Receptors for Maleylated Proteins Regulate Secretion of Neutral Proteases by Murine Macrophages

Abstract. *Receptors for maleylated or acetylated proteins as well as for α-2-macroglobulin–protease complexes on macrophages serve as scavengers by mediating the uptake of macromolecules from the extracellular compartment. Described in this report is a novel function of these receptors on macrophages: regulation of neutral protease secretion. The binding of maleylated bovine serum albumin to macrophages triggered secretion of three neutral proteases: neutral caseinases, plasminogen activator, and cytolytic proteinase. Release of acid phosphatase, however, was not induced. An important biological consequence of protease secretion by macrophages, tumor cytolysis, was also triggered by engagement of the receptor for maleylated bovine serum albumin. By contrast, the binding of α-2-macroglobulin–protease complexes to the macrophages suppressed secretion of all three proteases. Thus two receptors heretofore believed to serve principally as scavengers also regulate secretory functions of macrophages.*

An important function of the mononuclear phagocyte system is uptake of extracellular macromolecules by way of various scavenger pathways (*1*). For example, macrophages (Mϕ) have receptors that can bind and internalize certain lipophilic apoproteins, maleylated or acetylated proteins, α-2-macroglobulin (α2M) complexed to proteases, and fucose or mannose terminal glycoproteins (*1–4*). Such receptors are believed to be important in homeostasis because they facilitate uptake of various macromolecules, such as lysosomal enzymes, antigen-antibody complexes, protease-protease inhibitor complexes, low-density lipoproteins (LDL), and modified-LDL (*1–4*).

Differences in receptor expression on different populations of macrophages would not be surprising, because Mϕ in different stages of activation vary widely in their physical and biochemical properties (*5*). For example, Mϕ elicited by sterile inflammatory agents [such as thioglycollate broth (TG)] differ from Mϕ obtained from bacillus Calmette-Guerin (BCG)–infected mice in many respects, including the increased secretion of certain neutral proteases and the expression of tumoricidal activity (*6*). Indeed, activated murine Mϕ have a significantly decreased binding capacity for mannose-terminal glycoproteins [for example, ~ 3.2×10^4 receptors per Mϕ for TG-elicited Mϕ as opposed to ~ 3.5×10^3 receptors per Mϕ for BCG-activated Mϕ (*7, 8*)]. In addition, receptors for LDL have recently been reported to be diminished on human Mϕ exposed to lymphokine, a potent activator of Mϕ (*9*). The number and affinity of receptors for maleylated bovine serum albumin (Mal-BSA), α-2-macroglobulin–trypsin complexes (α2M-T), and lactoferrin on activated Mϕ, however, are similar when compared to Mϕ from sites of nonimmunologically mediated inflammation (*7*).

Since Mϕ can secrete neutral proteases copiously, including a novel neutral protease (cytolytic factor) essential for tumor cytolysis, and since such secretion is closely regulated (*6, 10*), we wondered if these scavenger receptors might regulate the secretion of neutral proteases from Mϕ. To test this hypothesis, we used Mϕ from animals that had been primed by infection with BCG (*11*). These primed Mϕ had a low basal level of secretion, but after receipt of a second signal—that is, exposure to Mal-BSA—they showed a dose-dependent increase in secretion of neutral caseinases, plasminogen activator, and cytolytic factor (Fig. 1, A to C). The increases in secretion of the three proteases mediated by Mal-BSA were of different magnitudes; this is not surprising since they are separate enzymes and since it is known that plasminogen activator and cytolytic factor are not secreted concomitantly (*12, 13*).

