Monoclonal T-Cell Proliferation and Plaque Instability in Acute Coronary Syndromes

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Background—Unstable angina (UA) is associated with systemic inflammation and with expansion of interferon-γ–producing T lymphocytes. The cause of T-cell activation and the precise role of activated T cells in plaque instability are not understood.

Methods and Results—Peripheral blood T cells from 34 patients with stable angina and 34 patients with UA were compared for the distribution of functional T-cell subsets by flow cytometric analysis. Clonality within the T-cell compartment was identified by T-cell receptor spectrotyping and subsequent sequencing. Tissue-infiltrating T cells were examined in extracts from coronary arteries containing stable or unstable plaque. The subset of CD4⁺CD28null T cells was expanded in patients with UA and infrequent in patients with stable angina (median frequencies: 10.8% versus 1.5%, \( P < 0.001 \)). CD4⁺CD28null T cells included a large monoclonal population, with 59 clonotypes isolated from 20 UA patients. T-cell clonotypes from different UA patients used antigen receptors with similar sequences. T-cell receptor sequences derived from monoclonal T-cell populations were detected in the culprit but not in the nonculprit lesion of a patient with fatal myocardial infarction.

Conclusions—UA is associated with the emergence of monoclonal T-cell populations, analogous to monoclonal gammopathy of unknown significance. Shared T-cell receptor sequences in clonotypes of different patients implicate chronic stimulation by a common antigen, for example, persistent infection. The unstable plaque but not the stable plaque is invaded by clonally expanded T cells, suggesting a direct involvement of these lymphocytes in plaque disruption. (Circulation. 2000;102:2883-2888.)

Key Words: angina • plaque • lymphocytes • cytokines • immune system

The spectrum of clinical syndromes caused by coronary atherosclerosis ranges from asymptomatic disease and stable angina (SA) to acute coronary syndromes (ACS), which include unstable angina (UA), acute myocardial infarction, and sudden cardiac death. The life-threatening complications of coronary artery disease arise as nonlinear events in an otherwise slowly progressive process. This nonlinearity has been attributed to a combination of factors, of which plaque disruption and superimposed thrombosis are considered to be the most important. Although the pathways leading to plaque rupture are incompletely understood, the participation of immune cells and inflammation in the process is now recognized. Activation of tissue-infiltrating inflammatory cells, such as macrophages, T cells, and mast cells, has been implicated to cause plaque vulnerability through the local production of tissue-digesting enzymes and procoagulant substances. Stimulation of inflammatory mechanisms in ACS is not, however, limited to the microenvironment of the plaque, because it also involves circulating cell populations. Patients with UA show increased frequency of lymphocyte and monocyte activation in the peripheral blood and elevated levels of acute-phase proteins, which are predictive for the clinical outcome.

We recently reported that monocyte activation in UA represents a downstream effect of an altered T-cell response characterized by the preferential production of interferon-γ (IFN-γ). IFN-γ–producing T cells in the peripheral blood were more frequent in patients with UA than in patients with SA. IFN-γ was derived from an unusual subset of CD4⁺ T cells, CD4⁺CD28null T cells, which was expanded in UA patients. An understanding of the pathophysiology of CD4⁺CD28null T cells and the mechanisms leading to their expansion may provide insights into the cause of ACS and into the roles played by T cells and macrophages in plaque rupture.

Methods

Study Population

Peripheral blood mononuclear cells (PBMCs) were obtained from 34 patients with chronic SA who underwent diagnostic coronary angiography (27 men, 7 women; mean age 64 ± 11 years) and from 34 patients admitted to the Mayo Clinic Coronary Care Unit who had a
diagnosis of UA, Braunwald’s class IIIB (20 men, 14 women; mean age 66±11 years).

Flow Cytometry
PBMCs were stained with phycoerythrin-conjugated anti-CD4 (Becton Dickinson) and FITC-conjugated anti-CD28 (Pharmingen) monoclonal antibodies and analyzed on a FACS Calibur flow cytometer (Becton Dickinson). The frequencies of CD4⁺CD28⁺ and CD4⁺CD28null T cells were determined with the use of WinMDI software (Joseph Trotter, Scripps Research Institute).

