T-Cell–Mediated Lysis of Endothelial Cells in Acute Coronary Syndromes

Takako Nakajima, DDS; Stephanie Schulte; Kenneth J. Warrington, MD; Stephen L. Kopecky, MD; Robert L. Frye, MD; Jörg J. Goronzy, MD; Cornelia M. Weyand, MD

Background—CD4 T lymphocytes accumulate in unstable plaque. The direct and indirect involvement of these T cells in tissue injury and plaque instability is not understood.

Methods and Results—Gene profiling identified perforin, CD161, and members of the killer-cell immunoglobulin-like receptors as being differentially expressed in CD4+CD28null T cells, a T-cell subset that preferentially infiltrates unstable plaque. Frequencies of CD161+ and perforin-expressing CD4 T cells in peripheral blood were significantly increased in patients with unstable angina (UA). CD161 appeared on CD4+CD28null T cells after stimulation, suggesting spontaneous activation of circulating CD4 T cells in UA. Perforin-expressing CD4+ T-cell clones from patients with UA exhibited cytotoxic activity against human umbilical vein endothelial cells (HUVECs) in redirected cytotoxicity assays after T-cell receptor triggering and also after stimulation of major histocompatibility complex class I–recognizing killer-cell immunoglobulin-like receptors. HUVEC cytolysis was dependent on granule exocytosis, as demonstrated by the paralyzing effect of pretreating CD4+CD28null T cells with strontium. Incubation of HUVECs with C-reactive protein (CRP) increased HUVEC lysis in a dose-dependent fashion.

Conclusions—In patients with UA, CD4 T cells undergo a change in functional profile and acquire cytotoxic capability. Cytotoxic CD4 T cells effectively kill endothelial cells; CRP sensitizes endothelial cells to the cytotoxic process. We propose that T-cell–mediated endothelial cell injury is a novel pathway of tissue damage that contributes to plaque destabilization. The sensitizing effect of CRP suggests synergy between dysregulated T-cell function and acute phase proteins in acute coronary syndromes. (Circulation. 2002;105:570-575.)

Key Words: apoptosis • endothelium • lymphocytes • coronary disease • plaque

Acute coronary syndromes (ACS) are primarily caused by erosion or rupture of atherosclerotic plaque, which gives rise to a superimposed thrombosis.1–3 The tissue defect on the plaque surface is closely associated with the presence of inflammatory infiltrate, and the emerging paradigm proposes that immune cells are involved in mediating tissue damage.4–7 Mechanisms of tissue injury that disrupt plaque surface are not precisely understood, but release of digestive metalloproteinases has been suspected to be a primary pathway.8,9 Beyond local secretion of metalloproteinases, the immune system has pathways aimed at destroying cells and matrix that unintentionally can lead to tissue damage. In an ongoing immune response, the mechanism for cell or particle removal is dictated by the nature of the antigen and effector cells present at the site. T lymphocytes and macrophages are major cellular components in vulnerable plaque.10,11 We have demonstrated that plaque-infiltrating T lymphocytes include a subset of functionally distinct CD4 T cells that lack CD28 expression.12 CD4+CD28null clonotypes can be isolated from culprit lesions but not from stable plaque. These T cells are capable of releasing large amounts of interferon (IFN)-γ13,14 and are the dominant population of IFN-γ-producing cells in peripheral blood of patients with unstable angina (UA).15 One of their functions seems to be the activation of monocytes and macrophages.16 Monocytes from patients with UA display a molecular fingerprint of ongoing IFN-γ stimulation. CD4+CD28null T cells could have direct involvement in plaque rupture by releasing IFN-γ, thereby controlling plaque-infiltrating macrophages, but they are distinct from classic helper T cells in several additional aspects.17,18 CD4+CD28null T cells cannot form the transcriptional initiator complex that controls expression of the major costimulatory molecule CD28; however, they have gained expression of other receptor families, including killer-cell immunoglobulin-like receptors (KIRs).14,19,20 In the present study, we show that CD4+CD28null T cells from patients with UA have killer-cell functions and can cause target-cell death through the release of the pore-forming enzyme perforin. Endothelial cells are susceptible to this T-cell–mediated injury. In the presence of C-reactive protein (CRP), at concentrations frequently found in patients at risk for coronary events, susceptibility of endothelial cells to T-cell–mediated cytotox-
Demographic and Clinical Characteristics

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*Fisher exact test used.

icity was increased, suggesting that CRP sensitized endothelial cells to cytotoxicity.

