Activated Monocytes Induce Smooth Muscle Cell Death
Role of Macrophage Colony-Stimulating Factor and Cell Contact
Puvi N. Seshiah, MD; Dean J. Kereiakes, MD; Sanjay S. Vasudevan, MD; Neuza Lopes, MD; Baogen Y. Su, PhD; Nicholas A. Flavahan, PhD; Pascal J. Goldschmidt-Clermont, MD

Background—Plaque disruption is the inciting event for coronary thrombosis and acute coronary syndromes. Multiple factors influence plaque rupture, including the loss of vascular smooth muscle cells (VSMCs). We hypothesized that monocytes/macrophages (MMs) activated by macrophage colony-stimulating factor (M-CSF) are responsible for VSMC death.

Methods and Results—VSMC apoptosis was markedly increased in the presence of both M-CSF and MMs (58.8 ± 3.3%) compared with VSMCs plus M-CSF without MMs (15.7 ± 1.5%, P < 0.0005), VSMCs plus MMs without M-CSF (22.7 ± 3.7%, P ≤ 0.0001), or control VSMCs alone (13.2 ± 2.1%, P = 0.0001). MM cell contact was required for M-CSF–stimulated killing of VSMCs, and MMs displayed an M-CSF concentration-dependent killing effect. Abciximab binds Mac-1 (CD11b/CD18) on MMs. When added to VSMCs exposed to MMs and M-CSF, abciximab (7 μg/mL) significantly reduced VSMC apoptosis (19.1 ± 2.2%, P ≤ 0.0003). Therapeutic doses of tiroxifiban (0.35 μg/mL) and eptifibatide (5 μg/mL), which inhibit platelet glycoprotein (GP) IIb/IIIa but not Mac-1, did not block activated MM-induced VSMC apoptosis (65.0 ± 3.4% and 51.3 ± 2.5%, respectively). A recombinant anti–CD-18 antibody had an effect similar to that of abciximab (16.5 ± 0.4%).

Conclusions—These data suggest that monocytes and physiological concentrations of M-CSF trigger VSMC apoptosis. Abciximab and specific inhibitors of the Mac-1 receptor can antagonize this process. (Circulation. 2002;105:174-180.)

Key Words: apoptosis ■ muscle, smooth ■ cell adhesion molecules ■ plaque ■ inflammation

Atherosclerotic plaque rupture with superimposed thrombosis is the main cause of acute coronary syndromes (ACS). Therefore, to preserve event-free survival, the challenge is not simply to suppress atherogenesis but to suppress life-threatening plaque rupture and ulceration in existing plaques. Three major determinants of plaque rupture are the size and consistency of the atheromatous lipid core, fibrous cap thickness, and the intensity of inflammation and repair within the core. A lipid core occupying ≥40% of the plaque area and a thin, collagen-poor fibrous cap increase the risk of plaque rupture and thrombosis. Caps of ruptured plaques are weakened when the macrophage density increases. Furthermore, macrophage-rich plaques are found more often in the coronary arteries of patients with unstable angina and non–Q-wave myocardial infarction than in those of patients with stable angina.

Human leukocyte antigen-DR expression, a marker of macrophage activation, is found more often on macrophages of ruptured plaques. Moreover, fibrous caps that have ruptured not only have twice as many macrophages as unruptured fibrous caps but contain half as many smooth muscle cells, indicating that the ratio of vascular smooth muscle cells (VSMCs) to monocytes/macrophages (MMs) is altered markedly in ruptured plaques. The proportion of VSMCs undergoing apoptosis is increased significantly in unstable angina plaques versus stable angina. Macrophage colony-stimulating factor (M-CSF) is a hematopoietic growth factor supporting survival, proliferation, and differentiation of mononuclear cells. Recent studies suggest that M-CSF is the strongest predictor of ACS. Both VSMCs and endothelial cells produce M-CSF when exposed to a variety of stimuli. Macrophages within atheromatous vessels seem to be the primary target for M-CSF. M-CSF is also produced by macrophages themselves and on engagement of their FcγR receptors. Elevated C-reactive protein (CRP) levels have been shown to be associated with ACS.
levels reflect the intensity of an inflammatory process involving macrophages, T and B cells, immunoglobulin (Ig) G deposition, and M-CSF production. CRP has a proinflammatory effect on endothelial cells and VSMCs.18 M-CSF is affected by CRP and interleukin (IL)-1β levels, suggesting a relation between atherogenic cytokines and acute-phase proteins in patients with ACS.17

We hypothesized that the common denominator to plaque destabilization might be M-CSF, and we proposed that M-CSF may promote VSMC killing by activating MMs, causing the consequent loss of VSMCs within vulnerable plaques.