T-Cell Receptor β-Chain Sequence Analysis of Peripheral T Cells
CD4⁺CD28⁺ and CD4⁺CD28null T cells were purified from PBMCs by cell sorting on a FACS Vantage flow cytometer. Total RNA of 1×10⁶ CD4⁺CD28⁺ and CD4⁺CD28null cells was extracted (Trizol, Life Technologies), and cDNA was amplified with primers specific for BV2, BV3, BV5S2, BV6, BV7, BV8, BV13S1, BV14, BV17, and BV18 and a BC-specific primer, as described. The BV families analyzed were chosen arbitrarily to cover ~50% of the total T-cell receptor (TCR) repertoire. Amplified products were labeled with the use of a primer extension assay with the appropriate end-labeled BC primer and were separated on a 5% denaturing polyacrylamide gel. Band intensities were analyzed to determine whether the distribution was gaussian, indicating polyclonality, or skewed by dominant bands, indicating clonal expansion. In previous studies, bands with intensities greater than the sum of 2 adjacent bands and accounting for ≥30% of the total product were predictive of clonality. Dominant bands were directly sequenced by automated sequencing (ABI Prism Sequence Detection System, Perkin Elmer Applied Biosystems).

TCR β-Chain Sequence Analysis of Tissue-Infiltrating T Cells
We obtained coronary artery specimens from the left anterior descending artery and the right coronary artery of a patient with a fatal myocardial infarction in the territory of the right coronary artery. Arterial fragments were confirmed by histology to contain stable and unstable plaques, and total RNA was extracted from the appropriate segments. A second sample with a fissured plaque was obtained from a patient undergoing atherectomy. TCR β-chain-specific cDNA was synthesized with the use of a BC antisense primer (CTGTGCACTCTCCTCCATTAC) and amplified with the use of BV-specific primers and a nested set of BC primers (GTGGGAGATCCTGCTTCTG, TTCTGATGGCTCAAACAC) and then sequenced.

Statistical Analysis
We used the Mann-Whitney U test (SigmaStat, SPSS) to compare the frequencies of CD4⁺CD28null T cells in the patients with SA and UA.

Results
Clonal Expansion of CD4⁺CD28null T Cells in Patients With UA
The CD28 molecule is pivotal for signaling in CD4⁺ T-cell activation, consequently, CD4⁺ T cells lacking CD28 expression are rare. As shown in Figure 1, CD4⁺CD28null T cells were infrequent in our cohort of 34 SA patients, with a median frequency of 1.5%. Their frequency was increased ~10-fold in our cohort of 34 UA patients (median 10.8%, P<0.001).

To explore the possibility that CD4⁺CD28null T cells in the UA patients were expanded in response to a defined stimulus, such as an antigen, we examined the diversity of T-cell antigen receptors. CD4⁺ T cells from 20 patients with UA were sorted into CD4⁺CD28⁺ and CD4⁺CD28null subpopulations, and TCR transcripts were amplified with BV- and BC-specific primer sets and fractionated on polyacrylamide gels. For CD4⁺CD28⁺ T cells, length classes of TCR transcripts followed a gaussian distribution characteristic of clonal T-cell populations. In contrast, transcripts from CD4⁺CD28null T cells frequently deviated from a gaussian distribution, with dominant bands suggestive of clonal populations (Figure 2). Seventy-two dominant bands were sequenced from these 20 patients, 59 of which yielded unequivocal sequences, confirming the presence of clonal T-cell populations. An average of 3 T-cell clones was seen in UA patients (range 0 to 11). Interestingly, the one patient who lacked clonal TCR sequences also had a lower frequency of CD4⁺CD28null T cells. The primer sets used covered ~50% of the CD4⁺ T-cell repertoire. Patients with unstable angina can therefore be estimated to carry an average of 6 clonally expanded CD4⁺ T cells in the repertoire. Comparison of clinical parameters between patients with low and high numbers of T-cell clonotypes demonstrated no features correlating with the extent of oligoclonality (data not shown). Individual T-cell clones differed in size, with large clones...
reaching 5% and small clones accounting for 0.5% of the circulating CD4^+ T-cell pool.

Persistence of Expanded CD4^+CD28null Clonotypes

We have previously shown that the frequencies of CD4^+CD28null T cells are stable over time. To examine whether clonal outgrowth was a transient event related to acute disease, we analyzed peripheral blood CD4^+CD28null T cells from 5 patients with UA 6 months after the initial study. As shown in Figure 3, clonal dominance of selected T cells in the CD4^+CD28null T-cell compartment was maintained for the majority of expanded clonotypes (16 of 18). Clonal identity was confirmed by sequencing.

