In vivo Imaging of Cutaneous T-Cell Lymphoma Migration to the Skin

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Abstract
Cutaneous T-cell lymphoma (CTCL) is characterized by the accumulation of malignant CD4+ T cells in the skin. Although the expression of adhesion molecules and chemokine receptors on CTCL cells has been studied extensively on ex vivo isolated cells, very little is known about the dynamics and mechanisms of CTCL trafficking in vivo. However, detailed knowledge of the molecular cues mediating CTCL migration may be used to interfere with their homing to the skin. We made use of real-time intravital epifluorescence video and two-photon microscopy to visualize malignant T cells from Sezary syndrome (SS), a leukemic variant of CTCL, in dermal microvessels in mouse ear skin. We found that SS cells rolled along dermal venules in a P-selectin- and E-selectin–dependent manner at ratios similar to CD4+ memory T cells from normal donors. We furthermore show that the chemokine CCL17/TARC, but not CCL27/CTACK, was sufficient to induce the arrest of SS cells in the microvasculature. However, a combination of both chemokines was required to induce extravasation of SS cells. Together, our experiments delineate the molecular adhesion cascade operant in SS cell homing to the skin in vivo. [Cancer Res 2009;69(7):2704–8]

Introduction
Cutaneous T-cell lymphoma (CTCL) is a low-grade non-Hodgkin lymphoma characterized by the clonal proliferation of malignant, epidermotropic CD4+ T-cells. Sezary syndrome (SS) is an erythrodermic leukemic variant of CTCL with high numbers of circulating tumor cells, and a poor prognosis (1). It is thought that the origin of CTCL cells is a mature, CD45RO+CD4+ memory T cell, which frequently lacks the expression of CD26 or CD7 (2–4). A key to understanding the biology and progression of CTCL would be to decipher the mechanisms involved in their migration to the skin. Although a number of phenotypic studies have characterized the expression of adhesion molecules and chemokine receptors on CTCL cells in vitro, there is little information on the molecular cues mediating their homing in vivo.

Skin trafficking of normal T lymphocytes has been studied in the past. Skin-homing memory T-cells can be identified by the expression of the cutaneous lymphocyte-associated antigen (5). Cutaneous lymphocyte-associated antigen serves as a ligand for E-selectin, which is constitutively expressed on the endothelium of dermal microvessels (6). Furthermore, several chemokine-chemokine receptor pairs, including CCL17/CCR4, CCL27/CCR10, and CCL1/CCR8 have been implicated in cutaneous T-cell trafficking (7). However, the precise role of these molecules in the individual steps of the multistep adhesion cascade has not been studied microscopically.

The majority of SS cells isolated from blood expresses CCR4, and cells migrate towards CCL17 in chemotaxis assays (8–10). Furthermore, immunohistochemistry has shown the expression of CCR10 by the vast majority of CTCL cells in the skin (9). CCL17 and CCL27 are both elevated in the serum of patients with CTCL, and serum levels correlate with disease activity (11, 12). CTCL cells also express other chemokine receptors, including CXCR4 and CXCR3 (13, 14), but the broad expression patterns of their respective ligands in a variety of vascular beds questions a role in specific skin homing. The function of these molecules described on CTCL cells has not been tested in vivo.

In the present study, we made use of intravital microscopy to investigate the behavior of SS cells directly within the vasculature of intact mouse skin. Our results identify the molecular steps involved in skin migration of SS cells.

Materials and Methods

Patients
Patients met International Society for Cutaneous Lymphoma criteria for SS (15). Healthy donors were recruited at the Wistar Institute. Informed consent was obtained from all donors in accordance with the University of Pennsylvania and the Wistar Institute ethics committee guidelines.

Reagents
Blocking antibodies against mouse P-selectin (clone SH1) and E-selectin (clone 9A9) were a kind gift from Barry Wolitzky, ChemBridge Research Laboratories, San Diego, CA. Recombinant human CCL17 and CCL27 was from R&D Systems.

Ear Skin Microscopy
Epifluorescence video microscopy. CD4+ cells from patients with SS or CD4+CD45RO+ cells from controls were labeled with CFSE (1 μmol/L; Molecular Probes/Invitrogen) and injected into anesthetized male C57BL/6 mice via the right carotid artery (5 × 107/mL in 50 μL bolus of saline solution). Intravital imaging of dermal vessels was performed using an IV-500 (Mikron Instruments) as previously described (16). Scenes were captured from three vessel trees per mouse. In blocking experiments, the same vessel trees were captured before and after antibody injection. Rolling and sticking fractions were determined as previously described (16).

