TNF-Induced Shedding of TNF Receptors in Human Polymorphonuclear Leukocytes: Role of the 55-kDa TNF Receptor and Involvement of a Membrane-Bound and Non-Matrix Metalloproteinase[1]

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A down-modulation of both the 55-kDa (TNF-R55) and the 75-kDa (TNF-R75) TNF receptors is observed in neutrophils exposed to a variety of stimuli. Proteolytic cleavage of the extracellular region of both receptors (shedding) and, with TNF, internalization of TNF-R55 and shedding of TNF-R75 are the proposed mechanisms. We have characterized the TNF-induced shedding of TNF receptors in neutrophils and determined the nature of the involved proteinase. Neutrophils exposed to TNF release both TNF receptors. A release of TNF receptors comparable to that observed with TNF was induced with TNF-R55-specific reagents (mAbs and a mutant of TNF) but not with the corresponding TNF-R75-specific reagents. A hydroxamic acid compound (KB8301) almost completely inhibited shedding of TNF-R55 and to a lesser degree shedding of TNF-R75. KB8301 also inhibited FMLP-induced shedding to a similar extent. Shedding was also inhibited by 1,10-phenanthroline, but this effect was considered nonspecific as the compound, at variance with KB8301, almost completely inhibited TNF and FMLP-induced PMN activation. Diisopropylfluorophosphate partially inhibited shedding of TNF-R75, suggesting the contribution of a serine proteinase to the release of this receptor. Shedding activity was not affected by matrix metalloproteinases inhibitors nor was it released in the supernatants of FMLP-stimulated neutrophils. These results suggest that TNF induces release of its receptors, that such a release is mediated via TNF-R55, and that a membrane-bound and non-matrix metalloproteinase is involved in the process. The possibility that ADAM-17, which we show to be expressed in neutrophils, might be the involved proteinase is discussed. ***The Journal of Immunology*****, 2000, 165: 2165–2172.**

Polymorphonuclear leukocytes (PMN)[3] are of crucial importance for host antibacterial defense but, when excessively or improperly stimulated, they become important effectors of tissue damage (1). The functions of these cells are modulated by a variety of mediators, including cytokines. TNF is recognized as one of the most potent of them; its effects range from induction of adhesion molecules on endothelial cells that favor leukocyte recruitment in the extravascular compartment (2) to PMN activation. TNF primes PMN for enhanced phagocytosis and respiratory burst activity (3, 4), activates adherence and spreading (5, 6), and triggers the release of reactive oxygen species (7, 8) and degranulation (9).

Two distinct species of surface receptors, one of 55 kDa (TNF-R55) and one of 75 kDa (TNF-R75), mediate biological responses to TNF (10). It is well established that the surface expression of both receptors decreases considerably when PMN are exposed to TNF and various agents including FMLP, the tumor promoter PMA, the Ca^{2+} ionofore A23187, endotoxin, and GM-CSF (11–13). The decrease in receptor expression occurs via a process known as shedding, whereby the extracellular portion of the receptors is proteolytically cleaved and released in a soluble form that maintains the ability to bind TNF (11, 14). With TNF, receptor down-modulation has been shown to occur via internalization of TNF-R55 and shedding of TNF-R75 (14). Receptor down-modulation may be a mechanism for PMN self-protection from excessive TNF-induced activation in the circulation, during their emigration into the tissues or at inflammatory sites. Indeed, it has been shown that preincubation of PMN with TNF or other stimuli results in PMN deactivation toward subsequent stimulation by TNF, and that this deactivation correlates to a reduction in TNF surface receptors (12).

A TNF-specific deactivation of granulocytes that, similar to in vitro observations, correlated with a decrease in receptor expression, has also been observed in vivo in subjects infused with TNF, in PMN from patients exposed to activated complement during hemodialysis (15), and PMN isolated from aseptic exudates (16). The released receptors, in turn, depending on their concentration relative to that of TNF, have been proposed either to compete with

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[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocytes; TNF-R55, 55-kDa TNF receptor; TNF-R75, 75-kDa TNF receptor; ADAM, a disintegrin and metalloproteinase; TACE, TNF-α converting enzyme; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMPs; DFP, diisopropylfluorophosphate; KB8301, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphthyl)alanine-*N*-methylamide; KB8845, (4-hydroxy-2*R*-isobutyl-3*S*-methylsuccinyl)-L-3-(5,6,7,8-tetrahydro-1-naphthyl)alanine-*N*-methylamide; KB7785, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-phenylglycine-*N*-methylamide; ^{125}I-TNF, ^{125}I-labeled TNF.

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their membrane-bound counterparts and limit TNF bioavailability (17) or to stabilize the ligand thus enhancing TNF signaling (18).