Although the greatest increases in secretion of the three proteases were observed when basal secretion was low (that is, when the Mϕ were primed), definite but smaller increases in protease secretion in response to Mal-BSA were observed when basal secretion was high (that is, when the primed Mϕ were activated) (data not shown). In contrast to enhanced secretion of neutral proteases, no increase in the secretion of one lysosomal acid hydrolase, acid phosphatase, was noted at any concentration of Mal-BSA tested (release of less than 10 nmole of acid phosphatase per milligram of Mϕ protein at all concentrations of Mal-BSA; intracellular content of 1700 nmole per milligram of Mϕ protein). Secretion of neutral proteases from Mϕ was not

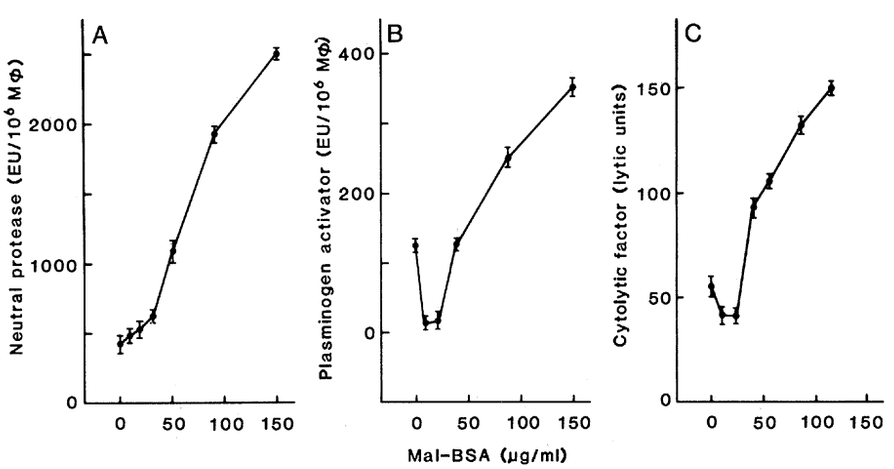

Fig. 1. Induction of secretion of the total neutral caseinases, plasminogen activator, and cytolytic factor by Mal-BSA. The Mϕ (2.5×10^5 per square centimeter) were prepared from animals primed with BCG according to a schedule that elicits Mϕ that are able to kill tumor cells when they are exposed to nanogram concentrations of endotoxin. These primed Mϕ were incubated for 4 or 48 hours in the absence of serum in a medium containing various amounts of Mal-BSA (*11*). The supernatants were collected in Siliclad tubes and centrifuged to remove nonadherent cells. Proteolytic activity was determined in 48-hour supernatants by using [^{3}H]casein as the substrate (*6*). The [^{3}H]casein, 0.1*M* phosphate buffer (*p*H 7.4), and Mϕ supernatant were incubated for 16 hours in microfuge tubes. The reaction was stopped with an excess of cold casein and proteins were precipitated with 6 percent trichloroacetic acid. The samples were then centrifuged and a portion of the supernatant containing ^{3}H-labeled acyl peptides (soluble in trichloroacetic acid) was added to a scintillation mixture and counted in a scintillation spectrometer. Acid phosphatase was quantified on similar samples (*24*). Plasminogen activator was measured in 48-hour supernatants by means of a similar assay (*25*), in the presence of human plasminogen. Released radioactivity in the control tubes (buffer and [^{3}H]casein) was subtracted from that released in the test samples. One unit of enzyme activity [(*EU*) for both neutral protease and plasminogen activator] is defined as 100 net counts per minute released per hour. Cytolytic factor was assayed in 4-hour supernatants as described previously (*6*) and lytic units were calculated as described (*12*). The results of three other experiments were similar.

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altered by triggering with various concentrations of mannose-BSA or lactoferrin (data not shown). In addition to its affinity for maleylated proteins and acetylated LDL, the Mϕ receptor for Mal-BSA binds a variety of polyanions, including the naturally occurring, fucose-containing, algal polysaccharide fucoidin (*3, 7*). Indeed, the receptor exhibits a particularly high affinity for fucoidin (*3, 7*). When fucoidin was incubated with BCG-activated Mϕ, slight increases in secretion of total neutral proteases and large increases in secretion of plasminogen activator and cytolytic factor were observed; these effects were dose-dependent (data not shown).

