In Vitro Migration of Lymphocytes through Collagen Matrix: Arrested Locomotion in Tumor-infiltrating Lymphocytes

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ABSTRACT

Antitumor immunity requires (a) extravasation of lymphocytes from the blood stream to interstitium, (b) locomotion through extracellular matrix to the site of the tumor, (c) effector cell recognition of the tumor target with cell/cell contact and binding of adhesion receptors, (d) T-cell receptor binding to histocompatibility and tumor antigens, and (e) tumor lysis. We hypothesize that the tumor microenvironment inhibits lymphocyte locomotion through extracellular matrix as one mechanism by which tumors may avert host defense. Lymphocyte locomotion was investigated in vitro using a three-dimensional collagen gel model. Fresh tumor-infiltrating lymphocytes (TIL) were obtained by enzymatic digestion of melanomas and renal cell carcinoma, and mononuclear cells were isolated by discontinuous Ficoll-Hypaque gradient. The lymphocytes were analyzed for motility from a point of origin between basal and overlay layers of collagen gel. Results showed that TIL migration was almost completely inhibited, compared with migration of normal and cancer patient peripheral blood leukocytes and lymphocytes from lymph nodes. Short-term (24-h) exposure of lymphocytes to cytokines during the assay in the collagen gel matrix had no effect on locomotor ability. Long-term (19, 30, or 35 days) culture of TIL in 200 units/ml of interleukin 2 reinstalled locomotor ability. Short-term exposure of any of the lymphocyte populations to interleukin 1-α, interleukin 1-β, interleukin 2, interleukin 3, interleukin 4, α-interferon, or γ-interferon had no effect on migration. Thus, TIL display a uniquely arrested ability to locomote through collagen gel. Inhibition of the locomotion of infiltrating effector cells is possibly a mechanism by which the tumor evades the host immune system.

INTRODUCTION

Host immunity to cancer occurs within two compartments: (a) the vascular system and lymphatics and (b) the ECM of the tumor microenvironment. For effective host immunity, T-cells home to the site of the tumor, extravasate from the bloodstream to the interstitium, and locomote through ECM in the tumor microenvironment. Subsequently, ancillary adhesion peptides on the effector cells bind to ligands on target cells, and binding of the T-cell receptor to histocompatibility and tumor antigens occurs (1–3). Thus, the ability of lymphocytes to migrate and accumulate at various sites in the body is a critical step in the effector phase of cellular immunity.

Trafficficking of lymphocytes through lymphoid organs, lymph, blood, and nonlymphoid organs is nonrandom, as determined using fluorescein isothiocyanate-labeled or radiolabeled sensitized and nonsensitized lymphocytes (4–6). There are three subpopulations of lymphocytes: (a) cells with preference for the intestinal lymphoid tissues; (b) cells directed to subcutaneous lymph nodes; and (c) cells that migrate preferentially through the skin or other peripheral nonlymphoid tissues. This trafficking is directed by specific lymphocyte surface receptors that bind to HEV, allowing access from the blood stream to lymph nodes (7–9). Despite the proposition that lymphocytes distribution is controlled through cell surface expression of adhesion molecules, little is known about regulation of lymphocyte locomotion after the cell leaves the confines of vascular or lymph endothelia and enters ECM at the site of inflammation or tumor. Indeed, the impaired movement of lymphocytes through ECM may contribute to failed immunity at the tumor site.

Addressing the local tumor immune response has been difficult owing to the complex nature of immune cell accumulation at the site of the tumor, the lack of correlation with systemic immune performance, and the difficulty in developing isolated in vivo models. We hypothesize that the neoplastic site is unfavorable to lymphocyte locomotion through ECM. To test this hypothesis a three-dimensional in vitro collagen model was adapted to assess lymphocyte locomotion. The purpose of this investigation was 2-fold: to determine the effect of microenvironment on the locomotor ability of TIL as compared with cancer patient PBL, normal PBL, and LNL, and to determine a possible biological mechanism of lymphocyte locomotion through interstitium by analyzing the effect of lymphokines on in vitro lymphocyte locomotion.