**Methods**

**Study Population**

Twenty-four patients with stable angina (SA), 22 patients admitted to the Mayo Clinic Cardiac Monitoring Unit with a diagnosis of recent-onset UA (Braunwald class IIIB), and 20 healthy age-matched individuals were included in the study (Table). Inclusion and exclusion criteria have been described.15 The protocol was approved by the Mayo Clinic Institutional Review Board.

**Flow Cytometry**

Peripheral blood mononuclear cells (PBMCs) and T-cell clones were stained with anti-CD4, anti-CD28 (both Becton Dickinson), and anti-CD161 (BD-Pharmingen) monoclonal antibodies (mAb)15 and permeabilized with PBS containing 0.1% Triton X-100, followed by staining with anti-perforin (Ancell) or isotype control mAb (Beckman Coulter) mAb. T-cell clones were then incubated with HUVECs at varying effector-target ratios. Supernatants were harvested after 4 hours, and calcine release was measured using a CytoFluor (PerSeptive Biosystems) fluorescence plate reader. Results are expressed as percentage of maximal lysis.

To inhibit granule-dependent cytotoxicity, T-cell clones were incubated with 25 mmol/L strontium chloride (Sigma) for 18 hours before the cytotoxicity assay.22 To inhibit Fas-Fas ligand–mediated cytotoxicity, HUVECs were treated with the nonstimulatory anti-Fas mAb (M3; Immunex) for 1 hour before incubation with the T cells. HUVECs were then treated with recombinant human CRP (10 μg/mL and 50 μg/mL; Calbiochem) or tumor necrosis factor (TNF)-α (25 ng/mL; R&D Systems) for 18 hours.

**Statistical Analysis**

Mann-Whitney U-test, Student’s t test, χ² test, and Fisher’s exact test were used where appropriate (SigmaStat, SPSS).

**Results**

**Aberrant Gene Expression in CD4 T Cells From Patients With UA**

To identify genes specifically activated in CD4⁺CD28null T cells, gene profiling comparing CD28⁺ and CD28null T-cell clones was used. CD161, perforin, and KIRs were differentially expressed in the CD28-deficient variants.20,23,24

To examine whether patients with UA have CD161-expressing CD4 T cells, PBMCs were analyzed by flow cytometry (Figure 1A). Patients with UA had significantly higher frequencies of CD4⁺CD161⁺ T cells compared with patients with SA (P=0.003) and age-matched controls (P<0.001). Expression of CD161 is very low on resting CD4⁺CD28null T-cell clones and is induced after activation with IL-12 (Figure 1B). Increased frequencies of CD4⁺CD161⁺ T cells suggest that a CD4 T-cell subset is activated in UA.

The difference between age-matched controls and patients with UA was even more striking for CD4⁺perforin⁺ T cells (Figure 2). Perforin-containing CD4 T cells were 19-fold expanded in patients with UA (P<0.001). Notably, a consid-
erable proportion of CD4 T cells in patients with SA (median, 4.7%) contained perforin (P=0.001).

Diversity of Perforin-Producing CD4 T Cells in Patients With SA and UA

Aberrant expression of perforin in CD4 T cells from patients with UA, and to a lesser degree in patients with SA, raised the question of how the appearance of perforin is related to the loss of CD28 expression in these patients. To address this, we determined the phenotype of perforin-expressing CD4 T cells by 3-color flow cytometric analysis. The population of perforin-expressing CD4 T cells was heterogeneous. Most perforin-producing CD4 T cells in patients with UA had lost expression of CD28 (Figure 3A). Perforin was also detected in CD4 T cells that had markedly reduced cell-surface expression of CD28 (CD28low). Perforin expression was essentially absent in CD4+CD28high T cells. In patients with SA, most perforin-producing CD4 T cells were CD28low, and in contrast to patients with UA, perforin-containing CD28null cells were rare.

The relationship between perforin gain and CD28 loss is summarized in Figure 3B, which shows results from 22 patients with UA, 24 patients with SA, and 20 age-matched control individuals. In control individuals, perforin expression was essentially absent in CD4+ T cells. CD4+ T cells from patients with UA consisted of CD28+ and CD28null subsets; expansion of CD28-deficient cells was typical for this patient group (P=0.005 compared with patients with SA). In patients with SA, most perforin-producing CD4 T cells were CD28low, and in contrast to patients with UA, perforin-containing CD28null cells were rare.