A critical test of the consequence of altered protease secretion would be to examine the cytolytic potential of primed Mϕ in response to the ligands. Primed Mϕ are not spontaneously cytolytic, but require exposure to a second signal, such as traces of endotoxin, to become fully cytolytic (*11, 14–16*). Primed Mϕ can complete the initial stage in cytolysis (that is, target binding) but cannot complete the second stage (that is, target injury mediated by secretion of lytic effectors such as cytolytic factor), until they are exposed to a second signal that triggers release of cytolytic factor (*11, 17*). When we examined BCG-primed Mϕ (*16*), we found that fucoidin, which is cleared by way of the receptor for Mal-BSA (*7*), triggered release of cytolytic factor (Fig. 2A) and completion of cytolysis (Fig. 2B). We next examined BCG-primed Mϕ from C3H/HeJ mice, which are genetically deficient in their ability to respond to endotoxin. Primed Mϕ from such mice are not able to complete cytolysis (*18*) and do not secrete cytolytic factor (*13*). Exposure of BCG-elicited Mϕ from C3H/HeJ mice to fucoidin, though not to endotoxin, triggered release of cytolytic factor (Fig. 2C) and completion of cytolysis (Fig. 2D). Thus, one critical biological function mediated by proteases can be shown to be regulated by the receptor for maleylated proteins. Thus the BCG-elicited Mϕ from C3H/HeJ mice are prepared for secretion of cytolytic factor and do so when confronted with a second signal.

We wondered whether protease secretion could be shut off by engagement of any of these receptors. We found that engagement of the receptor for α2M-T by its specific ligand resulted in decreased secretion of neutral proteases by BCG-activated Mϕ (Fig. 3). Decreases in secretion were also observed when Mϕ were incubated with increasing concentrations of native α2M (data not shown). We attribute this latter result to the formation of protease-inhibitor complexes by the combination of native α2M with proteases secreted from the Mϕ. The absolute degree of suppression was greatest when basal secretion was maximum (that is, when activated Mϕ were tested), but suppression was observed when basal secretion was low (that is, when primed Mϕ were tested) (data not shown). In addition, α2M-T was able to decrease tumor cytolysis in a dose-dependent fashion (data not shown).

Macrophage receptors, which recognize and mediate internalization of acetylated or maleylated proteins such as Mal-BSA (*1, 3*), are biologically important because LDL are strongly implicated in the pathogenesis of atherosclerosis and because Mϕ are thought to be a precursor of the foam cell, a prominent feature of the atherosclerotic lesion (*19, 20*). Although Mϕ clear native LDL poorly, incubation of Mϕ with an altered form of LDL can lead to facilitated uptake of the LDL, accumulation of lipids in the Mϕ, and development of Mϕ with the general appearance of foam cells (*21*). Our data thus demonstrate that both Mal-BSA and fucoidin induce augmented secretion of neutral proteases by

