T Lymphocytes in Human Atherosclerotic Plaques Are Memory Cells Expressing CD45RO and the Integrin VLA-1

Sten Stemme, Jan Holm, and Göran K. Hansson

The cellular composition of human atherosclerotic plaques has been analyzed in several immunohistochemical studies in recent years. These studies have shown that the main cell types of the plaque are macrophages, smooth muscle cells, and T lymphocytes. To further characterize the T-lymphocyte population in atherosclerotic plaques, human plaque tissue was digested enzymatically and the released cells were labeled with fluorescent antibodies and analyzed by flow cytometry. Fifteen patients undergoing carotid endarterectomy were studied. Sixty-four percent of plaque T cells expressed the low-molecular-weight form (CD45RO) of the leukocyte common antigen (CD45). Many of these cells expressed the integrin very late activation antigen-1 (VLA-1), which suggests that they are in a state of late activation. In contrast, only 1% of peripheral blood T cells from the same patients expressed VLA-1. Other markers of T cell activation, such as Ta1 (CD26) and HLA-DR, were also increased on plaque T cells. The interleukin-2 receptor (CD25), which is transiently expressed after activation, was present on only a small proportion of the cells. Taken together, this analysis of plaque lymphocytes shows that the majority of plaque T cells are memory cells, many of which are in a state of late or chronic activation. This T-cell phenotype may be the result of a preferential recruitment and/or retention of activated peripheral blood T cells or local antigenic stimulation of resting T cells. (Arteriosclerosis and Thrombosis 1992;12:206-211)

Methods

Cell Isolation

Atherosclerotic plaques were obtained from 15 patients, 12 men and three women, between 52 and 77 years of age, who were undergoing carotid endarterectomy for transient ischemic attacks. None of the patients suffered from any known chronic inflammatory disease. The specimens represented advanced lesions, usually involving most of the arterial circum-
were used, cells were incubated with the primary MAb, followed by staining with a secondary FITC-labeled goat anti-mouse immunoglobulin G (Becton Dickinson). The proportion of positive cells was determined among the CD3-positive cells. To classify cells as positive or negative, a threshold level was set for each antigen and then used throughout the study. For CD45RO and CD45RA, the level was set between that of the two populations (with high and low expression) occurring in peripheral blood. The level for HLA-DR-positive cells was set just below the fluorescence intensity of monocytes in peripheral blood. The level of CD26-positive cells was set to include the population with the highest expression in the approximately trimodal distribution seen in peripheral blood. For the remaining antigens, a threshold level was chosen that was able to exclude more than 90% of cells when stained with nonspecific antibody. To correct for background binding, each value obtained from staining with specific antibody was subtracted with values from staining with nonspecific antibody.

### Results

To establish the phenotype of T lymphocytes in human atherosclerotic plaques, cells were isolated from carotid endarterectomy specimens by digestion with collagenase, stained with MAbs to cell-surface markers, and analyzed by flow cytometry. T lymphocytes were identified with a phycoerythrin-labeled Leu-4 antibody directed to CD3, a membrane protein complex that is associated with the T-cell antigen receptor and that is expressed on all T cells. The same cells were then labeled with a panel of FITC-tagged MAbs directed to different lymphocyte surface antigens. This permitted a detailed characterization of the T-cell population. For comparison, T cells from the peripheral blood of patients were analyzed in an identical way.

Tests with collagenase treatment of phytohemagglutinin-activated peripheral blood mononuclear cells showed a negligible or no effect on reactivity of any of the antibodies used except for anti-CD4 (Leu-3a), for which the reactivity was substantially decreased. Residual CD4 reactivity, however, always allowed delineation of CD4+ T cells. The difference in phenotypes of plaque T cells and peripheral blood T cells and the fact that virtually no B cells were encountered among plaque lymphocytes (as assessed by MAb Leu-12 towards CD19, not affected by collagenase) indicate that contamination of peripheral blood cells in the plaque cell population was very low or absent. The representativity of the plaque T-cell samples obtained after collagenase digestion was supported by the correspondence of T-cell phenotypes with findings in previous immunohistochemical studies.1,2 In all patients, the majority of plaque T cells carried the common αβ-type antigen receptor. In two patients, the amounts of T cells expressing the γδ-type antigen receptor were 6% and 8%, respectively, which were slightly higher than those in peripheral blood (data not shown). Fifty-five percent of the plaque lymphocytes were CD8+ cytotoxic T cells, and the remainder were of the CD4+ helper pheno-

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### Table 1. Antibodies Used in the Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Anti-IL-2R</td>
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<td>C</td>
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<td>Control mouse IgG</td>
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</tbody>
</table>

*CD2-R, interleukin-2 receptor; VLA-1, very late activation antigen-1; IgG, immunoglobulin G; *CD, cluster of differentiation; BD, Becton Dickinson, Mountain View, Calif.; C, Coulter Immunology, Hialeah, Fla.; T, T Cell Sciences, Cambridge, Mass.; D, Dakopatts, Glostrup, Denmark.