Freshly isolated TIL from melanomas and renal cell carcinoma were analyzed for migration in collagen, either fresh or after culture in the presence of IL-2 and tumor-associated antigen. Freshly isolated TIL were significantly inhibited in their ability to migrate, as compared with PBL and LNL. However, TIL stimulated in culture with IL-2 and antigen migrated similarly to PBL. When biologically active amounts of cytokine were added directly into the medium of the assay, there was no effect on the distance of random migration of TIL, TIL cultured in IL-2, PBL, or LNL. In conclusion, TIL are unique among lymphocytes in their abated migratory ability. Inhibition is not constitutive, since long-term activation by IL-2 and antigen restores the migratory activity but short-term exposure does not improve locomotion.

MATERIALS AND METHODS

Lymphocytes. Buffy coats from healthy donors were purchased from the Regional Gulf Coast Blood Center (Houston, TX). PBL were isolated using Ficoll-Hypaque gradients by centrifugation at 850 to 1000 x g for 30 min and then washed with HBSS 3 times. TIL were obtained from seven tumor specimens removed from six patients with metastatic melanoma and one patient with metastatic renal cell carcinoma. Five of the specimens had an adequate number of TIL for the assay. LNL were obtained from three fresh pathological specimens. Two of the lymph nodes were infiltrated with melanoma, and one node was uninvolved with tumor in a patient with two of 11 axillary nodes positive for infiltrating melanoma. The tumor or lymph node tissue...
was minced sterilely and enzymatically digested with 2 mg/ml of collagenase D, 0.4 mg/ml of DNase I, and 0.4 mg/ml of hyaluronidase type V for 1 to 1.5 h in HBSS. All enzymes were obtained from Sigma Chemical Co. (St. Louis, MO). The cells in the effluent were then separated using Ficoll-Hypaque gradients to obtain the mononuclear cells fraction. TIL specimens were cultured with 200 units/ml of IL-2 in AIM-V serum-free medium, or RPMI 1640 medium, with 10% FBS, at 37°C in an atmosphere of 7% carbon dioxide and 93% air for 19, 30, or 35 days in the presence of tumor-associated antigen. Additionally, TXM 141 TIL were cultured in 200 units/ml of IL-3 and in 200 units/ml of IL-2 plus 10 units/ml of IL-4. Limited availability of TIL precluded culturing them in other cytokines.

Cryopreserved PBL from two patients with metastatic melanoma (TXM 56 and TXM 81) were utilized to assess their ability to migrate through collagen matrix because of limited availability of cancer patient peripheral blood specimens. There were sufficient cells to perform one assay with TXM 81 and four assays with TXM 56. To ensure that cryopreservation did not affect lymphocyte locomotory ability, normal donor PBL were divided into two aliquots, one cryopreserved for 72 h and the other held at 4°C until the assay was performed. The monoclonal cells were collected using Ficoll-Hypaque gradients and cryopreserved in 10⁷ cells/ml in 10% dimethyl sulfoxide and 90% FBS at -70°C. The cells were placed directly at -70°C from room temperature, and thawing was performed at room temperature. Eighty-five % of cryopreserved cells were viable as determined by trypan blue exclusion.

The cancer patient PBL and normal PBL were assessed for migratory ability in the collagen assay.

Cytokines. The recombinant IL-2 (1.5 x 10⁶ colony-forming units/ml of protein) was obtained from Hoffmann-LaRoche (Nutley, NJ). The other human recombinant lymphokines were purchased from Genzyme (Boston, MA). The specific activity of human recombinant IL-1α was 1000 units/ml (Lot 08731), of IL-1β was 1000 units/ml (Lots 07731 and 06634), of IL-3 was 10⁶ colony-forming units of protein (Lots 04733 and B9263), and of IL-4 was 10⁸ proliferation units/ml of protein. Human IFN-α and IFN-γ were purchased from Hoffmann-LaRoche. The activity of IFN-α was 10⁵ units/ml (Lot 3102) and of IFN-γ was 10⁵ units/ml (Lot N9207AX).