Methods

Reagents

Reagents were obtained from the following sources: recombinant M-CSF, anti–M-CSF mAb, clone-26730.11, and IL-1β (R&D Systems); abciximab (ReoPro), epifibatide, and aggrastat (hospital stores); cell culture media and supplements (Gibco-BRL and Clonetics); lymphoprep (Nycomed); anti–CD-14-FITC, clone-M5E2, and anti–CD-45-PE, clone-HI30, antibodies (Pharmingen); anti–α-smooth muscle actin (Sigma Chemicals); human monocyte isolation kit (Miltenyi Biotec); and pan-caspase inhibitor (ZVAD-fmk) (Calbiochem). Anti–CD-18 antibody was a generous gift from Hal Baron and Eugen Koren of Genentech.

Smooth Muscle Cell Culture

VSMCs (passage 3 to 7) were obtained from Clonetics (Bio Whitaker, Inc, Walkersville, Md) and from heart donors. The institutional review boards at Ohio State and Duke Universities approved all procedures involving human tissues. VSMC identity was confirmed by α-smooth muscle actin staining. VSMCs initially were grown in growth medium (SmGM2; Clonetics) with 5% serum, and at 60% to 70% confluence, the media was changed to a serum-free medium (50:50 of DMEM/F12 media with 5 mL of ITS, PSA, L-glutamine, and nonessential amino acids per 500 mL of solution). After 72-hour incubation, MM and VSMC co-cultures were stained live with the Vybrant Apoptosis kit (Molecular Probes), consisting of 2 nuclear stains, Hoechst 33342 (5 μg/mL) and propidium iodide (1 μg/mL). After incubation for 20 minutes at 37°C, coverslips were washed and the cell death index was estimated as previously described19 (apoptotic index = dead cells/[live cells + dead cells] × 100).

Statistical Analysis

Statistical analysis was performed with Systat 9 software from SPSS Software, Inc, and Prism 3.0 from Graphpad, Inc. Data were analyzed by ANOVA, and the Bonferroni multiple comparison post test was used to compare pairs of group mean ± SEM of individual experimental groups (groups compared are specified in each experiment). For statistical significance, a value of P ≤ 0.05 was considered significant.

Results

Monocytes Induce VSMC Apoptosis When Activated With M-CSF

The apoptosis rate of VSMCs exposed to both monocytes and M-CSF is increased markedly (results from 4 sets of experiments). Thus, the apoptotic index was 13.2 ± 2.1% for control VSMCs, 15.7 ± 1.5% for VSMCs cultured with M-CSF (100 ng/mL) but without monocytes, and 22.7 ± 3.7% for VSMCs co-cultured with monocytes without M-CSF. In contrast, VSMCs cultured with both M-CSF and monocytes display a markedly increased apoptotic index (58.8 ± 3.3%). The differences in apoptotic indexes between VSMCs cultured with monocytes and M-CSF versus all control conditions are significant (P < 0.0001) (Figure 1B). Similar results were obtained when VSMCs were grown in serum-supplemented media (5% serum), although the basal indexes of apoptosis were lower than in the absence of serum (Figure 1C).

It was confirmed that the death process was through apoptosis with the use of a pan-caspase inhibitor, ZVAD-fmk. When VSMCs were preincubated with ZVAD-fmk (10 μmol/L), apoptosis induced by co-culture with activated monocytes was completely abrogated. Thus, VSMCs cultured with monocytes and M-CSF exhibited an apoptotic index of 60.0 ± 2.1%, and the pan-caspase inhibitor ZVAD-fmk decreased this index to 15.4 ± 0.4% (P = 0.001), similar to that observed in smooth muscle cells cultured with monocytes alone (12.2 ± 1.6%) (Figure 2).

Effect of M-CSF Is Concentration-Dependent and Requires Direct Contact Between Monocytes and Smooth Muscle Cells

We tested concentrations of M-CSF ranging from 0.1 ng/mL to 100 ng/mL. Maximum VSMC apoptosis was obtained with

Abciximab, aggrastat, and epifibatide, 3 established parenteral glycoprotein (GP) IIb/IIIa blockers, were added to MM and VSMC co-cultures before exposure to M-CSF. Controls were used for VSMCs with abciximab, aggrastat, and epifibatide separately, with or without M-CSF. All experiments were done in triplicate.

