Isolation Based on L-Selectin Expression of Immune Effector T Cells Derived from Tumor-draining Lymph Nodes

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Abstract

The ability to generate a large number of tumor-reactive T lymphocytes is the most critical requirement for adoptive immunotherapy. Our laboratory has previously demonstrated that cells from tumor-draining lymph nodes (LNs) are an excellent source of tumor-reactive T lymphocytes. After activation with anti-CD3, these cells readily proliferate in low concentrations of interleukin 2 and acquire effector functions. The adoptive transfer of these cells is capable of mediating the regression of tumors established in the lung as well as in the brain. Here, we analyzed several adhesion molecules on the tumor-draining LN T cells and separated them based on L-selectin expression. The homing receptor L-selectin mediates adhesion to the luminal surface of specialized high endothelial venules, thus regulating lymphocyte recirculation through peripheral LNs. In response to progressive tumor growth, a small population of draining LN T cells down-regulated L-selectin and increased the expression of CD44 and lymphocyte function-associated antigen 1. In adoptive immunotherapy, purified T cells with low L-selectin (L-selectin−) expression constituted all the in vivo antitumor reactivity, whereas isolated high L-selectin (L-selectin+) cells were ineffective. Furthermore, reverse transcription-PCR analysis revealed that L-selectin+ cells expressed interleukin 2, IFN-γ, and tumor necrosis factor α mRNA upon in vitro stimulation with specific tumor cells. These results suggest that highly potent immune T cells can be isolated based on their pattern of adhesion molecule expression. The ability of the immune effector cells to transcribe cytokine genes when stimulated with tumor cells provides a basis for identifying similar cells for adoptive immunotherapy of cancer in humans.

Introduction

The systemic transfer of tumor-specific T lymphocytes is an effective means to eradicate progressively growing tumors (1, 2). This form of cancer treatment has been referred to as "adoptive immunotherapy," and the clinical application relies on the ability to isolate tumor-reactive lymphocytes from patients with malignancies and the development of methods to promote ex vivo activation and expansion of such T lymphocytes. In a variety of animal models, we have identified tumor-draining LNs1 to be an excellent source of tumor immune T cells (3). Following activation in vitro with anti-CD3 and IL-2, these cells are capable of mediating potent antitumor effects (4). Although activated T cells displayed an exquisite specificity toward the tumor that stimulated the draining LN, the polyclonal nature of the immune T cells down-regulated L-selectin and increased the expression of CD44 and lymphocyte function-associated antigen 1. In adoptive immunotherapy, purified T cells with low L-selectin (L-selectin−) expression constitutes all the in vivo antitumor reactivity, whereas isolated high L-selectin (L-selectin+) cells were ineffective. Furthermore, reverse transcription-PCR analysis revealed that L-selectin+ cells expressed interleukin 2, IFN-γ, and tumor necrosis factor α mRNA upon in vitro stimulation with specific tumor cells. These results suggest that highly potent immune T cells can be isolated based on their pattern of adhesion molecule expression. The ability of the immune effector cells to transcribe cytokine genes when stimulated with tumor cells provides a basis for identifying similar cells for adoptive immunotherapy of cancer in humans.

Materials and Methods

Mice. Female C57BL/6j (hereafter called B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained in a specific pathogen-free environment and were used at the age of 6–10 weeks.

Tumors. The MCA 205 fibrosarcoma, syngeneic to B6 mice, was induced with 3-methylcholanthrene (10). The tumor has been maintained in vitro by serial s.c. transplantation in syngeneic mice and was used within the 10th transplantation generation. Single-cell suspensions were prepared from solid tumors by enzymatic digestion as described previously (3, 10). An MCA 205 tumor cell line was established and maintained in vitro. The cultured cells were used as stimulators to induce cytokine gene expression. Another syngeneic fibrosarcoma, MCA 207, and a cloned melanoma, D5, derived from the B16-BL6 melanoma, were maintained in culture and used for specificity controls for RT-PCR.

Antibodies and Flow Cytometry. Hybridomas producing mAbs against the murine CD3-ε chain (145-2C11) and the murine L-selectin (MEL-14) were obtained from the American Type Culture Collection (Rockville, MD). PE-conjugated anti-Thy1.2 (30-H12), and PE-conjugated anti-L-selectin (MEL-14) mAbs were purchased from Pharmingen (San Diego, CA). Analyses of cell surface phenotypes were carried out by direct immunofluorescence staining of 0.5–1 × 10⁶ cells with conjugated mAbs. In each sample, 10,000 cells were analyzed by a FACScan flow microfluorometer (Becton Dickinson, Sunnyvale, CA).

