Perturbation of the T-Cell Repertoire in Patients With Unstable Angina

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Background—Monocytes are constitutively activated in unstable angina (UA), resulting in the production of IL-6 and the upregulation of acute phase proteins. Underlying mechanisms are not understood. To explore whether the production of the potent monocyte activator IFN-γ is altered in UA, we compared cytokine production by T lymphocytes in patients with UA (Braunwald’s class IIIB) and with stable angina (SA).

Methods and Results—Peripheral blood lymphocytes were collected at the time of hospitalization and after 2 and 12 weeks. Cytokine-producing CD4+ and CD8+ T cells were quantified by 3-color flow cytometry after stimulation with phorbol myristate acetate and ionomycin. UA was associated with an increased number of CD4+ and CD8+ T cells producing IFN-γ, whereas patients with SA had higher frequencies of IL-2+ and IL-4+ CD4+ T cells. Expansion of the IFN-γ+ T-cell population in UA persisted for at least 3 months. Increased production of IFN-γ in UA could be attributed to the expansion of an unusual subset of T cells, CD4+CD28null T cells.

Conclusions—Patients with UA are characterized by a perturbation of the functional T-cell repertoire with a bias toward IFN-γ production, suggesting that monocyte activation and acute phase responses are consequences of T-cell activation. IFN-γ is produced by CD4+CD28null T cells, which are expanded in UA and distinctly low in SA and controls. The emergence of CD4+CD28null T cells may result from persistent antigenic stimulation. (Circulation. 1999;100:2135-2139.)

Key Words: angina • ischemia • interleukins • immune system • lymphocytes

Coronary artery disease (CAD) is characterized by long periods of clinical stability with few, occasional abrupt changes that lead to acute coronary syndromes.1 Underlying mechanisms are unresolved, but recent studies suggest a role for inflammation.2 Inflammatory cells accumulate in the coronary lesions, possibly causing plaque instability. Activation of inflammatory pathways in unstable angina (UA), however, is not confined to the coronary lesions, but includes stimulation of circulating monocytes3,4 with increased production of IL-6 and subsequent induction of acute phase proteins.5–7

Monocyte activation can be mediated by several mechanisms, including IFN-γ released from activated T cells. To investigate the role of T cells in UA, we examined the ability of circulating CD4+ and CD8+ T cells to produce IFN-γ, IL-2, and IL-4. UA patients had an increased frequency of CD4+ IFN-γ+ and CD8+ IFN-γ+ T cells. Attempts to characterize the IFN-γ-producing T-cell subset led to the identification of an unusual subpopulation of CD4+ T cells, CD4+CD28null T cells, in UA patients. Such T cells were infrequent in patients with stable angina (SA). We have recently demonstrated that CD4+CD28null T cells represent proinflammatory cells with a high tissue-damaging potential.8 The expansion of CD4+CD28null T cells in UA suggests that these patients have a perturbation in the T-cell repertoire toward the production of IFN-γ, resulting in increased inflammatory responsiveness.

Materials and Methods

Population

Twenty-five patients with SA who underwent diagnostic coronary angiography between June and December 1997 (21 men; mean age, 64±10 years); 25 patients admitted during the same time interval with a diagnosis of UA (Braunwald’s class IIIB) (16 men; mean age, 67±10 years); 20 hospitalized control patients (14 men; mean age, 57±13 years); and 21 healthy individuals of the same age group (11 men; mean age, 62±11 years) were studied.

SA patients had no acute events or worsening of symptoms during the previous 6 months and no anginal episodes within the week preceding enrollment. UA patients had at least 2 episodes of angina at rest or 1 episode lasting >20 minutes during the preceding 48 hours, ST-segment shift diagnostic for myocardial ischemia during the angina attacks, and no elevation in serum creatine kinase on admission and during the first 24 hours of hospitalization. UA and
SA patients did not differ for demographic characteristics and for angiographic findings, except for a previous history of angina (data not shown).

Design of the Study

Blood

Peripheral blood samples were taken immediately after hospitalization, 7 to 14 days after hospital discharge, and 3 months after the first sample. In 9 SA and 8 UA patients, blood samples were obtained simultaneously from the aortic root and the coronary sinus during diagnostic coronary angiography before contrast medium injection and drug administration.

Laboratory Assays

Flow Cytometry

Frequencies of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD4⁺CD8⁻ T cells in peripheral blood mononuclear cells (PBMC) were determined by flow cytometry using the following monoclonal antibodies (mAb) anti-CD3 (fluorescein isothiocyanate-conjugated), anti-CD4 (peridinin chlorophyll protein-conjugated), anti-CD8 (peridinin chlorophyll protein-conjugated) (Becton Dickinson, San Jose, Calif), and anti-CD28 (fluorescein isothiocyanate-conjugated) (Pharmingen, San Diego, Calif) as described.⁹ Cytokine production by T-cell subsets was assessed after 4-hour in vitro activation with 10 ng/mL phorbol myristate acetate and 1 μg/mL ionomycin (Sigma Chemical Co, St Louis, Mo) in the presence of 10 μg/mL Brefeldin A (Epitope Technologies, Madison, Wis). After staining for cell surface markers, cells were fixed and cytoplasmic cytokines were stained with phycoerythrin-labeled anti-cytokine mAb (anti-IFN-γ, anti-IL-2, and anti-IL-4) (R&D Systems, Minneapolis, Minn) as recommended by the manufacturer. Nonspecific staining with isotype-matched control mAb was <1%.