Perforin-Expressing CD4 T Cells Can Induce Endothelial Cell Death

Having demonstrated that patients with UA possessed CD4 T cells that contained perforin, we wanted to test whether such T cells had cytolytic capability. CD4 T cells were cloned by limiting dilution from PBMCs of patients with UA. CD4+CD28null and CD4+CD28low T-cell clones were selected. Representative T-cell clones are shown in Figure 4A. Because endothelial cells have a critical role in atherosclerotic plaque rupture, HUVECs were chosen as targets. Results for 6 different T-cell clones are shown in Figure 4B. All CD4+CD28null T-cell clones efficiently lysed the endothelial cell targets in an anti-CD3 redirected cytolysis assay. At an effector-target ratio of only 10:1, 60% to 70% specific lysis was obtained. As expected, the T-cell clones failed to kill HUVECs when anti-CD3 mAb was not anchored on the target cell (not shown). All CD4+CD28null T-cell clones lacked cytolytic capability, even at high effector-target ratios.

To investigate differences in cytolytic activity between the 2 different kinds of T-cell clones, we compared amounts of perforin in the cytolytic CD4+CD28null and the noncytolytic CD4+CD28low clones. Although the noncytolytic clones unequivocally contained perforin, the concentrations were one tenth of those in cytolytic T cells (Figure 4C). From these data, we concluded that patients with UA carry a specialized subset of CD4+CD28null T cells that have gained ability to produce high levels of perforin and to mediate target cell injury, including the death of endothelial cells. An intermediate state with low expression of perforin and CD28, as seen in patients with SA, is not sufficient to transform CD4 T cells into killers.

CD4+ Cytolytic Cells Lyse Endothelial Cells Through the Granule Exocytosis Pathway

Cytotoxic T lymphocytes can lyse target cells by 2 independent pathways, the exocytosis of granules containing perforin...
and the interaction of Fas ligand on the killer cell with Fas on the target cell. \(25\) We examined whether perforin expression was the mechanism for target cell damage. Strontium ions (\(\text{Sr}^{2+}\)) induce degranulation of cytoplasmic granules and deplete perforin stores. \(22\) Pretreatment of CD4\(\text{CD}28\) null T-cell clones with \(\text{Sr}^{2+}\) inhibited target cell lysis by 50% (Figure 5). In these experiments, 25 mmol/L \(\text{Sr}^{2+}\) produced optimal inhibition of target cell destruction without affecting T-cell viability (data not shown). The effect of granule depletion on target cell lysis demonstrated that granule exocytosis was the major, but not only, pathway through which CD4 T cells mediated endothelial cell damage. Addition of a noncytotoxic anti-Fas mAb in the cytolytic assay did not additionally rescue endothelial cells from apoptotic cell death (data not shown), suggesting that residual cytotoxic activity is not attributable to Fas-Fas ligand interaction.

**CD4**\(\text{CD}28\) null T Cells Mediate Endothelial Cytotoxicity in the Absence of T-Cell Receptor Triggering

Experiments shown in Figure 4 document the cytotoxic potential of CD4 CD28 null T cells. However, cytotoxic activity in vivo would require the presentation of specific antigen on endothelial cells. CD4 CD28 null T cells did not kill HUVECs spontaneously, suggesting that the appropriate antigen was not expressed. In the absence of a known antigen, we explored whether nonspecific mechanisms could trigger cytotoxic activity. Prime candidate molecules were KIRs, identified by gene profiling as being selectively expressed on CD4 CD28 null T cells. Stimulatory KIRs have been implicated in controlling cytotoxic activities of natural killer cells. Stimulatory KIR2DS2 on the surface of CD4 CD28 null T-cell clones were cross-linked by mAb GL183. This signal was sufficient to induce effective lysis of HUVECs (Figure 6).

**CRP Sensitizes Endothelial Cells to T-Cell–Mediated Cytotoxicity**

Recently, CRP was described as having direct proinflammatory effects on human endothelial cells. \(26\) We examined whether CRP affected the responsiveness of endothelial cells to perforin-mediated cytotoxicity. Incubation with CRP consistently increased sensitivity of HUVECs to T-cell cytotoxicity. Results from two representative experiments are shown in Figure 7A. Cytotoxicity levels were higher when HUVECs were exposed to \(10\) \(\mu\)g/mL CRP, and cytotoxicity additionally increased with doses of \(50\) \(\mu\)g/mL CRP. In contrast, susceptibility of HUVECs to perforin/granzyme toxicity remained unaffected by TNF-\(\alpha\), a potent activator of endothelial cells. Figure 7B summarizes 7 independent experiments using 7 different clones derived from 3 individuals. Results are expressed as percent increase in cytotoxicity after CRP (\(P<0.001\)) and TNF-\(\alpha\) (\(P=0.85\) preincubation of endothelial cells.