Tumor-draining LN Cells and Anti-CD3/IL-2 Activation. B6 mice were inoculated s.c. with 1.5 × 10⁶ MCA 205 tumor cells on both flanks. Twelve to 13 days later, tumor-draining inguinal LNs were harvested, and single-cell suspensions were prepared mechanically as described previously (4, 10). LN cells were activated on 24-well plates precoated with the anti-CD3 mAb. Each
well contained 3–4 × 10⁶ cells in 2 ml CM. CM consists of RPMI 1640 supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, and 0.5 μg/ml fungizone (all from Life Technologies, Inc.) and 5 × 10⁻⁵ M 2-mercaptoethanol (Sigma Chemical Co.). After 2 days of incubation at 37°C in 5% CO₂, activated cells were suspended in 4 units/ml human recombinant IL-2 (Chiron, Emeryville, CA) at 1–2 × 10⁷/ml and cultured in gas-permeable culture bags (Baxter Healthcare Co., Deerfield, IL) for 3 days as described previously.⁴

**Fractionation of T Cells.** T cells in the LN cell suspension were concentrated by passing through nylon wool columns (Robbins Scientific Co., Sunnyvale, CA). After a 45-min incubation at 37°C, the first 15-ml pass-through fraction contained 90–95% T cells. Purified T cells were further fractionated into two subpopulations based on the expression of L-selectin. Tumor-draining LN T cells were first incubated for 20 min at 4°C with the L-selectin hybridoma ascites fluid at a 1:1000 dilution. Four to 5 × 10⁶ cells in 3 ml CM were plated on a T-25 flask, which was precoated with mouse antirat immunoglobulin Ab (MicroCoclett Mouse Anti-Rat T-25 cell culture flask; Applied Immune Sciences, Inc., Santa Clara, CA). After a 1-h incubation at 4°C, nonadherent (L-selectin⁺) cells were collected by gentle rocking. These cells were incubated on a new mouse antirat Ab-coated flask to yield highly purified (>90%) L-selectin⁻ cells. Adherent cells were collected from the first incubation flask with a cell scraper after rinsing twice with PBS. More than 90% of the recovered adherent cells were L-selectin⁻.

**Adaptive Immunotherapy.** B6 mice were inoculated intracranially in the right hemisphere with 1 × 10⁴ MCA 205 tumor cells in 10 μl HBSS to establish brain metastases.³ Three to 4 days after tumor inoculation, mice were sublethally irradiated (500 rads), followed by i.v. infusion of effector T cells. Mice were followed for evidence of intracerebral tumor growth, and survival time was recorded. Significant differences of survival time between groups were analyzed by Wilcoxon rank-sum test. Two-sided P < 0.05 was considered significant.

**RNA Preparation and RT.** MCA 205, 207, and D5 tumor cells were treated with 2 × 10⁶/ml/well in CM in 24-well plates. After attachment during a 2-h incubation at 37°C, 2 × 10⁶ effector cells in 1 ml CM were added. After an additional 3 h of incubation, cells were harvested by gentle agitation and pipetting. The cell preparation contained minimum contamination of tumor cells. Total RNA was extracted from the cells using the TRIzol isolation reagent (Life Technologies) according to the manufacturer’s instructions. cDNA was synthesized from 3 μg RNA in a volume of 20 μl containing 5 mM MgCl₂ solution, 1× PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 units/μl RNase inhibitor, 2.5 μM oligo(dT), (all from Perkin Elmer, Foster City, CA), 1 mM deoxynucleotide triphosphate mix (Life Technologies), and RNase-free, double distilled H₂O. Synthesis of cDNA was performed in a Perkin Elmer GeneAmp 2400 PCR system as follows: RT at 42°C for 15 min; denaturing at 99°C for 5 min; annealing at 5°C for 5 min; and extension at 72°C for 7 min. Samples of cDNA were stored in −20°C until further use.