Collagen Gel Assay. Type I rat tail collagen was used to investigate lymphocyte motility in a three-dimensional collagen gel model previously described by Ratner et al. (10). An adaptation of their model was used in our investigation (Fig. 1). Briefly, 1 ml of cold liquid collagen solution in 10× RPMI 1640 medium was transferred to each 25-mm-diameter tissue culture well and then incubated for 15 min at 37°C to polymerize the collagen. Lymphocytes were mixed with cold collagen solution at 2.5 x 10⁷ cells/0.1 ml. Twenty μl of cell suspension were transferred to the base layer of collagen gel and incubated 10 min to form a 10-mm disk. An overlay of polymerized 3-mm-thick collagen was precast in 18-mm diameter culture well and transferred to the base layer overlying the lymphocytes. Leading front migration distance was measured using an inverted light microscope.

RESULTS

Locomotor Ability of Normal and Cancer Patient PBL. TXM 56 and TXM 81 PBL obtained from melanoma patients and normal PBL were assessed for migration in collagen. The results after 24-h incubation in the collagen assay are shown in Fig. 2. There was no statistically significant difference in the distance migrated by normal PBL held at 4°C, cryopreserved normal PBL, or cryopreserved cancer patient PBL. Moreover, there was no statistically significant difference in the mean migration distance of this cryopreserved PBL population compared with 14 other normal donors tested (Table 1). Thus, the PBL of cancer patients migrate at an apparently normal rate.

Locomotor Ability of LNL. Freshly isolated LNL were obtained from pathological specimens from three patients with metastatic melanoma. The 24-h mean migration through collagen, as assessed by leading front distance into the overlay layer of collagen, was 790 ± 44 μm and into the basal layer was 790 ± 44 μm. These values are not statistically significantly different from normal PBL migration (Fig. 3). Thus, lymphocytes from two distinct lymphoid compartments randomly migrate in collagen in vitro without prior stimulation with mitogen or lymphokine. Furthermore, enzymatical digestion of the lymph nodes did not alter the ability of the lymphocytes to migrate.

Phenotypes of TIL. The vast majority of TIL in freshly isolated samples were T-cells (CD3). RC6 TIL, from a renal cell carcinoma, were 76.5% CD3+, 72% CD4+, 41% CD8+, 8%
Fig. 2. Migration of cancer patient PBL in collagen. TXM 81 and TXM 56 are PBL specimens from two patients with metastatic melanoma. These cells were cryopreserved in our laboratory. An aliquot of normal PBL was cryopreserved for comparison. There was no statistically significant difference between migration of normal PBL, cryopreserved PBL, or cancer patient PBL. There were only enough TXM 81 cells to perform one assay; four assays were performed with TXM 56. NL, normal. Columns, mean; bars, SEM.

Table 1 Migration of lymphocyte populations in the presence of cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Direction</th>
<th>Normal PBL</th>
<th>TIL</th>
<th>Cultured TIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Over*</td>
<td>804 ± 87 (14)</td>
<td>271 ± 45 (5)</td>
<td>790 ± 36 (3)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Basal</td>
<td>763 ± 93 (14)</td>
<td>276 ± 86 (4)</td>
<td>791 ± 28 (3)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Over</td>
<td>667 ± 62 (2)</td>
<td>483 ± 17 (1)</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>Basal</td>
<td>746 ± 38 (2)</td>
<td>597 ± 32 (1)</td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>Over</td>
<td>698 ± 53 (2)</td>
<td>533 ± 12 (1)</td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>Basal</td>
<td>742 ± 59 (2)</td>
<td>475 ± 25 (1)</td>
<td></td>
</tr>
<tr>
<td>IFN-α</td>
<td>Over</td>
<td>829 ± 64 (12)</td>
<td>332 ± 53 (4)</td>
<td>966 ± 52 (2)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Basal</td>
<td>791 ± 92 (12)</td>
<td>429 ± 71 (3)</td>
<td>907 ± 41 (2)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Over</td>
<td>879 ± 74 (12)</td>
<td>197 ± 52 (3)</td>
<td>946 ± 37 (2)</td>
</tr>
<tr>
<td>IL-9</td>
<td>Basal</td>
<td>783 ± 84 (12)</td>
<td>364 ± 74 (3)</td>
<td>917 ± 23 (2)</td>
</tr>
<tr>
<td>LNL</td>
<td>Over</td>
<td>777 ± 118 (12)</td>
<td>320 ± 41 (4)</td>
<td>1007 ± 41 (2)</td>
</tr>
<tr>
<td>TIL</td>
<td>Over</td>
<td>747 ± 106 (12)</td>
<td>438 ± 54 (3)</td>
<td>893 ± 56 (2)</td>
</tr>
</tbody>
</table>

* Migration into the overlay or basal layer of collagen.
* Mean ± SEM.
* Numbers in parentheses, number of replicates in each experimental group.
* P < 0.001 compared with normal PBL.