**Figure 4.** Cytotoxicity of CD4 T-cell clones against endothelial cells. A, CD4 T cells were sorted from PBMCs of patients with UA and cloned. CD28<sup>+/-</sup> and CD28<sup>low</sup> clones were selected. Representative clones are shown. B, In a redirected cytotoxicity assay, using avidin-conjugated anti-CD3 immobilized on biotinylated target cells, CD4 CD28<sup>null</sup> T-cell clones (●) effectively lysed endothelial cells; CD28<sup>low</sup> T-cell clones (○) were unable to kill. C, CD4 T-cell clones were stained with anti-perforin and anti-CD28 mAb, and fluorescence profiles were analyzed by flow cytometry. Expression of perforin in noncytotoxic CD28<sup>null</sup> clones was several-fold lower than in cytotoxic CD28<sup>null</sup> clones.

**Figure 5.** CD4 T-cell cytotoxicity is mediated by perforin and not Fas. T-cell clones were preincubated with 25 \(\mu\)mol/L strontium for 18 hours and cocultured with calcine-loaded biotinylated HUVECs and avidin-conjugated anti-CD3 mAb in a 4-hour assay. Cytotoxicity was determined as in Figure 4. Results for 2 representative cytotoxic clones are shown. Strontium treatment diminished target cell lysis by 50%, equivalent to at least a 4-fold reduction in the effector-target ratio. Open bar indicates untreated; solid bar, treated.

**Figure 6.** Antigen-independent cytotoxicity of CD4 T-cell clones. CD4 CD28<sup>low</sup> T-cell clones expressing KIR2DS2 were tested for cytotoxicity in a redirected cytolysis assay using T-cell receptor triggering with anti-CD3 (○), anti-KIR2DS2 (●), and soluble anti-CD3 (●). Signaling through KIR2DS2 was sufficient to induce endothelial cell lysis.
Discussion

Patients with plaque instability have a remarkable subset of CD4 T cells with the ability to directly injure target cells. We demonstrate that CD4 T cells accumulated in unstable plaque have cytolytic machinery and, in response to triggering of their antigen receptor or aberrantly expressed KIRs, kill endothelial cells. Perforin-mediated damage of cell membranes may be a critical mechanism in tissue injury that leads to plaque rupture.

Gene profiling identified differential expression of perforin in a specialized subset of CD4 T cells, which led to the exploration of this effector molecule in UA. As expected, perforin-containing granules were infrequently detected in CD4 T cells from healthy donors. In patients with UA, these cells accounted for up to 30% of all CD4 T cells. Perforin-expressing cells had either completely lost the CD28 molecule, or surface expression of CD28 was 10-fold reduced. CD4\(^{CD28^{low}}\) T cells have recently come to our attention because they produce large amounts of IFN-\(\gamma\). Their frequency in peripheral blood seems to be a biological marker for plaque instability. Our experiments, however, documented that these T cells lacked cytolytic ability. One possible explanation is that their perforin levels were present in patients with UA. Functional tests, however, demonstrated that these T cells lacked cytolytic activity. In our experiments, human serum was not required to facilitate this unique effect, suggesting a direct binding of CRP to endothelial cells.

Data presented here emphasize that endothelial cells are susceptible to cytotoxicity of CD4\(^{CD28^{null}}\) T cells. It is conceivable that endothelial cell death could play an immediate role in plaque rupture. Obviously, lysis of macroendothelial cells covering the plaque could directly lead to tissue erosion and plaque rupture. For this to occur, cytolytic CD4 T cells must make contact with the endothelial cell layer of the macrovorum. It has been argued that flow conditions in medium-size arteries produce high shear stress and prevent adherence of lymphocytes to the endothelial surface. Instead, lymphocytes may use low-flow capillaries or venules for the transition from lumen into tissue. The site of entrance for intraplaque T cells has not been determined, but a dense network of capillaries is newly formed within the expanding plaque. Plaques prone to rupture are highly vascularized, and excessive neocapillarization has been considered to be characteristic of vulnerable plaques. These microvessels provide ideal conditions for macrophages and lymphocytes to invade tissue and likely also represent the primary target for activated cytotoxic CD4 T cells. Endothelial cell injury in these growing microcapillaries could induce tissue ischemia and could also lead to intraplaque hemorrhage. We propose a novel mechanism of plaque disruption caused by immune-mediated damage of neocapillaries.

Future efforts will focus on characterizing signals necessary to elicit T-cell cytotoxicity. Disease-relevant antigens, possibly derived from microbial organisms, could be presented on the endothelial cell surface, initiating the cytolytic machinery of T cells with corresponding antigen receptors.
Alternatively, CD4+CD28null T cells have acquired a mechanism of antigen-independent activation. Stimulation of KIR receptors by the appropriate HLA-C molecules on the endothelial cell may trigger cytotoxicity in vivo even in the absence of antigen.

**Acknowledgments**

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**References**

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