**PCR Amplification of cDNA and Analysis of Amplified Products.** The following pairs of 5' and 3' primers were purchased from Life Technologies:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>GTGGGCCGCTTACAGCCACA</td>
<td>CGGTTTGGCTTGGGTTGTC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>ATGAGCACAGAAAGCATGATC</td>
<td>TACAGGCTTGTCACCTGAATT</td>
</tr>
<tr>
<td>CD3</td>
<td>GTCAACAGCGCACCCACTTC</td>
<td>CATGATC and TACAGGCTTGTCACCTGAATT</td>
</tr>
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**Results and Discussion.** In a normal B6 mouse, the inguinal LNs are small (~1 mm in diameter) and contain approximately 2 × 10⁶ cells/LN when mechanically disrupted. Inoculation s.c. on the posterolateral flank of mice with 1.5 × 10⁶ MCA 205 tumor cells resulted in apparent hyper trophy of the inguinal LNs 12 days later, to 3–4 mm in diameter, and routinely, 2–3 × 10⁶ cells could be harvested from each LN. Despite an increase in cellularity, tumor-draining LNs contained 35–40% Thy-1.2⁺ cells compared with ~70% T cells in the resting inguinal LNs. Cells from these tumor-draining LNs were passed through nylon wool columns to concentrate T cells. They were stained with anti-L-selectin mAb and analyzed by flow cytometry to determine whether there were changes in the proportions of cells bearing the LN-specific homing receptor L-selectin. As depicted in Fig. 1, T cells of normal LNs were mostly L-selectin⁻, with a small (~15%) fraction of cells staining weakly with anti-L-selectin mAb (L-selectin⁻). However, this L-selectin⁻ cell population increased to 24 ± 8% as the result of stimulation by the growing tumor. Compared with L-selectin⁻ T cells, these L-selectin⁻ cells also showed increased expression of LFA-1 and CD44 (data not shown). Both molecules are known to be associated with T cell activation (11, 12). Thus, the increased number of L-selectin⁻ T cells suggests a response to the growing tumor.

In previous studies, stimulation of MCA 205 tumor-draining LN cells with anti-CD3 activated by culture in low concentrations (e.g., 4 units/ml) of recombinant IL-2 resulted in the growth of T cells and generation of specific immune effector cells capable of mediating the regression of established tumors (4, 13). Analysis of L-selectin expression on 5-day activated LN cells revealed an increased proportion...
of cells with down-regulated L-selectin (Fig. 1). However, it was not clear whether this increase was due to the in vitro activation of L-selectin+ cells or preferential proliferation of L-selectin− cells.

To test whether this characteristic modulation of surface adhesion molecules reflected an immune response to the growing tumor, LN T cells were separated based on their expression of L-selectin by mAb panning. Before separation, T cells were enriched by passing through a nylon wool column, resulting in 92 ± 3% Thy1.2+ cells. In four independent experiments, the L-selectin-positve and -negative fractions were 94 ± 3% and 96 ± 4% pure, respectively. On activation with anti-CD3 for 2 days and IL-2 for an additional 3 days, L-selectin− cells proliferated more vigorously than L-selectin+ cells. On average, L-selectin− cells increased 7.8 ± 2.1-fold, compared with a 3 ± 1.5-fold increase observed in L-selectin+ cells. These cells were then tested for their in vivo antitumor reactivity by the systemic transfer to mice bearing 3–4-day established intracranial MCA 205 tumors. As shown in Fig. 2, the transfer of 15 × 10^6 activated cells derived from unfractionated tumor-draining LNs significantly (P < 0.006) prolonged the survival of tumor-bearing mice, but only 2 of 10 mice were cured of the disease in two experiments. Cells derived from the purified L-selectin− population did not demonstrate any antitumor effects. Mice that received the transfer of 5 × 10^6 (experiment 1) or 15 × 10^6 (experiment 2) activated L-selectin− cells died with a median survival time identical to that of untreated mice. However, cells derived from the L-selectin− population proved to be most active. With as few as 2 × 10^6 cells transferred, all treated mice survived beyond the 60 days of observation. Although an intracranial tumor was used as a model system because of our interest in developing clinical protocols for glioma, activated L-selectin− LN cells were highly effective in eliminating established pulmonary metastases (data not shown). These results clearly indicate that, similar to previous observations in immune responses to allogeneic skin graft, virus infections, and immunization with proteins (9, 14, 15), the small subset of LN T cells becoming L-selectin− represents the immune effector cells responding to the growing tumor. By down-regulation of L-selectin, these cells are well equipped to deviate from migrating via the classic route of recirculation from the blood stream through LNs and, rather, to circulate through peripheral nonlymphoid tissues.

The purified L-selectin− LN cells also showed increased LFA-1 and CD44 expression (data not shown). Increased LFA-1 especially with high avidity to interact with intercellular adhesion molecule 1 may facilitate adhesion and extravasation to endothelial cells (16). The increased CD44, by interacting with hyaluronate, would effect the motility of lymphocytes. Some data suggest that CD44 also has a role in adhesion to activated endothelium (17). Thus, these molecules may promote traffic to the tumor site.

The down-regulation of L-selectin on antigenically stimulated T cells is not a permanent alteration. After cessation of a primary immune response to minor histocompatibility antigens, some of the activated effector T cells revert to small resting memory cells with concomitant reexpression of L-selectin (18). In our study, with highly purified (≥90%) L-selectin− LN cells, activation by the anti-CD3/IL-2 method resulted in the reexpression of L-selectin in approximately 30% of cells (data not shown). It is possible that some effector T cells converted to L-selectin− memory-like cells. However, separation of in vitro-activated, tumor-draining LN cells shortly before adoptive immunotherapy revealed that only L-selectin− cells were therapeutically effective (data not shown). These results indicate that T cells responding to a growing tumor became L-selectin−, and they remained L-selectin− during culture. These L-selectin− cells constituted all antitumor activities in the activated tumor-draining LN cells.