Experiments were done with the use of recombinant human IL-1β (100 to 1000 pg/mL) in place of M-CSF. Anti–human M-CSF neutralizing antibody (1 μg/mL) was added along with IL-1β, and the VSMCs plus monocytes were co-cultured for 72 hours. Isotype control, IgG2a (clone 20102.1), was used for VSMCs cultured with monocytes and IL-1β.
50 ng/mL and 100 ng/mL, whereas half-maximal killing occurs at a physiological dose of 5 ng/mL (Figure 3). Our data are consistent with a process in which MMs, on engagement of their M-CSF receptor, can trigger the cell death program of VSMCs. Next, we sought to define whether activation of the cell death program of VSMCs required physical contact between VSMCs and activated MMs. Monocytes were separated from the VSMCs with a porous insert (3-μm-diameter pores), and when stimulated with M-CSF (100 ng/mL) were unable to induce VSMC apoptosis (21.0 ± 1.0%) compared with VSMCs co-cultured and in direct contact with activated monocytes (66.3 ± 1.8%) (P ≤ 0.001) (Figure 3).

**Activation of Monocyte Killing Activity Toward VSMCs Is Specific to M-CSF**

To test if the activation of the observed monocyte-induced killing is specific to M-CSF, we studied another common monocyte-activating cytokine, IL-1. IL-1 is known to induce monocyte adherence. We repeated co-culture experiments by using IL-1 instead of M-CSF. IL-1 at physiological concentrations (100 pg/mL) did not induce monocyte-induced VSMC apoptosis. VSMCs cultured with monocytes and IL-1 (100 pg/mL) exhibited an apoptotic index of 10.5 ± 2.4%, which was similar to that of control VSMCs and VSMCs cultured with unactivated monocytes, whereas M-CSF activation of monocytes significantly increased VSMC apoptosis (60.0 ± 3.0%).
Markedly higher doses of IL-1 (500 pg/mL and 1 ng/mL) did cause increased monocyte-induced killing of VSMCs (59.5 ± 3.5% and 59.0 ± 3.8%, respectively) (Figure 4). However, monocyte killing of VSMCs in response to high doses of IL-1 was dependent on the endogenous production of M-CSF induced by IL-1. Thus, when VSMC killing by monocytes was triggered by IL-1 (500 pg/mL and 1000 pg/mL), such killing was completely abrogated in the presence of 1 μg/mL of anti-M-CSF–blocking antibody (17.0 ± 2.1% and 13.6 ± 1.9%, respectively; P < 0.001 and P = 0.001, respectively) (Figure 4). A nonspecific isotype-matched control antibody, IgG2a (1 μg/mL), was used and did not block IL-1–induced (1000 pg/mL) VSMC apoptosis (55.0 ± 2.0%) (Figure 4). These experiments, taken together, indicate that M-CSF could function as a rate-limiting cytokine in the process of monocyte killing of VSMCs. IL-1, even at physiological levels, has been shown to induce the production of M-CSF by VSMCs and monocytes.10,11,18

Chimeric Monovalent Antibody That Cross-Reacts With Mac-1, Abciximab, Blocks M-CSF–Induced Monocyte Killing of VSMCs

Engagement of the α6β2 receptor (CD11b/CD18 or Mac-1) plays a central role in multiple activities of macrophages and granulocytes in inflammatory processes, including atherosclerosis. The chimeric monovalent and humanized monoclonal antibody Fab fragment abciximab binds with high affinity to the activated conformation of Mac-1.20 Abciximab at therapeutic concentration (7 μg/mL) was added to VSMCs alone or to co-cultures of VSMCs and monocytes, with and without M-CSF. A significant decrease in the apoptotic index of VSMCs occurs when abciximab is added to VSMCs co-cultured with monocytes in the presence of M-CSF (P < 0.0003, relative to identical conditions except for the absence of abciximab) (Figure 5). Abciximab also binds α6β3 (GP IIb/IIIa or fibrinogen receptor) and the activated conformation of the α6β2 (the vitronectin receptor).21 Therefore, we tested the possibility that other GP IIb/IIIa blockers, such as eptifibatide and tirofiban, also could reduce the pro-apoptotic effect of M-CSF–activated macrophages on VSMCs. These inhibitors are highly efficient at blocking...
platelet aggregation but, unlike abciximab, do not interfere with Mac-1 binding. Neither tirofiban nor eptifibatide detectably inhibited the M-CSF–induced monocyte killing of VSMCs. The cell death index in the presence of tirofiban (therapeutic concentration, 0.35 μg/mL) was 65.0 ± 3.4% and for eptifibatide (therapeutic concentration, 5 μg/mL) was 51.3 ± 2.2% (Figure 5), not significantly different from that of VSMCs exposed to M-CSF–activated MMs (51.9 ± 1.6%, P = 0.76). However, the difference between the apoptotic indexes obtained in the presence of tirofiban or eptifibatide versus abciximab (19.1 ± 2.3%) was significant (P = 0.0006 and P ≤ 0.001, respectively) (Figure 5).