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**Flow Cytometry**

Cells were incubated with antibodies at previously determined optimal concentrations at 4°C for 30 minutes and washed once in PBS between steps. The antibodies used are listed in Table 1. After a final wash in PBS, the cells were fixed with 1% paraformaldehyde in PBS and analyzed in a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.).

For determination of their state of activation, T cells were double-labeled with phycoerythrin-conjugated Leu-4 (anti-CD3) and one of a panel of fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (MAbs). When unconjugated MAbs were used, cells were incubated with the primary MAb, followed by staining with a secondary FITC-labeled goat anti-mouse immunoglobulin G (Becton Dickinson). The proportion of positive cells was determined as described.7 In brief, tissue specimens consisting of the plaque and inner media were meticulously cleared of peripheral blood and any thrombotic material. To destroy lymphocytes that might remain adherent to the surface, the plaque was washed four times in 20 ml distilled water. The tissue was then minced into fine pieces and digested for 3 hours at 37°C with collagenase type I (Sigma Chemical Co., St. Louis, Mo.) at 900 units/ml in phosphate-buffered saline (PBS) with 5 mg/ml bovine serum albumin (radioimmunoassay grade, Sigma), 0.2 mM CaCl2, and 1 mg/ml glucose. The resulting cell suspension was filtered through a 150-mesh nylon net, washed in PBS, and stained for flow cytometry. Peripheral blood mononuclear cells from the patients and healthy blood donors were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation.

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**Flow Cytometric Analysis of Plaque T Cells**

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type. In peripheral blood, 46% of T cells were CD8+, but this difference was not statistically significant.

**Memory Cell Phenotype of Plaque T Lymphocytes**

Immunologically naive T cells differ from memory cells in their expression of the CD45 antigen. The CD45, or leukocyte common antigen (LCA), is a family of all-surface tyrosine phosphatases expressed on all types of leukocytes. The protein is encoded by a single gene, and the different forms are obtained through alternative splicing of the large primary transcript. Naive T lymphocytes never previously activated express the 205-220-kd form CD45RA of LCA, which is recognized by the MAb 2H4. However, after the first round of activation of the T cell through exposure to its specific antigen, the splicing of the primary transcript is altered so that the 180-kd form, CD45RO, which is recognized by the antibody UCHL1, is expressed instead. Reactivity to UCHL1 can thus be used as a marker for previously activated T lymphocytes.

The numbers of CD45RO+ and CD45RA+ T cells were inversely correlated in plaque cells as well as in the peripheral blood of patients (Figure 1). The pattern of CD45 isoform expression was, however, distinctly different in the plaque compared with that in blood. The mean proportion of CD45RO+ T cells was 49% in peripheral blood but was increased to 64% among plaque T cells (Figure 2). A similar discrepancy, even more pronounced, was seen in the expression of CD45RA. Forty-four percent of T cells in peripheral blood expressed CD45RA compared with only 12% in plaque T cells (Figure 2).

These data indicate that the majority of plaque T lymphocytes belong to the memory-T-cell subset. This implies that they have previously undergone activation, but it remains unclear whether the activation step occurred before or after cell entry into the plaque.

**Markers of Activation in Plaque T Lymphocytes**

CD45RO is expressed on activated T cells as well as on resting memory T cells. CD45RO expression, therefore, gives no information about the degree of activation at the time of analysis. To determine this, a panel of five different antibodies recognizing surface antigens expressed at different time points after T cell activation was used.

The high-affinity interleukin-2 receptor, the receptor for autocrine T-cell growth factor interleukin-2, is not expressed on resting T cells but is induced within 24 hours after activation. The present study showed that interleukin-2 receptor expression was low among plaque T cells, and the frequency of positive cells did not differ significantly from that in peripheral blood (Figure 3). This suggests that the number of proliferating T cells is low in advanced human atherosclerotic plaques.

Other cell-surface proteins show slower kinetics of expression after T-cell activation. In cell culture, CD26 (Tal) expression is induced 2-5 days after activation with phytohemagglutinin. HLA-DR and

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**Figure 1.** Representative histograms showing CD45RO (upper panel) and CD45RA (middle panel) expression in T cells from plaque (left) and peripheral blood (right). Cell suspensions from plaque or peripheral blood mononuclear cell preparations were double-labeled with phycoerythrin-conjugated anti-CD3 and FITC-labeled UCHL1 (CD45RO), 2H4 (CD45RA), or control mouse immunoglobulin G antibody and analyzed by flow cytometry. CD3-positive cells were gated and plotted in histograms, with cell numbers on the y axis and the logarithm of FITC fluorescence intensity on the x axis. FITC, fluorescein isothiocyanate.