Sharing of TCR Sequences in Expanded CD4^+CD28null Clonotypes From Multiple Patients

Comparative analysis of TCR sequences expressed by expanded clonotypes can provide information about the stimulus driving the outgrowth of these T cells. A finding of structurally similar TCRs in multiple patients with UA would indicate exposure of these patients to the same antigen(s). The 59 clonal TCR sequences were analyzed for the use of TCR-BV and TCR-BJ gene elements (Figure 4). The distribution of BV gene segments from clonally expanded T cells was different from that of unselected T-cell populations, with BV3S1/S2 the BV element most common by far, accounting for 24% of all expanded clonotypes (compared with an expected 5% in a random sample). In addition, BV14S1 and BV8S1/S2 were encountered frequently, whereas BV17 and BV6S2/S3 were found in only a small proportion of clonal T cells. Nonrandom use of TCR gene segments was even more pronounced for BJ genes. More than 35% of the TCR sequences included a rearranged BJ2S1 gene, 22% a BJ2S7 gene, and 15% a BJ2S3 gene. BJ2S2 and BJ2S5 gene segments were not found, and BJ genes of the BJ1 family were explicitly infrequent. The BV3S1/S2-BJ2S1 TCR gene segment combination accounted for 11% of TCR sequences, compared with an expected 1% in a random T-cell population.

The structure of the TCR that directly contacts the antigenic peptide is the junctional N-D-N region at the interface of TCR-BV and TCR-BJ gene segments. To further investigate the hypothesis that antigen recognition underlies clonal expansion, we compared nucleotide and amino acid sequences of the junctional region of expanded clonotypes. Receptor homology was defined as the sharing of ≥60% of all amino acids. Among the 59 TCR sequences, similarities were seen in 10 sequences derived from 7 patients (Table 1). One patient carried 2 clonotypes and another had 3 clonotypes that expressed N-D-N sequences very similar to those identified in other UA patients. The group of related TCR sequences included 3 receptors of the overrepresented of BV3S1/S2-BJ2S1 rearrangement. All TCR sequences with shared amino acid sequences displayed heterogeneity at the level of nucleotide sequences.

CD4^+CD28null Expanded Clonotypes Infiltrate Unstable Plaque

If CD4^+CD28null clonotypes respond to antigenic stimulation and are involved in precipitating acute ischemic complications, they should be present in the arterial wall. Culprit and nonculprit lesions of postmortem coronary specimens were examined for the representation of TCR sequences, and TCR
TABLE 1. Amino Acid Sequence Homologies of TCR β-Chains From Expanded Clonotypes Derived From Different Patients With Unstable Angina

<table>
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<tr>
<th>Donor</th>
<th>BV N-D-N</th>
<th>BJ</th>
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<tr>
<td>BE</td>
<td>3S1/S2 CASS</td>
<td>LGGTY</td>
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<tr>
<td>LM</td>
<td>3S1/S2 CASS</td>
<td>PTGGTY</td>
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<td>RM</td>
<td>3S1/S2 CASS</td>
<td>LGDDTY</td>
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<td>SW</td>
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<td>LM</td>
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<td>UA</td>
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<td>BL</td>
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<td>UA</td>
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<td>WE</td>
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transcripts in the tissue specimens were amplified and sequenced. As shown in Figure 5, coronary artery tissue with a stable plaque was free of tissue-infiltrating T cells, whereas the arterial wall segment of the lesion that caused the fatal myocardial infarction did produce a signal. In this patient’s peripheral blood, CD4+CD28null T cells were highly expanded and included 5 clonal TCR sequences (Table 2). Two of these 5 sequences, from a BV3 and included 5 clonal TCR sequences. Two of these BV14 TCR sequences. First lane is DNA size marker. Bands in contrast, unstable plaque in RCA contained BV2, BV3, BV8, and BV14 TCR sequences. LAD did not yield TCR sequences. In these segments were amplified with BV- and BC-specific primers. Amplification products were subjected to sequence analysis. Stable lesion from LAD did not yield TCR sequences. In contrast, unstable plaque in RCA contained BV2, BV3, BV8, and BV14 TCR sequences. First lane is DNA size marker. Bands in BV2 LAD lane are nonspecific amplification products and do not represent a clonal T-cell population.

Figure 5. Infiltration of selected CD4+CD28null T cells into unstable plaque. Coronary artery tissue was collected from a patient who died of acute myocardial infarction in the territory supplied by the right coronary artery (RCA). TCR spectrotopy demonstrated clonal expansions of BV2/S2, BV3/S1/S2, BV8/S1/S2, and BV14/S1 CD4+CD28null T cells in peripheral blood. Arterial segments containing culprit plaque in RCA and lumen-compromising plaque in left anterior descending coronary artery (LAD) were identified by histology. Tissue extracts from these segments were amplified with BV- and BC-specific primers; amplification products were subjected to sequence analysis. Stable lesion from LAD did not yield TCR sequences. In contrast, unstable plaque in RCA contained BV2, BV3, BV8, and BV14 TCR sequences. First lane is DNA size marker. Bands in BV2 LAD lane are nonspecific amplification products and do not represent a clonal T-cell population.

levels with receptor sequences corresponding to those found in his peripheral CD4+CD28null T cells (Table 3).