Because PMN rapidly and extensively release their TNF receptors after adherence or exposure to various agonists (11–13), it has been suggested that these cells might be a significant source of increased circulating TNF receptors observed in physiologic and pathologic conditions such as exercise (19), experimental endotoxemia (20), a clinical model of postoperative sepsis (21), and high-dose TNF in isolated limb perfusion (22). In light of these observations, the comprehension of the mechanisms that regulate shedding in PMN might provide the basis for experimental approaches aimed at modulating systemic responses to TNF.

We undertook this study to characterize the TNF-induced release of TNF receptors and to determine the nature of the proteinase(s) involved in the process. Our data show that TNF induces the release of both receptors, that such a release is mediated by TNF-R55, and that a metalloproteinase is involved in the process. The finding that the releasing activity is cell associated and unaffected by inhibitors of matrix metalloproteinases (MMP) suggests that it may belong to the ADAM (a disintegrin and metalloproteinase) family of membrane-bound metalloproteinases. On the basis of the available evidence, the possibility that TACE (TNF-α converting enzyme) might be the TNF receptors "sheddase" of stimulated PMN is discussed.

Materials and Methods

Reagents

BSA fraction V, FMLP, and streptavidin-R-PE conjugate were from Sigma (St. Louis, MO); Percoll was obtained from Pharmacia (Uppsala, Sweden). Immunoassays for human soluble TNF-R55 and TNF-R75 were performed using ELISA kits obtained from R&D Systems (Minneapolis, MN). All solutions were made in endotoxin-free water for clinical use.

Proteinases inhibitors

α_1-Antitrypsin, chymostatin, 1,10-phenanthroline, phosphoramidon, leupeptin, thiorphan, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Sigma; diisopropylfluorophosphate (DFP) was purchased from Acros Organics (Fair Lawn, NJ); [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphthyl)alanine-*N*-methylamide (KB8301) (23), (4-hydroxy-2*R*-isobutyl-3*S*-methylsuccinyl)-L-3-(5,6,7,8-tetrahydro-1-naphthyl)alanine-*N*-methylamide (KB8845), and [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-phenylglycine-*N*-methylamide (KB7785) (24) were gently provided by Dr. K. Yoshino (Nippon Organon, Osaka, Japan). TIMP-1 and TIMP-2 were prepared as described elsewhere (25).

Abs and cytokines

mAb H398 (subclass IgG2a), a TNF-R55-specific and competing mAb (26), was a generous gift of Dr. P. Scheurich (University of Stuttgart, Stuttgart, Germany); mAb utr-1 (subclass IgG1), a TNF-R75-specific and competing mAb (27), was kindly provided by Dr. M. Brockhaus (Hoffmann-LaRoche, Basel, Switzerland); mAb huTACE-M222 (subclass IgG2a), a human TACE-specific mAb (28), was kindly donated by Dr. R. Black (Immunex, Seattle, WA). mAb 2F7 and mAb 16E8 (29, 30), which are rat anti-human ADAM-12 disintegrin-like and cystein-like domains, respectively, and antiserum 119, a rabbit anti-human ADAM-12 disintegrin-like domain polyclonal Ab, were generously provided by Dr. F. Loechel (University of Copenhagen, Copenhagen, Denmark). Affinity isolated biotin-conjugated goat anti-mouse IgG and affinity isolated FITC-labeled goat anti-rat IgG, utilized as secondary Abs in FACS experiments, were obtained from Sigma and Tago (Burlingame CA), respectively. Pure recombinant human TNF, expressed in *Escherichia coli*, was obtained from Bissendorf Biochemicals (Hannover, Germany). p55TNF, the human TNF mutant that specifically recognizes TNF-R55, and p75TNF, the human TNF mutant that specifically recognizes TNF-R75 (31), were generously donated by Dr. H. Loetscher (Hoffmann-LaRoche).

Neutrophil isolation

Blood was drawn from healthy volunteers and anticoagulated with 4 mM EDTA. Neutrophils were isolated according to a single-step separation procedure (32) with slight modifications, as previously described in detail (33). The resulting cell population contained 96–98% neutrophils, 2–4% eosinophils, and 1–2% mononuclear cells. The cells were separated and suspended in 140 mM NaCl, 5 mM KCl, 5 mM HEPES, 5 mM glucose, and 0.2% BSA, pH 7.4 (HBS^--BSA) at room temperature and in the absence of divalent cations to avoid neutrophil aggregation and activation. All experiments were carried out in HBS^--BSA supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBS-BSA).

U937 cells

U937 cells, a human monoblast-like cell line, were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, and 50 μg/ml gentamicin at 37°C in an atmosphere of 95% air and 5% CO_2.