**Figure 2.** Bar graph showing distribution of CD45RO+ and CD45RA+ T cells in plaque and peripheral blood. T cells were identified with phycoerythrin-conjugated anti-CD3 and were double-labeled with FITC-tagged antibodies. A threshold level for positive cells was determined for each antigen and then applied on all samples. Error bars represent SEM, n=13. FITC, fluorescein isothiocyanate.
Peripheral blood
Plaque

IL-2R CD38 HLA-DR CD26 VLA-1

FIGURE 3. Bar graph of cells positive for a panel of activation markers in T cells from plaque and peripheral blood. Experimental conditions were as described in the legend to Figure 2. Error bars represent SEM, n=12. Differences between mean values of blood and plaque T cells are significant for HLA-DR, CD26, and very late activation antigen-1 (VLA-1) (p<0.001, Mann-Whitney U test). IL-2R, interleukin-2 receptor.

CD38 (OKT10) show a similar pattern of expression, starting 5–6 days after antigen stimulation.14 These surface markers are gradually downregulated approximately 1 week after cessation of stimulation in vitro. They can thus be considered markers of relatively recent activation.

In the present study, the proportion of CD26+ T cells was doubled in plaque cells compared with that in peripheral blood, and the proportion of HLA-DR+ T cells was also significantly increased (Figure 3). The expression of these surface antigens indicates that the proportion of recently activated T cells is increased in the plaque compared with that in the peripheral blood.

In contrast to the antigens discussed above, VLA-1 is expressed 2–3 weeks after stimulation with mitogen and remains on the cell surface for a longer time.15,16 In the present study, more than 31% of plaque T cells were VLA-1+ (Figures 3 and 4), whereas only 1% of T cells in peripheral blood expressed VLA-1. The surface phenotype of T lymphocytes from peripheral blood of the patients did not differ significantly from that of T lymphocytes from healthy blood donors (data not shown).

Discussion

The present study demonstrates that the T-cell populations in advanced atherosclerotic plaques are distinctly different from those in peripheral blood from the same individuals. Forty-four percent of the T cells in blood expressed the high-molecular-weight (CD45RA) form of LCA that is characteristic for naive T cells. In contrast, only 12% of plaque T cells were of the naive phenotype. Similarly, 64% of plaque T cells exhibited the memory (CD45RO) phenotype compared with 49% in the blood. The selective accumulation of memory-type T cells argues strongly against entrapment of inactive circulating cells as a significant mechanism for recruitment of T cells in advanced atherosclerotic lesions. Instead, they suggest that T cells are activated locally in the plaque, or alternatively, that circulating activated or memory T cells may be selectively recruited to the plaque. The dominance of memory T cells in the plaque per se does not indicate that local activation occurs. The expression of other phenotypic markers suggests, however, that many of these T cells are activated or maintained in an activated state in the plaque.

The differential expression of activation markers with different kinetics revealed an interesting aspect of the T-cell activation state in the plaque. The interleukin-2 receptor, which is rapidly induced after activation but is then quickly downregulated, was not increased in plaque T cells. HLA-DR and CD26, which can be considered as activation markers with intermediate-kinetic characteristics, were increased approximately twofold. In contrast, the CD38 antigen was not significantly increased in plaque T cells. After in vitro activation, the expression of CD38 paralleled the expression of HLA-DR.14 This discrepancy may indicate that the surface expression pattern of this antigen in vivo differs from that in vitro. Finally, VLA-1 was increased more than 10-fold in plaque tissue compared with that in the patients’ blood. Taken together, this pattern suggests that a large proportion of plaque T cells are in a state of late or chronic activation. These phenotypic characteristics resemble those observed for T cells in other chronic inflammatory diseases, including rheumatoid arthritis,17–20 multiple sclerosis,21 sarcoidosis,22 and Graves’ disease23 and may reflect common mechanisms in the development of the inflammatory infiltrates in these disorders.

The expression of VLA-1 is interesting not only as a marker of activation but also because it may be of
The present study confirms and extends previous immunohistochemical observations that the atherosclerotic plaque contains activated T lymphocytes. An important new observation is that the majority of plaque T cells are of the memory phenotype. T cells of this differentiation stage have a high capacity for lymphokine production, and many plaque T cells exhibited phenotypic signs of late activation, which further supports the hypothesis that they are releasing lymphokines in the plaque. Finally, the expression of integrin receptors by these cells suggests that matrix interactions may be important for the regulation of the local inflammatory response in atherosclerosis.

Acknowledgment

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References

10. McDevitt HO: MHC class II antigens: Their potential role in atherogenesis. *Arterioscler Thromb* 1988;8:393–399
35. Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S: Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J Immunol* 1988;140:1401–1407

**KEY WORDS** • atherosclerosis • T lymphocytes • flow cytometry • activation
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