Intravital two-photon microscopy. Ear skin was prepared as described previously (17). Chemokines (100 ng in 2 μL saline) or saline were injected intradermally prior to adoptive cell transfer. Carboxy-SNARF-1 (10 μmol/L; Invitrogen)–labeled SS cells were injected into the carotid artery (106 cells/mL; 50 μL bolus) followed by FITC-labeled Dextran (molecular weight, 500,000 Da; 200 μL of a 2 mg/mL stock) 5 min later. Imaging was performed on a Prairie Technology Ultima System. For time series, image stacks were

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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acquired every 25 to 40 s for up to 90 min. Firm adherence was assumed if cells arrested for more than two consecutive frames. As an independent measure, the total number of arrested cells in the mouse ear was counted under epifluorescent light 120 min after injection of chemokines.

Cells were used within 2 h of isolation. All procedures involving experimental animals were performed under approval by Wistar’s Institutional Animal Care and Use Committee.

**Immunofluorescence Staining**

Staining was performed on 7-μm frozen sections from mouse ear skin with anti-human CD3 (Dako), mouse collagen IV (Chemicon), TCVR7.1 (Immunotech/Beckman Coulter), and ICAM-1 (Abcam). Sections were examined using a LSM 520 confocal microscope (Zeiss).

**Statistical Analysis**

Statistical analysis was performed by Student’s t test or one-way ANOVA with Bonferroni post-test using GraphPad Prism 4 (GraphPad Software). P < 0.05 was used as level of significance.

**Results and Discussion**

**Behavior of SS cells in the microvasculature of the mouse ear.** The characteristics of patients with SS and the cells used in this study are shown in Supplementary Table S1. Flow cytometry was used to evaluate the expression of molecules implicated in T-cell homing to the skin on SS cells as compared with CD4⁺CD45RO⁺ memory T-cells from healthy donors. When compared with controls, SS cells exhibited similar expression of CCR4 [67.3 ± 10.9% (SS) versus 48.7 ± 3.8% (control); P = 0.19], CCR10 (18.9 ± 3.7% versus 13.4 ± 1.3%; P = 0.25), E-selectin ligands (36.5 ± 8.0% versus 25.1 ± 6.1%; P = 0.38), P-selectin ligands (42.5 ± 8.3% versus 37.7 ± 5.5%; P = 0.71), L-selectin (53.3 ± 11.8% versus 53.8 ± 7.9%; P = 0.98), CD44 (98.7 ± 0.8% versus 99.5 ± 0.3%; P = 0.45), and LFA-1 (96.2 ± 1.6% versus 98.9 ± 0.4%; P = 0.29). A significant difference was observed in the expression of α4 integrin, which was detected in 83.8 ± 3.3% of control cells but only in 13.5 ± 3.9% of SS cells (P < 0.0001). Together, SS cells reveal a similar panel of homing receptors on their surface as memory T-cells from healthy individuals (Supplementary Fig. S1).

In order to gain further insight into the mechanisms of SS cell migration to the skin, we tracked CFSE-labeled CD4⁺ T-cells from patients with SS or CD4⁺CD45RO⁺ memory T-cells from healthy donors in the dermal microvasculature using epifluorescence video microscopy. In noninfamed dermal vessels, control cells displayed a rolling fraction of ~25%, whereas no sticking occurred (Fig. 1A). SS cells showed comparable numbers of rollers, but a higher sticking fraction of 0.5 ± 0.4% (Fig. 1A). We have previously shown that, under homeostatic conditions, leukocytes in the dermal microvasculature roll in a P-selectin and E-selectin-dependent manner (16). As shown in Fig. 1B, blocking of murine P-selectin nearly abolished the number of rolling SS cells as compared with the isotype antibody (1.8 ± 0.6% versus 25.2 ± 2.7%; P < 0.001). Blocking of E-selectin reduced the rolling fraction to ~35% of the control value (9.3 ± 3.5% versus 25.2 ± 2.7%; P < 0.01). These data establish a functional role of selectins in the specific interactions between human CTCL cells and the dermal vasculature.

**Role of CCL17 and CCL27 on the trafficking behavior of SS cells in the skin.** Firm adherence of rolling leukocytes requires a chemokine-mediated activation of cell surface integrins resulting in a transition from low to high affinity. To determine the mechanisms of firm adherence and potential extravasation of SS cells, we made use of intravital two-photon microscopy (17). Consistent with the results above, under baseline conditions, SS cells frequently rolled along postcapillary venules, whereas sticking was rarely observed [(Figs. 1C and 2A; Supplementary Movie S1A); note that the sticking fraction is measured here as a percentage of slow rolling cells, and is thus higher than in Fig. 1A]. Injection of CCL17 resulted in a 3-fold increase in the number of sticking cells (81.67 ± 6.1% versus 27.7 ± 1.7%; P < 0.01; Figs. 1C and 2B; Supplementary Movie S1B). Notably, in contrast with CCL17, CCL27 did not increase firm adherence (41.3 ± 13.2%; P > 0.05 versus control; Figs. 1C and 2C; Supplementary Movie S1C). In addition, the combination of both chemokines was not superior to the injection of CCL17 alone (75.8 ± 6.1%; Fig. 1C).