Leu16*, and 12% LeuM3*. There were no detectable CD16* cells present. M9 TIL, from a metastatic melanoma, were 85% CD3*, 44% CD4*, 30% CD8*, and 0% CD16*. After 20 days of culture in IL-2, M9 TIL were 99% CD3*, 68% CD4*, 30% CD8*, <2% CD16*, 0% Leu16*, and 0% LeuM3*. Based on the results of two-color analysis, the majority of CD4* T-cells were CD4* CD8*, and the majority of CD8* were CD4* CD8*.

TXM 141 TIL, from a metastatic melanoma, were phenotyped in our laboratory after culture for 15 days in IL-2. These TIL were 79% CD3*, 14.3% CD4*, 50.5% CD8*, and 5.2% CD16*. The percentage of false-positive staining on TIL with control monoclonal antibodies was less than or equal to 1%.

Locomotor Ability of TIL. In contrast to the locomotion of all other lymphoid populations, TIL locomotion through collagen matrix was significantly decreased by more than 75% compared with that of normal PBL, LNL, and cancer patient PBL (P < 0.001) (Fig. 4). It is unlikely that the inhibited TIL migration is due to the enzymatic dissociation procedure because LNL obtained by enzymatic digestion migrated normally. Furthermore, a melanoma TIL specimen was 80% viable after 24-h incubation in collagen. Thus their inability to migrate is probably not due to TIL cell death, and lymphocytes from the tumor microenvironment are functionally different from lymphocytes deployed systemically.

Locomotor Ability of TIL Cultured in Cytokine. To determine if the inhibition of TIL locomotion is an intrinsic characteristic of this cell population, we next analyzed whether stimulation of TIL in culture would reinstate their locomotor ability. Freshly isolated TIL from three patients with metastatic melanoma were cultured with 200 units/ml of IL-2 for 19, 30, or 35 days, the same time period used to culture TIL for immunotherapy. An attempt was made to culture the TIL in IL-3, but the cells were not viable. TIL cultured in 200 units/ml of IL-2 plus 10 units/ml of IL-4 for 15 days had as many viable cells as did cells cultured with IL-2 alone. The viable cultured TIL were assessed for migration in the collagen gel assay. After 24-h incubation, there was a significant increase in the leading
Effect of Cytokine on Lymphocyte Locomotion. The recovery of locomotor activity in TIL cultured with IL-2 suggested that (a) IL-2 modulates cell surface structures that interact with the collagen, (b) proliferation induced by IL-2 favors migratory lymphocytes, thereby selecting the most motile cells, or (c) proliferating cells are more motile than nonproliferating cells. The prospect that modulation of cell surface structures results in improved migration was tested by introducing cytokines directly to the assay medium. IL-1α, IL-1β, IL-2, IL-3, IL-4, IFN-α and IFN-γ were individually included in the medium of the assay. The assay was incubated for 24 h, and leading front migration into the overlay and into the basal layer was assessed. The migration distances of fresh TIL, TIL stimulated in culture with IL-2 for 19 days, and normal PBL were not significantly different with this short-term exposure to cytokine (Fig. 6). Table 1 shows the mean values and standard errors for all experiments performed. For two of the TIL specimens, there were only sufficient numbers of cells to perform the assay in quadruplicate without cytokine in the medium. When the migration distances for all TIL were averaged, it appears that there is a significant difference in TIL migration with cytokine in the medium. However, in individual experiments the difference was not significant when the assays containing cytokine were compared with the controls for each experiment. Also, in one experiment the basal layer was damaged, so the number of replicates for the migration into the overlay in the no cytokine, IL-2, and IL-4 groups was larger. Only one TIL specimen contained a sufficient number of cells to perform the assay with all seven of the cytokines tested.