Monocyte Stimulation With IFN-γ

One million PBMC were incubated for 18 hours at 37°C with and without 200 U/mL IFN-γ (BioSource International, Camarillo, Calif). Cell surface expression of HLA class I molecules or monocytes was determined by flow cytometry using the mAb W6/32 (ATCC, Bethesda, Md) and anti-CD14 mAb (Becton Dickinson, San Jose, Calif).

Statistical Analysis

The Mann-Whitney U test and the Kruskal Wallis 1-way ANOVA were used for comparisons between groups. Correlations were determined using Spearman’s rank correlation test. The remaining variables were compared using Student’s t test for paired and unpaired variables or the Fisher exact test, as appropriate. In addition, we used bootstrap sampling to estimate the distribution of the differences between the medians of the groups of SA and UA patients.

Results

The Functional Profile of Circulating T Lymphocytes Distinguishes Patients With SA and UA

Absolute lymphocyte numbers in patients with SA and UA and age-matched controls were not different. After in vitro activation, a median of 12.4% of the peripheral CD4⁺ T cells in normal donors and of 12.4% in patients with SA showed cytoplasmic staining for IFN-γ compared with ≈56% in patients with UA (median frequency 26.4%, P < 0.001) (Figure 1). Conversely, the median frequency of CD4⁺IL-2⁺ T cells was higher in patients with SA (28.4%) than in the control (19.7%) and UA cohorts (16.1%, P < 0.001). SA patients had the highest levels of IL-4–producing CD4⁺ T cells producing IL-2 and IL-4.
cells (median 2.4%). Increased IFN-γ expression in UA was also seen for CD8 T cells. The frequencies of CD8^+ cells that synthesized IFN-γ was higher in UA patients (58.2%) than in controls (38.7%) and SA patients (38.5%, P<0.001). IL-2 and IL-4 expression by CD8 T cells was similar in all 3 study cohorts. Increased frequencies of IFN-γ-producing cells in UA patients were not related to passing through the coronary circulation. Frequencies in samples harvested from the coronary sinus and from the aortic root during coronary angiography failed to show a significant difference (data not shown).

Expansion of IFN-γ-Producing T Cells Is Not a Consequence of Acute Chest Pain

In SA patients, the frequencies of cytokine-producing T cells were stable. In UA donors, the frequencies of CD4^+ IFN-γ^+ and CD8^+ IFN-γ^+ cells declined 1 to 2 weeks after discharge but returned to their original high levels after 3 months, although the patients were free of symptoms. CD4^+ IL-2^+ and CD4^+ IL-4^+ T cells were characterized by a reciprocal course with an intermittent increase at 7 to 14 days after discharge and a return to initial frequencies after 3 months (Figure 2).

Expansion of CD4^+CD28null T Cells in Patients With UA

Production of high amounts of IFN-γ is typical for an unusual subset of T cells, CD4^+CD28null cells which are infrequent in normals. To address the question whether UA patients carry CD4^+CD28null T cells, PBMC were analyzed by 2-color cytometry (Figure 3). Median frequencies of CD4^+CD28null T cells were 9-fold higher (9.0%) in UA than in SA patients (P<0.001). Analysis of the distribution of the differences of the medians after bootstrap sampling yielded a 99% CI ranging from 4.1 to 19.4, confirming that the medians differed significantly (P<0.01). Frequencies of UA patients were also significantly increased when compared with hospitalized control patients (P=0.001). Also, the expansion of CD4^+CD28null T cells persisted after hospital discharge. Thirteen UA and 14 SA patients were reanalyzed 63 to 221 days after symptom stabilization to rebound to the original levels at 3 months, although the patients were free of symptoms at that time. No longitudinal changes were found in SA patients.

Figure 2. Longitudinal monitoring of cytokine-producing T cells. Frequencies of cytokine-producing T cells were determined at 3 time points: hospital admission (adm), 7 to 14 days after hospital discharge (7–14 d), and 3 months (3 m). Data presented as box plots. Percentage of CD4^+ and CD8^+ T cells producing IFN-γ significantly decreased in patients with UA 7 to 14 days after symptom stabilization to rebound to the original levels at 3 months, although the patients were free of symptoms at that time. No longitudinal changes were found in SA patients.