These results were further corroborated by counting the total number of fluorescent cells in the vascular bed using epifluorescence microscopy 2 hours after the injection of cells. Thus, CCL17 lead to an increase in the number of arrested cells (116.8 ± 18.85 versus 38.5 ± 9.2 cells/(10⁶ injected cells/ear in the control group; P < 0.01), whereas CCL27 did not reveal any change (25.8 ± 3.9 cells/ear; P > 0.05; Fig. 1D).

Although neither injection of CCL17 nor CCL27 alone was associated with extravasation of SS cells, the combination of both chemokines resulted in transmigrating cells as well as extravasated cells located in the dermis (6.8 ± 2.8% of the cells arrested; Fig. 3). As an independent confirmation of their extravascular localization,
Figure 2. Effects of CCL17 and CCL27 on the adherence of SS cells to dermal microvessels. Saline (A), CCL17 (B), or CCL27 (C) was injected intradermally into the ear skin; 5 min later, SNARF-labeled CD4+ T cells from SS patients were adoptively transferred followed by injection of FITC-Dextran. Time-lapse movies of cells in the dermal vascular plexus were captured by two-photon microscopy. The pictures show three time points from representative cells (time in min:s). Blue, second harmonic generation signals from extracellular matrix fibers. Bars, 25 μm.

Figure 3. SS cells extravasate after combined application of CCL17 and CCL27. CCL17 and CCL27 were injected intradermally followed by injection of SS cells as in Fig. 2. A, representative cells that are partially located in the vessel wall (transmigrating cells). B, representative cells that localize to the perivascular tissue (left, an overlay of the image stack; right, XY section with the associated YZ and XZ planes); C, representative cells that are located in the dermal collagen. Bars, 25 μm.
immunofluorescence staining for human CD3 and murine collagen-4 was performed. Comparable to intravital imaging, CD3+ cells were observed in the perivascular tissue (Fig. 4A and B). A mean number of 5 ± 3 cells per section was observed (n = 10 sections).

To ensure that the extravasated cells did not selectively comprise the remaining CD4+ nontumor cells, we performed costaining for CD26 and CCR10 on the samples from the patients (patients 6, 7, and 8) that were used in the extravasation experiments. Eighty-four percent of all CCR10+ cells were CD26+, demonstrating a preponderance of malignant CCR10-positive cells. In addition, extravasated cells from patient 6, in whom 100% of circulating CD4+ cells expressed vβ7.1, stained positively for this TCR (Fig. 4C), thereby confirming the extravasation of SS cells.

As injection of chemokines could also induce endothelial activation, and therefore mediate indirect effects on SS cell recruitment, we assessed ICAM-1 expression on the venular endothelium. We did not find a significant difference between sections from saline-injected or CCL27 plus CCL17-injected mice (data not shown).

Our data show that a combination of CCL17 and CCL27 is capable of inducing extravasation of SS cells into the dermis, consistent with a nonredundant function of these chemokines in CTCL accumulation in the skin. Thus, CCL17 seems to mediate firm arrest of SS cells in dermal venules, whereas CCL27 is necessary for their transmigration. One caveat with the interpretation of these results is that, in our patients, the percentage of CCR10+ SS cells was lower (~20%) than CCR4-expressing cells (~65%). Thus, it may be argued that the lack of firm adherence of SS cells after CCL27 application could be due to the lower number of CCR10+ cells. However, it is interesting to note that both in the case of SS cells and normal memory CD4+ cells, CCR10 is expressed at much lower levels in peripheral blood as compared with cells within the skin. Therefore, a plausible scenario would be that CCR10+ tumor cells have a selective advantage over CCR4 single positive cells in their accumulation in the skin.

Prolonged observation of extravasated SS cells showed that they did not migrate further through the dermis. A possible explanation could be that the presence of exogenous chemokines interfered with other promigratory cues in the tissue, or that the SS cells home to distinct microenvironments within the dermis where they became sessile. Further experimentation will be necessary to elucidate the mechanisms behind this phenomenon.

Taken together, we have established a model for the observation of the intravascular behavior of Sezary cells as well as their transmigration across the vessel wall. This has enabled us to define the specific role of adhesion molecules and chemokines in the homing of SS cells to the skin, the primary location of CTCL. Increased understanding of these trafficking pathways may be used to interfere with the skin homing of CTCL cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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