DISCUSSION

Impaired TIL locomotor capability may be a mechanism of inhibition of host defense against tumor. TIL isolated from human melanomas and renal cell carcinoma displayed retarded migration through collagen in vitro compared with normal donor PBL, LNL, and cancer patient PBL, which migrated similarly to normal PBL. The in vitro collagen matrix has been previously used to analyze leukocyte and tumor cell migration (10, 12-16). Collagen fibers are a substantial component of the ECM that lymphocytes must traverse to reach tumor. Polymerized collagen provides a three-dimensional and physiological environment for assessment of cell migration in vitro (12, 17). In contrast to two-dimensional migration on modified plastic surfaces (18-22), the three-dimensional assay may provide an approximation of the tissue environment of the tumor. Gelled collagen is transparent; therefore, migration may be assessed by light microscopy (21). Because of its physical and chemical similarity to in vivo interstitium (23), the gel is compatible with freshly isolated or cultured lymphocytes. Schor et al. (16) showed that human PBL spontaneously and reproducibly migrate through collagen matrix in a random omnidirectional pattern. Murine LNL migrate randomly (10, 15, 21), and locomotion varies with the cell cycle. Migration distance increases in lymphocytes stimulated with concanavalin A (10). Murine splenic lymphocytes cultured 72 to 80 h with recombinant IL-2 have twice the number of motile cells, and they...
migrate more than twice the distance of uncultured cells (15). In this investigation, culturing human TIL for 19 to 35 days reinstated migratory ability. The inhibited migration in freshly isolated TIL suggests that either a suppressor cell or a soluble substance is present in the tumor microenvironment that may affect effector/target cell contact. We believe that the ability of TIL to migrate through matrix is a critical step in vivo effector function.

The immune response to tumor involves a series of lymphocyte actions, including lymphocyte trafficking, homing to the site of the tumor, extravasation from the bloodstream into interstitium, migration through ECM, binding of effector cell adhesion receptors to target cell surface ligands, activation of effector cell via T-cell receptor binding to tumor and histocompatibility antigens, and tumor cell lysis. Lymphocytes traffic in a nonrandom manner (4–6). Each subpopulation of lymphocytes recirculates by continuous passage from blood through lymphoid organs and lymph and back to blood. The process of lymphocyte homing is regulated at many levels, and the first level of control is via recognition of, binding to, and extravasation through HEV in peripheral lymph nodes, mucosa of the intestine, or sites of chronic inflammation (24). Binding to HEV is organ specific and dependent on specific homing receptors, such as the homing-associated cell adhesion molecule, the peripheral lymph node homing receptor, or lymphocyte function-associated molecule 1, on the cell surface that recognize mucosal vascular addressin, peripheral lymph node addressin, or both, respectively (25). When a repertoire of clonal lymphocyte specificities circulate, tumor antigen is recognized by a particular clone with subsequent expansion of that clone, which is antigen dependent (2). It is at this level that matrix may serve as an obstacle to interaction of lymphocytes with tumor.

Once extravasation occurs, it is possible that adhesion receptors such as the very late activation integrin supergene family of molecules that bind to components of ECM (i.e., collagen, fibronectin, and laminin) are involved in lymphocyte locomotion, but as yet, preliminary data using antibodies to α- and β-chains suggest that very late activation molecules may not be required for lymphocyte locomotion. One mechanism by which tumor may avert the host immune system is to inhibit lymphocyte migration, thus preventing the initial binding of effector to tumor target. Gunji and Gorelik (26) showed that fibrin coagulation, it is possible that the tumor is suppressing TIL locomotion, thus preventing the initial binding of effector to the tumor site. In conclusion, the tumor microenvironment has several down-regulatory effects on the infiltrating immune cells, and our study shows that one mechanism may be inhibition of lymphocyte locomotion through ECM. In immunotherapy models, simultaneous i.v. infusion of lymphokines with stimulated TIL may have no immediate effect on the infiltrating ability of the lymphocytes, whether they are TIL already at the site of the tumor or ex vivo-stimulated TIL used in adoptive immunotherapy. Future immunotherapeutic strategies against cancer may address in vivo stimulation of lymphocyte locomotion to enhance host immunity.

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