We further tested the contribution of Mac-1 engagement for monocyte-induced killing of VSMCs by using a human recombinant anti–CD-18 antibody. This anti–CD-18 antibody at a dose of 3.5 μg/mL was able to inhibit M-CSF–induced monocyte killing (16.5 ± 0.4%) when compared with M-CSF–activated monocytes co-cultured with VSMCs in the absence of anti–CD-18 (51.9 ± 1.8%) (P = 0.00004). This inhibition was similar to that observed with abciximab (19.1 ± 2.2%) (Figure 5). These results obtained with a recombinant anti–CD-18 antibody further support the requirement of Mac-1–mediated cell-to-cell contact between M-CSF–activated monocytes and VSMCs to allow for execution of the death pathway that results in VSMC killing.

**Discussion**

Plaque disruption is the major inciting event for ACS. The factors that determine plaque vulnerability to rupture include matrix degradation by MMs, decreased VSMC cellularity, and increased MM representation within the atherosclerotic plaque. Loss of VSMCs is probably due to increased apoptosis. The mechanism for increased macrophage accumulation within threatening plaques has not yet been established. In the absence of supporting factors such as M-CSF, monocytes are genetically engineered to undergo programmed cell death within 24 to 48 hours after their release from the bone marrow. Thus, M-CSF and perhaps other factors such as the Fc portion of immunoglobulins within the vessel wall could explain the increase in the MM survival and presence within the plaque.

We have demonstrated a novel mechanism for VSMC apoptosis that is induced by monocytes when activated with M-CSF. M-CSF thus plays a central role in activating monocytes and increasing their proliferation. These activated monocytes, in turn, could cause VSMC loss and thereby weaken the coronary plaque.
M-CSF has emerged as one of the strongest risk factors for adverse outcomes in patients with stable angina. It is the only GP IIb/IIIa blocker that is addressed through direct comparative trials between abciximab and other GP IIb/IIIa blockers. However, their data differ from ours: In their study, monocytes were allowed to mature into macrophages for 6 days before the VSMCs were added to the wells containing macrophages, and they observed maximal killing after 6 to 8 days in culture. In our co-culture, when freshly isolated monocytes were added directly to the VSMCs and activated with M-CSF, maximal killing was observed in 2 to 3 days. Boyle et al did not study the effect of M-CSF on monocyte killing, and it can be surmised that the monocytes that they had used must have been activated. We also show that the physiological control of monocyte-activated killing by M-CSF, although probably present to avoid uncontrolled monocyte-induced destruction of vascular tissues, may represent a final common pathway for the cytokine contribution to plaque destabilization.

Abciximab is believed to be primarily a platelet GP IIb/IIIa receptor blocker; however, studies have determined that abciximab binds also to both activated αvβ3 and αvβ1 receptors. Because αvβ3 receptors are present on granulocytes and monocytes, the latter being implicated in atherosclerosis and plaque disruption, we hypothesized that abciximab could potentially inhibit the accelerated smooth muscle cell death induced by activated monocytes.

We show that abciximab can inhibit the killing of VSMCs by M-CSF–activated MMs. This effect could be mediated by Mac-1 binding of abciximab on the surface of monocytes, thereby preventing the adhesion of monocytes to ligands such as ICAM-1 displayed by VSMCs. The effect of an anti–CD-18–blocking antibody effect that was similar to that of abciximab on VSMC killing by M-CSF–activated macrophages supports the participation of the Mac-1 receptor in the process.

The effect of abciximab on circulating inflammatory markers after percutaneous coronary intervention has been studied. The use of abciximab reduced levels of inflammatory cytokines by 30% to 100%. This effect was attributed to the cross-reactivity of abciximab with αvβ3 (vitronectin) and Mac-1 receptors and seemed to be specific for abciximab only. Furthermore, abciximab has been shown to reduce long-term mortality rates in patients after percutaneous coronary intervention. It is the only GP IIb/IIIa blocker that was shown to have a significant effect on long-term mortality rates. However, it would be speculative to suggest that such a differentiating effect might be attributed entirely to the Mac-1–blocking property of abciximab. The clinical relevance of alternative receptor effects of abciximab can only be addressed through direct comparative trials between abciximab and other GP IIb/IIIa blockers.

Acknowledgments

This study was supported by National Institutes of Health grants GM-53236, HL-56091, and HL-52315 to Dr Goldschmidt-Clermont. Dr Goldschmidt-Clermont is an Established Investigator of the American Heart Association.

References


Activated Monocytes Induce Smooth Muscle Cell Death: Role of Macrophage Colony-Stimulating Factor and Cell Contact
Puvi N. Seshiah, Dean J. Kereiakes, Sanjay S. Vasudevan, Neuza Lopes, Baogen Y. Su, Nicholas A. Flavahan and Pascal J. Goldschmidt-Clermont

_Circulation_. 2002;105:174-180
doi: 10.1161/hc0202.102248
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/2/174

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/