Radioassay of TNF receptor expression

The radioiodination of TNF was performed as previously described (33). After incubation for 15 min in the presence or in the absence of TNF, neutrophils were cooled in ice and centrifuged for 5 min at 200 $\times$ *g*. All subsequent steps were conducted at 4°C to prevent cell activation and recycling and/or shedding of TNF receptors. The cells were then incubated with a glycine-HCl buffer (50 mM glycine and 125 mM NaCl (pH 3.0)) for 1.5 min to remove any TNF bound to the receptors. Preliminary experiments revealed that this procedure removes all cell-associated ^{125}I-labeled TNF (^{125}I-TNF) without affecting cell viability as shown by others (14, 34). To stop the acid treatment, the cells were diluted with HBS-BSA and centrifuged, and the pellets were resuspended in HBS-BSA. Each cell suspension was divided in aliquots and used for receptors measurement. Expression of total TNF receptors was measured after addition of 2.5 ng/ml ^{125}I-TNF (sp. act. 33.0 or 45.0 μCi/μg). For expression of TNF-R55, mAb utr-1 (10 μg/ml) was included in the incubation mixture, in addition to ^{125}I-TNF, to block binding of TNF to TNF-R75. For expression of TNF-R75, mAb H398 (10 μg/ml) was included in the incubation mixture, in addition to ^{125}I-TNF, to block binding of TNF to TNF-R55. Nonspecific binding was determined by addition of a 200-fold excess of cold TNF. After incubation for 2.5 h under agitation on a rocker platform, the unbound TNF was removed by centrifugation (30 s at 13,000 $\times$ *g*, 4°C) and two washings with HBS-BSA. The pellet-containing bottom parts of the 1.5-ml microfuge tubes used in the experiments were then cut off and counted in a gamma counter.

Assay of TNF receptor releasing activity in PMN supernatants

PMN (10^7/ml) were incubated without and with FMLP (5×10^{-8} M) for 20 min at 37°C under constant agitation. After cooling on ice, the cells were centrifuged (13,000 $\times$ *g* for 3 min) and the supernatants collected. In parallel experiments, U937 cells were collected, centrifuged at 250 $\times$ *g* for 7 min, and suspended in HBS-BSA. Aliquots of supernatants corresponding to 7.5×10^6 cells, from resting and FMLP-treated PMN were then incubated with 10^6 U937 cells for 20 min at 37°C. After centrifugation, expression of TNF receptors on U937 cells was measured by flow cytometry.

Immunofluorescence flow cytometry

U937 cells or PMN after the different treatments were cooled at 4°C, divided in aliquots, and incubated for 45 min with the Abs of various specificities: mAb H398 (2 μg/ml) for TNF-R55, mAb utr-1 (2 μg/ml) for TNF-R75, mAb M222 (3 μg/ml) for TACE, and mAbs 2F7 and 16E8 (5 μg/ml) for ADAM-12. After two washes with ice-cold PBS, the cells were incubated for 30 min with biotinylated goat anti-mouse IgG and, after two additional washes, were incubated for another 20 min with a streptavidin-R-PE conjugate. For mAbs 2F7 and 16E8, a FITC-conjugated goat anti-rat IgG secondary Ab was used. After washing, the cells were suspended in PBS containing 0.75% formaldehyde and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

Measurement of solubilized TNF receptors

A commercially available ELISA kit (R&D Systems) was used to measure the TNF receptors released in the supernatants obtained from PMN after the various treatments. In this assay an immobilized anti-TNF-R55 or anti-TNF-R75 mAb is used to capture the specific receptor, which is then detected by a peroxidase-conjugated polyclonal Ab. According to the manufacturer, in this immunoassay, TNF does not show any significant cross-reactivity and exhibits only a low level interference (10% decrease in the observed value using TNF at 5 ng/ml).

In all experiments the spontaneously solubilized receptors before the start of each experiment (t_0) were subtracted from the final results.

Data analysis

ID_{50} values were determined by fitting the data to a sigmoidal curve using GraphPad Prism 3.0 for Windows 98 (GraphPad Software, San Diego, CA). Student's *t* test on paired data was used to calculate statistical significance.

Results

TNF induces shedding of both TNF-R55 and TNF-R75

PMN were incubated in the absence and in the presence of TNF, and TNF receptor expression and shedding were measured. Fig. 1*A* shows that TNF causes down-modulation of the expression of its receptors, and that this is accompanied by their release in the incubation medium (Fig. 1*B*). In quantitative terms, the release of TNF-R75 is higher than that of TNF-R55. Also, resting release of TNF-R75 is more marked than the corresponding release of TNF-R55. The total amount of receptors measured in Triton X-100 extracts of PMN was 69.0 ± 3.4 and 101.9 ± 17.3 (pg/10^6 PMN ± SD, $n = 3$) for TNF-R55 and TNF-R75, respectively. Thus, considering that the extra amount of shed receptors in the presence of TNF (TNF minus resting) is 16.1 ± 6.7 for TNF-R55 and 45.0 ± 16.4 for TNF-R75 (Fig. 1), it turns out that TNF causes the release of about 23% of TNF-R55 and 44% of TNF-R75. Fig. 2 shows that TNF-induced release of both receptors becomes apparent after a lag time of 5 min from the addition of the cytokine, increases rapidly afterward, and reaches its maximum after 15 min of incubation. In contrast, release from resting PMN linearly increases with time, at least up to the 60 min of incubation of our experiments.