Discussion

The recognition that the majority of ACS evolve from only mildly to moderately obstructive atherosclerotic plaques led to an appreciation of nonmechanical factors contributing to the clinical manifestations of coronary artery disease. Our finding that an unusual subset of T lymphocytes, CD4+CD28null T cells, is expanded in patients with UA but not in patients with SA suggests that these cells are involved in the acute events complicating otherwise stable and slowly progressive coronary disease. This T-cell population breaks several rules that apply to classic CD4 helper T cells. CD4+CD28null T cells contain monoclonal T-cell populations that persist over time at a large clonal size. Sequence homologies of TCR β-chains isolated from clones of multiple patients support the notion that these clones are expanded in response to exogenous antigen(s) common to patients with UA. CD4+CD28null T-cell clonotypes are accumulated in lesions with plaque rupture, suggesting that they are involved in the events leading to plaque instability, rupture, superimposed thrombosis, and induction of acute ischemia.

Diversity of the TCR repertoire is a fundamental principle of the immune system, and mechanisms that maintain diversity are critical in sustaining immunocompetence. In response to antigen, T cells proliferate and expand clonally. Clonal expansion of antigen-specific T cells is pivotal for a successful immune response but must be transient. Mechanisms are in place to prevent outgrowth of individual clones, limit T-cell proliferation, and induce clonal downsizing. Persistent clonal expansion is therefore a significant finding and may indicate defects in mechanisms of clonal downsizing. Evidence for defects in apoptotic pathways has been provided for CD4+CD28null T cells. The reduced propensity of CD4+CD28null T cells for apoptosis has been associated with overexpression of the survival protein Bcl-2, which suggests that these T cells might have developed properties that allow their escape from normal pathways of clonal downsizing.

Alternatively, long-term stimulation with antigen could cause the expansion and persistence of clonal CD4+ populations. This interpretation is suggested by the high degree of similarity in the amino acid sequence of TCR β-chains from clones of several patients. Obvious candidate antigens are microbial proteins, such as bacterial or viral products. Several recent lines of evidence have suggested a role for chronic infection in the pathogenesis of ACS. Although proof for a direct involvement of such infectious organisms in plaque inflammation is missing, persistent Chlamydia pneumoniae, Helicobacter pylori, or cytomegalovirus infections are suspected to have a role in the manifestations of coronary artery disease. Intracellular pathogens might conceivably survive in macrophages and reach immunogenicity in the atherosclerotic plaque. Alternatively, these cells could recognize self-antigens that become immunogenic in the atherosclerotic plaque. Interestingly, CD4+CD28null T-cell clones isolated from patients with vascular complications of rheumatoid arthritis have been shown to react to adherent cells, suggesting, as one possibility, a defect in self-tolerance.
T-Cell Receptor Sequences in Unstable Plaque of a Patient Who Underwent Atherectomy

Table 3 shows the accumulation of selected CD4+CD28null T-cell clones in unstable plaque of a patient who died of acute myocardial infarction. The table compares the T-cell receptor sequences found in peripheral blood, coronary artery tissue, and unstable plaque. The sequences are divided into polyclonal and specific clonal types.

For instance, in peripheral blood, BV3S1/S2 is polyclonal with the sequence SPAGTYN, and BV5S1/S2 is also polyclonal with the sequence SLGTGYN. In the coronary artery tissue, these sequences are detected, indicating clonal expansion in these sites.

In the unstable plaque, BV3S1/S2 is not detected, while BV5S1/S2 is detected with the sequence SLGTGYN. This suggests that these sequences are selectively enriched in the unstable plaque compared to the blood or artery tissue.

Acknowledgments

This work was supported in part by the Mayo Foundation and the Fondazione Internazionale di Ricerca per il Cuore, Rome, Italy, and by grants from the National Institutes of Health (RO1-AR41974, RO1-AR42527, and RO1-EY11916) and the American Heart Association (MHA 161). We thank Kristin Cornell, RN, for assistance in patient enrollment and Toni L. Higgins and James W. Fulbright for assistance in manuscript preparation.

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Circulation. 2000;101:2883-2888
doi: 10.1161/01.CIR.101.25.2883

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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