; N-acetylglucosamine-6-O-sulfotransferase 2; β1,4 galactosyltransferase I and III (β4GalT-I and β4GalT-III), α2,3-sialyltransferase III and IV (ST3Gal-III and -IV), and FucT-VII. The primer sequences were as follows (5′-3′): core 2 β1, 6 N-acetylglucosaminyltransferase, ATCCGAAA CACCTCTCTTTTCTGGC and GGTCAGTGTTTTAATGTCTCCAAAG[18]; N-acetylglucosamine-6-O-sulfotransferase 2, GCACACTAGTCATAAAGG GTGTGC and TTGCGTGCAGATACCACGAAAGGC[18]; β4GalT-I, AAG CAGAACCCAAATGTGAAGATG and GGGCGAGATATAGACATGC CTC[18]; β4GalT-III, TCTACCACCTGCACCCCTTCTTGC and GCTGTGAT GTTGGTATAAAGAGGC[18]; ST3Gal-III, ATGGAGGCGTTCTTGCCA ACAAG and ATGCGAACGGTCTCATAGTAGTG[18]; ST3Gal-IV, TTGAA CAATGCCCCAGTGGCTGG and TCTTGGGAGACATTATGGCCTGAC[18]; FucT-VII, CCC ACC GTC GCC CAG TAC CGC TTC and CTG ACC TCT GTG CCC AGC CTC CCG T[19]; and β-actin, GTGGGGCGCCCCAG GCACCA and CTCCTTAATGTCACGCACGATTTC. A series of standard dilutions of a plasmid were used to quantify these messages. Specific signals for all transcripts were readily detected in human CLA^+ T cells. Standard dilutions were amplified with the pGEM-T Easy Vector System (Promega, Madison, Wis) from the PCR amplifiers above. Analysis was performed at 40 cycles, which was within the linear amplification range. PCR analyses were conducted twice, and the specificity of PCR products was confirmed by means of sequence analysis. The mRNA level of each glycosyltransferase was normalized to mRNA levels of β-actin.

Cell lysate preparation and immunoprecipitations

For lysate preparation, $CD3^+$ T cells cultured with 1,25D(3), RA, or diluent control were washed 3 times in ice-cold PBS and lysed in buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.02% NaAzide, 20 μg/mL phenylmethylsulfonyl fluoride, complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, Ind), and 2% Nonidet P-40 (NP-40; 250 μL/100 × 10^6 cells). After a 2-hour incubation on ice, insoluble cellular debris was pelleted by means of centrifugation for 30 minutes at 10,000*g* at 4°C, and solubilized protein lysate was collected and quantified by using the Bradford protein assay (Sigma).

For immunoprecipitation of PSGL-1, 2 μg of the anti-PSGL-1 mAb KPL-1 or mouse IgG isotype was added to cell lysates, which had been precleared with recombinant protein G–agarose (Invitrogen, Carlsbad, Calif). The antibody-lysate mixture was added to protein G–agarose and incubated overnight at 4°C on a rotator. Immunoprecipitates were washed 5 times with