TNF-induced shedding is mediated by TNF-R55

Initially, we tested the effect of receptor-specific reagents on receptor shedding. Fig. 3 shows that, after 15 min of incubation, p55TNF, a TNF-R55 specific TNF mutant, induces a release of both receptors, which is comparable to that induced by TNF. In contrast, p75TNF, a TNF-R75-specific mutant, does not exert any effect, even after 30 min of incubation (data not shown). The possibility that shedding could have occurred after TNF-R75 engagement (but the released receptors are not detectable because of an increased lability) was excluded by showing that receptor expression measured by immunofluorescence flow cytometry remained unchanged over a 30-min period (data not shown). Consistent with these findings are the results obtained with receptor-specific mAbs showing that mAb H398 (a TNF-R55 specific and competing mAb) induces release of TNF-R75 (TNF-R55 could not be measured due to interference by the mAb with the ELISA) while mAb utr-1 (a TNF-R75 specific and competing mAb) does not affect release of TNF-R55 (TNF-R75 could not be measured due to interference by the mAb with the ELISA). In additional experiments we observed that Fab fragments prepared from the TNF-R55-specific and competing mAb H398, which do not affect TNF receptors expression, completely prevented TNF-induced down-modulation of both receptors (data not shown).

Effect of proteinase inhibitors

A panel of proteinase inhibitors was initially screened for their effect on TNF-induced receptor solubilization. Table I shows that, among them, the metalloproteinase inhibitors 1,10-phenanthroline and the peptide-hydroxamates KB8301 and KB7785 strongly inhibited TNF-induced release of both TNF receptors. The inhibitory effect of KB8301 and KB7785 appears to be dependent on the hydroxamic acid-chelating moiety since KB8845, which lacks this moiety, is ineffective. Shedding of TNF-R55 was almost totally inhibited by KB8301 and 1,10-phenanthroline and less markedly by KB7785. Shedding of TNF-R75 was less markedly inhibited than shedding of TNF-R55 by all three compounds. The other inhibitors tested were ineffective, with the possible exception of DFP, which slightly but reproducibly inhibited TNF-R75 release only. Hydroxamic acid-based compounds and 1,10-phenanthroline have been extensively used to assess the involvement of metalloproteinases in shedding of various receptors, including selectins, CD43, and TNF receptors themselves (35–37) However, attention has not been paid to the possible effects of these inhibitors on the intracellular signaling pathways triggered by receptor engagement. We therefore tested the effect of KB8301 and 1,10-phenantholine on TNF-induced PMN activation as measured by the increase in O_2^- production, adherence, and expression of β_2 integrins. Table II shows that 1,10-phenanthroline virtually abolishes TNF-induced cell activation, whereas KB8301 has no effect. Similar results (i.e., strong inhibition with 1,10-phenanthroline or no effect with KB8301) were obtained after stimulation with FMLP (data not shown). A strong inhibitory effect on PMN activation was also observed using the nonchelating analogue 1,7-phenanthroline (data not shown). As these results point to a nonspecific effect of 1,10-phenantroline, it was decided not to use this inhibitor in subsequent studies.

Fig. 4 shows the dose-response curve of the effect of KB8301, the most effective shedding inhibitor. From the data of these experiments a ID_{50} values of 0.22 and 0.84 μM were calculated for TNF-R55 and TNF-R75, respectively. The results of the figure also demonstrate that the less-pronounced inhibition of TNF-R75

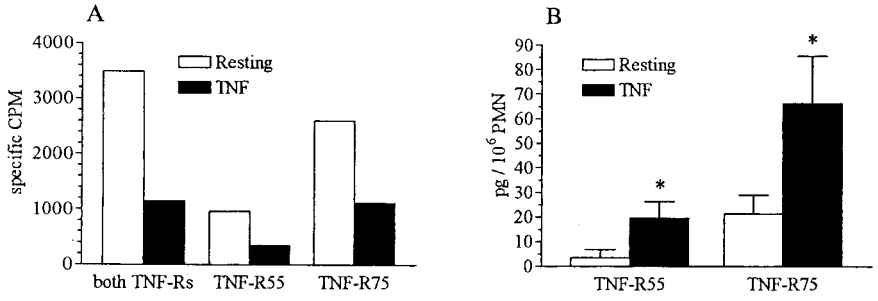

FIGURE 1. TNF receptor expression (*A*) and shedding (*B*) in PMN at rest or after exposure to TNF. PMN (5×10^6/ml in HBS-BSA), after equilibration for 10 min at 37°C under constant agitation, were incubated with TNF (2.5 ng/ml) for 15 min. After cooling on ice, PMN were centrifuged ($400 \times g$ at 4°C for 7 min), and the supernatants were collected and used for the measurement of the solubilized receptors as described in *Materials and Methods*. To quantify receptor expression, the cells in the pellet were washed with an acidic buffer to remove cell-associated TNF, and receptors expression was measured with ^{125}I-TNF as described in *Materials and Methods.* The results are from one experiment representative of two similar ones for receptors expression, and the means of the results of 10 experiments for receptor shedding are shown. Bars indicate SD. *, $p < 0.001$ vs the corresponding resting PMN.