Note is the observation that the transfer of 2 × 10^6 purified L-selectin− cells had superior in vivo antitumor reactivity to 15 × 10^6 unfractionated cells (Fig. 2), which contained ~40% L-selectin− cells (Fig. 1). These results suggest that the antitumor activity of purified L-selectin− cells is far greater than that of cells mixed with L-selectin+ cells. It is possible that the L-selectin− T cells exerted a suppressive effect on the function of L-selectin− cells. The suppressive nature of L-selectin− cells needs to be confirmed experimentally by mixing various proportions of purified cells before in vitro activation and before adoptive transfer into tumor-bearing mice.

To define further the function of the L-selectin− cell population in the tumor-draining LNs, purified cells were activated, and the resulting cells were stimulated with tumor cells for 3 h before RT-PCR quantitation of IL-2, IFN-γ, and TNF-α gene expression (Fig. 3A). To obtain more precise information on the quantitation of PCR, we
calculated the intensities of the bands as percentages of that induced by anti-CD3 (Fig. 3B). Because anti-CD3 stimulation represents a biologically active event, and all populations of T cells were induced to express strikingly similar levels of PCR products, the cytokine mRNA induced by the stimulation with tumor cells can be meaningfully compared between different T-cell preparations. Without restimulation, none of the cells had detectable IL-2 gene expression. However, l-selectin− but not l-selectin+ or unfractionated cells expressed the IL-2 gene product on restimulation with MCA 205 tumor cells. The activated L-selectin+ cells constitutively expressed TNF-α and IFN-γ genes, but their expression was greatly up-regulated on tumor cell stimulation. The TNF-α gene expression was also detected in L-selectin+ cells on tumor stimulation, but the greatest enhancement was observed in L-selectin− cells. These lymphokine gene inductions in the L-selectin+ cell population appeared to be immunologically specific, because antigenically distinct MCA 207 and D5 tumor cells failed to up-regulate their expression. Calculating based on percentages of anti-CD3-activated gene products, L-selectin+ cells stimulated with MCA 207 or D5 tumor cells yielded 1 and 26% relative TNF-α and 48 and 32% relative IFN-γ gene products, respectively. Compared with 36 and 41% constitutive expression of TNF-α and IFN-γ genes, respectively, stimulation with antigenically distinct tumor cells did not result in the up-regulation of expression of these lymphokine genes. Transcripts of β-actin demonstrated similar intensities in all samples, indicating the consistency of mRNA purification and cDNA synthesis. Taken together, these results indicate that the l-selectin− T cells are immunologically more active than l-selectin+ T cells. The ability to express multiple lymphokine genes by the l-selectin− cells suggests that tumor regression after adoptive transfer of these cells may involve functional responses of these lymphokines. To support this view, the activated tumor-draining LN cells lack in vitro cytotoxicity despite their potent in vivo antitumor reactivities (4).

Although the feasibility of adoptive immunotherapy for cancer has been demonstrated with the use of nonspecific lymphokine-activated killer cells and tumor-infiltrating lymphocytes, the lack of available in vitro assays that can faithfully predict in vivo therapeutic efficacy makes it difficult to consistently isolate or generate tumor-reactive lymphoid cells for the treatment of humans (19, 20). In this study, l-selectin− cells showed tumor-specific IL-2, TNF-α, and IFN-γ gene expression when encountered with tumor cells. It is also clear that the l-selectin− cells contributed to all of the antitumor activities. These observations are particularly important when considering the potential utility to identify antitumor effector cells suitable for clinical adoptive immunotherapy.

Although several adhesion molecules, such as CD44 and LFA-1, are expressed at high levels on l-selectin− tumor-draining LN cells, the differential expression of l-selectin is probably the best single molecular marker for identifying and purifying functional antitumor effector cells. First, the proportion of l-selectin− cells in the draining LN T cells is small (~25%), thus affording an efficient means of selecting antitumor effector lymphocytes from the whole population. Second, separation based on the nonreactivity of cells with a mAb would induce the least perturbation of their cell membranes. Third, an analogous l-selectin molecule has been described in humans, and availability of the mAb makes it theoretically possible to separate human T cells based on l-selectin expression (21). Preliminary studies indicate that the proportion of l-selectin− cells in normal human LN T cells is approximately 11%. Our current clinical protocols of adoptive immunotherapy of malignancy will allow us to further study the immunological reactivities of l-selectin− T cells in tumor-draining LN as well as LN draining autologous tumor vaccines.

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