Figure 3. Increased frequencies of CD4^+CD28null T cells in patients with UA. Frequency of CD28null T cells within the CD4^+ compartment was compared in 25 patients with SA and 25 patients with UA. CD4^+CD28null T cells were significantly expanded in UA patients (P<0.001). Patients with SA did not differ from age-matched normal controls. 
days after the initial visit. The frequencies of CD4+CD28null T cells at the 2 time points correlated highly \((R=0.81, P<0.001)\), indicating that the expansion was not related to the acute event or the hospitalization.

The frequency of CD4+CD28null T cells correlated \((R=0.64, P=0.002)\) with the number of IFN-γ–secreting cells (Figure 4). In addition, analysis of cytoplasmic IFN-γ in CD4+CD28+ and CD4+CD28null T cells by 3-color cytometry in 16 UA patients showed that the increased IFN-γ production in UA could be attributed to the CD4+CD28null T cells, whereas the CD4+CD28+ subset produced normal amounts.

**Monocytes of Patients With UA Remain Responsive to IFN-γ**

Levels of HLA class I expression, a monocyte activation marker upregulated by IFN-γ, was determined in 10 patients (4 with SA and 6 with UA) following 18 hours of incubation with or without 200 U of human recombinant IFN-γ. Results of a representative experiment are shown in Figure 5. In all donors, CD14+ cells expressed higher levels of HLA class I molecules following stimulation (mean fluorescence intensity 439.5 versus 296.5, \(P<0.01\)), demonstrating that monocytes of UA patients remain responsive to IFN-γ.

**Discussion**

Immune cells infiltrating coronary lesions, including macrophages, T lymphocytes, and mast cells have been suspected to contribute to plaque instability.10–12 The current investigation suggests that aberrations of the global immune system are of functional importance for this plaque inflammation. Data presented here establish that a subset of UA patients have expanded unusual CD4+ T cells that excessively produce IFN-γ on stimulation. Circulating CD4+CD28null cells might infiltrate the coronary plaque and induce inflammation, particularly if stimulatory antigen is locally expressed. Also, the well established constitutive activation of circulating monocytes in UA could be viewed as a consequence of excessive IFN-γ production by these cells.

Emergence and expansion of CD4+CD28null cells indicates a defect in T-cell homeostasis in UA. Generation of these T cells could be genetically controlled and/or result from long-term antigenic stimulation. Accumulating data favor long-term antigen recognition and thus provide indirect evidence for a role of antigen persistence in UA. Expanded CD4+CD28null cells are highly oligoclonal, suggesting ongoing antigenic stimulation.13,14 Obvious candidate antigens are derived from microorganisms chronically infecting the host. Release of IFN-γ by CD4+CD28null cells would be in line with anti-microbial reactivity. As the interaction of T cells with antigen is highly specific, CD4+CD28null T-cell clones should provide excellent reagents to search for relevant antigens in UA.

The association of T-cell repertoire perturbations with UA but not with SA raises the possibility that committing to certain immunopathways may have detrimental effects for the host. Although responding to persistent antigen should be beneficial, expansion of CD4+CD28null cells appears to be correlated with negative consequences. CD28null cells lack transcription of CD40 and are therefore unable to support B-cell differentiation and antibody production.15 Concomitantly, they have acquired expression of perforin and granzyme B equipping them with cytolytic abilities.8 Independent from costimulatory signals provided by their microenviron-

Figure 4. Correlation between IFN-γ production and increased frequencies of CD4+CD28null T cells in UA. Frequencies of CD4+CD28null T cells were compared with numbers of IFN-γ-producing T cells in SA \((n=25)\) and UA patients \((n=21)\). A significant correlation was found in UA \((R=0.64, P=0.002)\) but not in SA patients \((R=0.30, P=0.12)\).

Figure 5. IFN-γ responsiveness of monocytes in SA and UA patients. Levels of HLA class I molecule expression on CD14+ monocytes were determined by flow cytometry following 18 hours of stimulation with 200 U/mL of IFN-γ (solid line) or medium alone (dotted line). Results of a representative experiment are shown. Control staining with isotype matched antibodies is shown as the gray overlay.
ment, CD⁴⁺CD₂⁸null cells produce high amounts of the proinflammatory cytokine IFN-γ. And finally, these unusual lymphocytes have a tendency to leave the circulation and infiltrate the tissue. We propose that a subset of patients, developing instability of otherwise benign and slowly progressing CAD, have systemic alterations in the immune system manifesting as the emergence of unusual T cells, possibly triggered by chronic microbial infection. The functional profile of expanding T cells, however, is biased against antibody-mediated host defense and favors tissue infiltration, cell-mediated cytotoxicity, and secretion of high amounts of IFN-γ. Migration of CD⁴⁺CD₂⁸null T cells into plaques, possibly initiated by the local deposition of microbial antigens, would elicit tissue destructive immune pathways such as IFN-γ–mediated activation of metalloproteinase-secreting macrophages, cytolysis of smooth muscle cells, and damage of endothelial cells. This model proposes a direct relationship between plaque instability and effector functions of CD⁴⁺CD₂⁸null T cells.

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