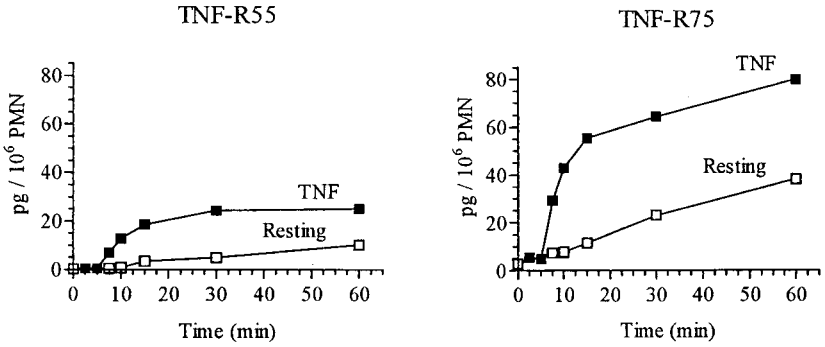

FIGURE 2. Time course of TNF receptors release from PMN at rest or after exposure to TNF. PMN (5×10^6/ml in HBS-BSA) were preincubated for 10 min at 37°C under constant agitation. TNF (2.5 ng/ml) was then added and incubations were stopped at various times by cooling on ice. After centrifugation, supernatants were collected and used for soluble receptors measurement as described in *Materials and Methods*. The results of one experiment representative of two similar ones are reported.

release by KB8301 cannot be overcome by increasing its concentration. Fig. 5 shows that KB8301 also inhibits shedding of TNF-R55 and TNF-R75 induced by FMLP, with a profile similar to that observed with TNF, i.e., almost complete inhibition of TNF-R55 release, and partial inhibition of TNF-R75 release.

Characteristics of the TNF receptor releasing activity

PMN contain a collagenase (MMP-8) and gelatinase B (MMP-9), two zinc-dependent endopeptidases of the MMP family that are strongly inhibited by peptide hydroxamates (38–41). We used two physiologic inhibitors of MMPs, i.e., TIMP-1 and TIMP-2 (42), to see if MMPs are involved in the shedding process. Table III shows that neither inhibitor affected TNF-induced receptor shedding. Since peptide hydroxamates are known to inhibit zinc metalloproteinases (41), we tested the effect of Zn^{2+} on inhibition of shedding by KB8301. Using 0.1 mM excess $ZnCl_2$ we were unable to counteract the inhibitory activity of KB8301 (data not shown).

We next sought to determine whether the shedding activity is released from PMN. To this end, we used U937 cells as a target, as they express substantial amounts of both TNF receptors and matalloproteinases and serinproteinases have been shown to be involved in their cleavage (36). Operatively, we measured receptor expression in these cells after incubation with supernatants obtained from PMN stimulated with FMLP, a potent secretagogue and inducer of TNF receptor shedding (11, 12). Table IV shows that a substantial down-regulation of both TNF-R55 and TNF-R75 occurs in U937 cells after TNF treatment, indicating that a sheddase similar to that functioning in PMN is operative in these cells, whereas the expression of both receptors is not altered following incubation with supernatants from either resting or FMLP-treated PMN. This suggests that the proteinase is membrane bound, although we cannot formally exclude that the inability to detect any shedding activity in PMN supernatants might reflect the instability of the hypothetically released sheddase, which might have completely lost its activity during the period of time (about 5 min for cooling on ice and centrifugation) needed to prepare the supernatants before assaying their activity, which seems really very unlikely.

Toward a more precise definition of the sheddase

The previous results indicate that a membrane-bound non-MMP is involved in TNF-induced shedding of TNF receptors. Members of the ADAM family of metalloproteinases, a class of membrane bound proteins that contain both disintegrin and metalloproteinase domains, recently have been shown to be involved in the shedding of the ectodomains of several membrane proteins (43, 44). We have assessed the expression in PMN of two of them known to possess proteinase activity, i.e., ADAM-17 (TACE) and ADAM-12 (45). Fig. 6 shows that PMN express ADAM-17 to an extent comparable to TNF receptors, whereas ADAM-12 was undetectable using either mAbs, as shown in the figure, or polyclonal Abs (data not shown).