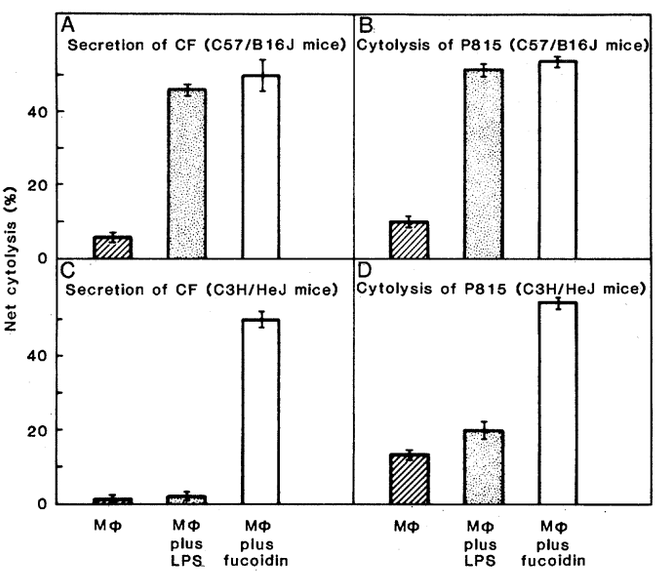

Fig. 2. Induction of tumoricidal activity by engagement of the Mal-BSA receptor with fucoidin. The BCG-primed Mϕ, from C57/B16 (A and B) or C3H/HeJ (C and D) mice, were elicited and plated as described in Fig. 1. They were allowed to adhere for 2 hours before being washed. P815 mastocytoma tumor targets labeled with ^{51}Cr were added (3×10^4 per 6-millimeter microtiter well) and incubated for 16 hours alone, with endotoxin (lipopolysaccharide, *LPS*; 10 ng/ml), or with fucoidin (100 μg/ml) (*11*). The supernatants (100 μl) were removed and the released radioactivity determined in a gamma counter. The percentage net cytolysis is defined as $(E - S)/M$, where E is the radioactivity (counts per minute) in the experimental samples, S is the radioactivity released spontaneously, and M is the maximum radioactivity released from frozen and thawed cells. The results of three other experiments were similar.

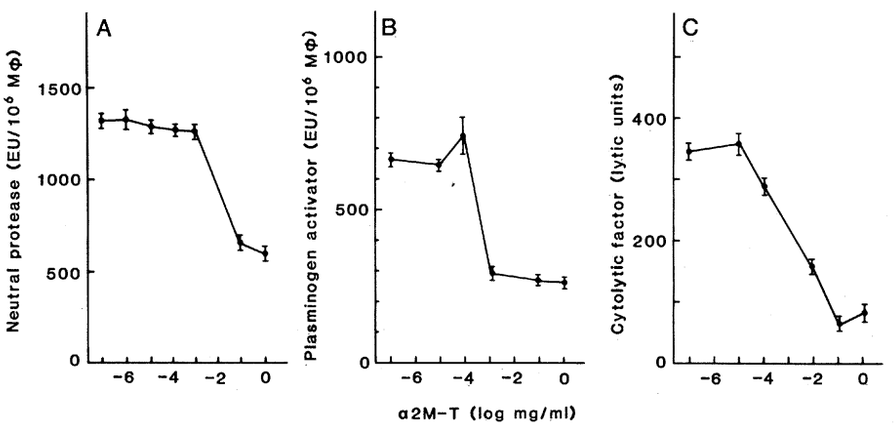

Fig. 3. Decreased secretion of neutral proteases by Mϕ cultured with α2M-T. The Mϕ (2.5×10^5 per square centimeter), were elicited from animals injected with BCG according to a regimen designed to activate the Mϕ so that they would be capable of spontaneous tumor cytolysis and secretion of high levels of neutral protease (*6, 11*). These Mϕ were incubated for 3 hours at 37°C in tissue culture medium with various amounts of α2M-T. The cultures were washed three times and incubated again in the absence of serum for 4 hours for determination of cytolytic factor or for 48 hours for determination of neutral protease and plasminogen activator. The results of three other experiments were similar.

the Mϕ. Given the recent demonstration of biological conversion of native LDL to a form recognized, taken up, and degraded by Mϕ by way of the Mal-BSA receptor, one potential source of this ligand in vivo has already been identified (*22*). The relevance of the novel function for this receptor remains to be established. The increased secretion of neutral proteases conceivably could contribute to the formation of atheromatous lesions, either directly by causing tissue damage or indirectly by mediating prolonged inflammation. Alternatively, increased fibrinolysis mediated by augmented secretion of plasminogen activator, a potent lytic agent of thrombi, could retard atherogenesis (*23*). We have already observed one biological consequence of the interaction of maleylated-acetylated proteins with Mϕ—the induction of tumor cytolysis. The full range of biological responses induced by engagement of scavenger receptors on a variety of cells remains to be established.