Discussion

Regulation of the availability of cellular TNF receptors may be an important protective mechanism against excessive TNF activity. Proteolytic cleavage (shedding) is the major and best-documented mechanism of TNF receptor down-modulation that may on the one hand reduce cellular sensitivity to TNF, and on the other hand limit bioavailability of TNF by providing soluble forms of the receptors that would compete for the cytokine with the cellular receptors. TNF orchestrates all stages of inflammation and plays a pivotal role in immune response to infection (46). However, excessive TNF production may turn TNF activities from beneficial to extremely injurious. In fact, acute release of large amounts of TNF in the circulation is followed by the characteristic manifestations of septic shock, i.e., decrease in capillary resistance, capillary leakage, falling in cardiac output, diffuse coagulation with necrosis of vital organs, and cardiopulmonary collapse (47–49). PMN are crucial targets of TNF activity, and if exposed to high TNF doses they may become key effectors of its toxic effects. The present study was undertaken to characterize the mechanisms involved in the solubilization of TNF receptors in PMN exposed to TNF. Our results clearly show that the down-modulation of receptor expression induced by TNF is associated with shedding of considerable amounts of both TNF-R55 and TNF-R75 (Fig. 1). This finding

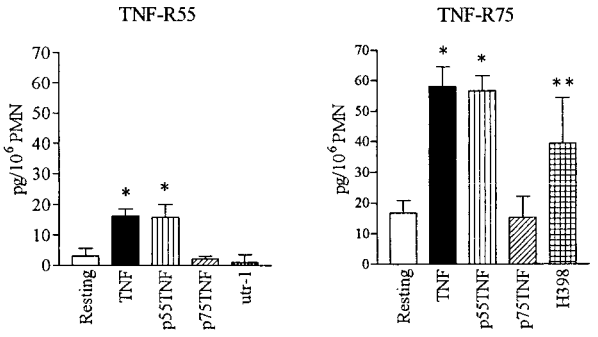

FIGURE 3. Effect of receptor-specific reagents on the shedding of TNF-R55 and TNF-R75. PMN (5×10^6/ml in HBS-BSA), after prewarming for 10 min at 37°C, were incubated for 15 min with TNF or receptor-specific reagents (mAbs and mutants of TNF). After cooling and centrifugation, the soluble receptors were measured in the supernatants: TNF (2.5 ng/ml), p55TNF (15 ng/ml), p75TNF (45 ng/ml), mAb utr-1 (5 μg/ml), and mAb H398 (5 μg/ml). The data are means of the results of three to five experiments. Bars indicate SD. *, $p < 0.002$ vs resting PMN; **, $p < 0.02$ vs resting PMN.

Table I. *Effect of proteinase inhibitors on TNF-induced shedding of TNF receptors*[a]

Inhibitor	Target Proteinase	Soluble TNF-R55 (% inhibition)	Soluble TNF-R75 (% inhibition)
KB8301 (5 μM)	Metallo	91.5 ± 8.8 (16)[b]	56.3 ± 20.4 (15)
KB7785 (5 μM)	Metallo	67.8	39.3
KB8845 (5 μM)	Control	−11.8	−5.1
1,10-Phenanthroline (1 mM)	Metallo	94.6 ± 10.1 (9)	69.9 ± 17.7 (8)
EDTA (5 mM)	Metallo	9.2 ± 16.9 (3)	3.5 ± 10.1 (3)
Thiorphan (40 μM)	Metallo-endopeptidases	−0.3	7.3
Phosphoramidon (100 μM)	Metallo-endopeptidases	−12.5	−3.6
DFP (1 mM)	Serine	7.7 ± 6.1 (5)	21.8 ± 6.3 (4)
Chymostatin (50 μM)	Serine, chymotrypsin-like	13.0	−7.2
TLCK (75 μM)	Serine, trypsin-like	7.1	−0.9
Leupeptin (15 μM)	Serine, trypsin-like; cysteine	6.9	0.0

[a] PMN (5×10^6/ml in HBS-BSA), after prewarming for 10 min at 37°C, were incubated with the inhibitors for 3 min and then 2.5 ng/ml TNF was added for an additional 15 min of incubation. After cooling, PMN were centrifuged, and soluble receptors were measured in the supernatants as described in *Materials and Methods*.

[b] Data are expressed as percent inhibition of control ± SD. The number of experiments is indicated in parentheses. When not indicated, the data are from one experiment representative of two similar ones. Controls were (in pg/10^6 PMN ± SD, n = 16) 18.2 ± 5.7 for TNF-R55 and 47.9 ± 11.5 for TNF-R75.