WILLIAM J. JOHNSON
Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710
SALVATORE V. PIZZO
MICHAEL J. IMBER
Departments of Pathology and *Biochemistry, Duke University Medical Center*
DOLPH O. ADAMS
Departments of Pathology and *Microbiology-Immunology, Duke University Medical Center*

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Coronary Vascular Reactivity After Acute Myocardial Ischemia

Abstract. *Exogenous thrombin produced a biphasic response (a potent dose-related vasodilatation followed by vasoconstriction) in nonischemic canine coronary arteries. The vasodilatation was not blocked by propranolol, atropine, or indomethacin, but was completely blocked by heparin or denudation of the intimal endothelial cells. A similar loss of vasodilating response to thrombin occurred in ischemic coronary arteries with a concomitant enhancement of vasoconstriction. This study indicates that altered responses to thrombin in coronary arteries with damaged endothelium may play an important role in the pathogenesis of coronary vasospasm.*

Much of the recent interest in the pathogenesis of coronary artery vasospasm and its role in the development of acute myocardial infarction (*1, 2*) stems from a report by Prinzmetal (*3*) that a variant form of angina pectoris may be precipitated from coronary vasospasm without detectable severe coronary obstruction. Many investigators have attempted to identify the mechanism as well as the factors that provoke vasospasm. Several naturally occurring spasmogenic factors, such as noradrenaline, serotonin, and thromboxane A_2 and other derivatives of prostaglandins, have been postulated to play an important role in the modulation of coronary arterial tone (*1, 4, 5*). To date, however, none of these has been shown to be the primary factor in the initiation or maintenance of coronary vasospasm. Recently, thrombin, a naturally occurring factor that is readily activated during vascular injury, was shown to be a potent vasoconstrictor and to be responsible for the development of the cerebral vasospasm (*6*). To determine whether thrombin plays a similar modulating role in the coronary circulation, I studied the effects of thrombin on the coronary vascular reactivity of ischemic and nonischemic canine coronary arteries.

Experimental myocardial infarction was induced in male mongrel dogs (23 to 30 kg) by a temporary coronary occlusion and reperfusion model as described (*7*). The development, as well as the extent, of myocardial ischemic injury during the 90-minute coronary occlusion and 1 to 2 hours of reperfusion was estimated by recording the changes in the epicardial electrocardiogram and was subsequently confirmed by staining of the ventricular muscle with nitro-blue-tetrazolium and examining the loss of intracellular dehydrogenases (*7*). This model of myocardial ischemia generally results in a transmural myocardial necrosis that constitutes approximately 20 to 30 percent of the total left ventricular mass. After completion of the temporary myocardial ischemia, the heart was excised, and the epicardial coronary arteries, with an outside diameter of approximately 1.5 to 2.5 mm, were isolated from the ischemic (left anterior descending) and the nonischemic (left circumflex) coronary arteries. The coronary vessels were cleaned of adhering fats and adventitial tissues, and ring preparations of these vessels were prepared as described (*8*). All vessels were passively stretched to generate a resting or basal tension of 2.0 g and were allowed to equilibrate in 30 ml of Krebs-Henseleit solution at 37°C with continuous oxygenation (95 percent O_2 and 5 percent CO_2) for 60 to 90 minutes before the start of the experiment (*9*).

The effects of purified bovine plasma thrombin (Sigma) on the contractile activity of the nonischemic coronary arteries are shown in Fig. 1. In contrast to a potent vasoconstrictory effect of thrombin as reported in isolated cerebral arteries (*6*), the addition of 5.0 units of thrombin to isolated canine coronary arteries resulted in an immediate vasodilatation followed by gradual recovery of the basal tone. The vasodilating response to thrombin was directly proportional to the resting or basal tone, as evidenced by the proportionally greater dilation when the basal tone was increased from 2.0 to 4.0 g (Fig. 1B). Similarly, addition of thrombin (0.5 to 5.0 units) to coronary arteries previously contracted with either 20 m*M* KCl (Fig. 1D) or 3×10^{-7} *M*

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