differs from what previously reported by Porteu and Hieblot (14) who showed that TNF-induced down-modulation of its receptors from PMN is due to shedding of TNF-R75 and internalization of TNF-R55. That internalization of TNF-R55 takes place was, in fact, verified in our laboratory too (Ref. 50 and data not shown), thus indicating that both internalization and shedding are involved in the down-modulation of this receptor. The failure by Porteu and Hieblot (14) to detect shedding of TNF-R55 may well be explained by the low sensitivity of their ELISA for the soluble receptor, which may have led to underestimation of TNF-R55, the less abundant (33) and less extensively shed (present paper) receptor. Concerning the question regarding which of the two receptors mediates shedding, the observations that TNF-R55-specific but not TNF-R75-specific mAbs and mutants of TNF trigger release of both receptors (Fig. 3) and that TNF-induced receptor release could be inhibited by Fab fragments from a TNF-R55-specific and competing mAb, categorically point to TNF-R55 as the receptor that transduces the signal leading to shedding of both receptors. These results, combined with the observations that all PMN responses to TNF so far studied are mediated by TNF-R55, further emphasizes the notion that this receptor is probably central to the modulation of all TNF-induced PMN activities.

The nature of the proteinase involved in shedding is unknown. Data from a study conducted with PMN militate against a role of serine or thiol proteinases in this respect since inhibitors of these classes of proteinases did not affect FMLP-induced shedding (11). In cell systems different from PMN, hydroxamic acid-based compounds were found to inhibit shedding of both TNF receptors (51–53), suggesting that zinc-dependent metalloproteinases are involved in the process, since hydroxamic acid-based compounds are potent inhibitors of this class of proteinases (41). Our results (Table I) show that TNF-induced shedding of its receptors is inhibited by the metal chelators KB8301 and KB7785 but not by the metalloendopeptidase inhibitors thiorphan and phosphoramidon or by EDTA. Insensitivity to phosphoramidon and thiorphan indicates that membrane metalloproteases of clan MA (54), which include the granulocyte Ags CD10 and CD13, have no role in the process. The lack of effect of EDTA, which appears intriguing at first sight, will be discussed more in detail below. KB8301 inhibited almost totally the release of TNF-R55 and, to a lesser degree, release of TNF-R75. In fact, ~40% of the released TNF-R75 "evaded" inhibition at all inhibitor's concentrations (Fig. 4), suggesting that a proteinase insensitive to this inhibitor contributes to shedding of this receptor. The observation that among the other inhibitors used only DFP partially inhibited TNF-R75 release suggests that this proteinase is a serine proteinase. The identity of the proteinase, which accounts for the DFP-inhibitable shedding upon TNF stimulation and which turns out to be the responsible for the high levels of spontaneous (resting) release of this receptor (Figs. 1 and 2) will be described elsewhere (C. Gasparini and P. Dri, manuscript in preparation). Thus, a metalloproteinase is involved in TNF-induced shedding of its receptors. The finding that KB8301 caused an inhibition of FMLP-induced shedding similar to that observed with TNF (Fig. 5) suggests that activation of a metalloproteinase is

Table II. *Effect of the metalloproteinase inhibitors KB8301 and 1,10-phenanthroline on TNF-induced PMN activation*[a]

Additions	O_2^- Production (nmol/10^6 PMN)	Adherence (%)	β_2 Integrin Expression (MCF)[c]
Resting	5.8 ± 4.2[b]	12.3 ± 7.6	58.0 ± 9.9
TNF	55.6 ± 10.8	78.5 ± 15.7	105.7 ± 16.2
TNF + KB8301	57.2 ± 9.6	76.2 ± 13.2	108.3 ± 18.7
TNF + 1,10-phenanthroline	1.9 ± 1.3	6.5 ± 3.2	57.4 ± 12.1

[a] PMN (1.5×10^6/ml), after prewarming for 10 min at 37°C, were incubated with 5 μM KB8301 and 1 mM 1,10-phenanthroline for 3 min. Thereafter, O_2^- production, adherence, and β_2 integrin expression were measured exactly as described (63) after incubation for 30 min without (resting) and with 2.5 ng/ml TNF.

[b] Data are means ± SD of three to five experiments.

[c] Mean channel fluorescence.

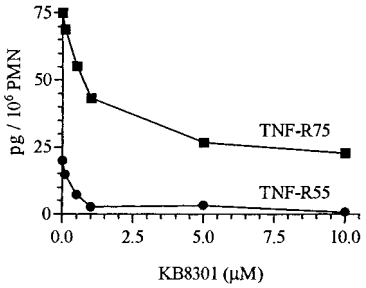

FIGURE 4. Dose response of the effect of KB8301 on TNF-induced release of TNF receptors. PMN (5×10^6/ml in HBS-BSA), after prewarming for 10 min at 37°C, were incubated for 3 min with KB8301 and for an additional 15 min with TNF (2.5 ng/ml). After cooling and centrifugation, the soluble receptors were measured in the supernatants. The results of one experiment representative of two similar ones are reported.

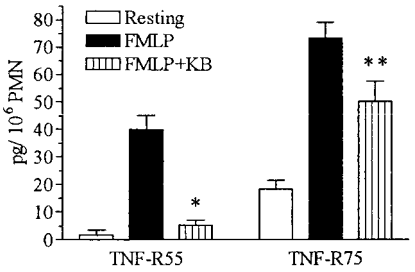

FIGURE 5. Effect of KB8301 (KB) on FMLP-induced shedding of TNF receptors. PMN (5×10^6/ml in HBS-BSA), after preincubation for 10 min at 37°C, were incubated for 3 min with KB8301 (5 μM) and for additional 15 min with FMLP (5×10^{-8} M). After cooling and centrifugation, the soluble receptors were measured in the supernatants. The means of the results of three experiments are reported. Bars indicate SD. *, $p < 0.02$ vs FMLP-treated PMN; **, $p < 0.05$ vs FMLP-treated PMN.

also involved in receptor shedding induced by ligands other than TNF.

Two sets of data help to better classify the enzyme responsible for the stimulated release of TNF receptors in PMN. First, the results reported in Table III which show that TIMP-1 and TIMP-2 do not affect TNF-induced receptor shedding rule out the possibility that MMPs might be involved in the process, and second, the failure to detect in supernatants from FMLP-stimulated PMN any receptor releasing activity (Table IV) suggests that the proteinase is membrane bound. Thus, the involved sheddase is a non-matrix and membrane-anchored metalloproteinase. These characteristics are distinctive for ADAMs, a large group of metalloproteinases, included in family M12, subfamily B (reprolysins) of clan MB, according to a recent classification (55). A number of ADAMs have been identified and molecularly cloned so far in mammals and many of them predicted to encode potentially active metalloproteinases. However, few of them have been shown to possess catalytic activity and to be expressed in granulocytes. For example, ADAM-8 is expressed in granulocytes but has no known catalytic activity (56). ADAM-9, which was shown to be expressed in peripheral blood leukocytes by Northern blotting (57), did not cleave a peptide spanning the cleavage site of TNF-R55 and cleaved a TNF-R75 peptide at a site different from that involved in receptor processing (58). ADAM-10, whose presence in peripheral blood leukocytes has also been documented by Northern blotting, was shown to possess pro-TNF-α processing activity and to cleave a peptide containing the proposed cleavage site of pro-TNF-α, but not peptides containing the proposed cleavage sites of TNF-R55 or TNF-R75 (59). The recently described ADAM-12 (29) with functional proteolytic activity (45) was also excluded as a possible candidate sheddase as we could not find evidence of expression in PMN (Fig. 6). A remarkable member of this family of metallo-

Table III. *Effect of MMP inhibitors TIMP-1 and TIMP-2 on TNF-induced shedding of TNF receptors*[a]

Additions	TNF-R55 (pg/10^6 PMN)	TNF-R75 (pg/10^6 PMN)
TNF	24.4 ± 3.6[b]	50.4 ± 4.7
TNF + TIMP-1	19.8 ± 1.1	50.5 ± 3.7
TNF + TIMP-2	22.3 ± 1.2	51.8 ± 3.0

[a] PMN (5×10^6/ml in HBS-BSA), after prewarming for 10 min at 37°C, were incubated for 3 min with 2 μg/ml TIMP-1 or 1.6 μg/ml TIMP-2 and then for an additional 15 min with 2.5 ng/ml TNF. After cooling on ice, PMN were centrifuged and soluble receptors measured in the supernatants.

[b] Data are means ± SD of three experiments.

Table IV. *Effect of TNF and of supernatants from FMLP-treated PMN on the expression of TNF receptors in U937 cells*[a]

Incubation Conditions	TNF-R55	TNF-R75
Control	50.1 ± 8.9[b]	39.5 ± 9.4
+ TNF (10 ng/ml)	36.5 ± 4.7	26.7 ± 3.7
+ Supernatants from resting PMN	42.5 ± 4.9	43.0 ± 7.8
+ Supernatants from FMLP-treated PMN (t_0)	44.2 ± 8.4	43.0 ± 6.9
+ Supernatants from FMLP-treated PMN (t_{20})	42.0 ± 7.9	44.9 ± 10.7

[a] PMN (10^7/ml in HBS-BSA), after prewarming for 10 min at 37°C, were incubated without (resting PMN) or with FMLP (5×10^{-8} M) for 20 min (t_{20}) at 37°C. FMLP (5×10^{-8} M) was also added to an aliquot of PMN kept on ice (t_0). After cooling on ice and centrifugation (3 min at 13,000 × *g*), the supernatants were collected, brought to 37°C, and added to U937 cells (10^6 cells plus an aliquot of supernatants corresponding to 7.5×10^6 PMN equivalents, in a final volume of 350 μl). The control contained 10^6 U937 cells in 350 μl of HBS-BSA. After incubation for 20 min, the U937 cells were centrifuged and expression of TNF receptors was measured by immunofluorescence flow cytometry as described in *Materials and Methods*.

[b] The data are means ± SD of the mean channel fluorescence of three to four experiments.

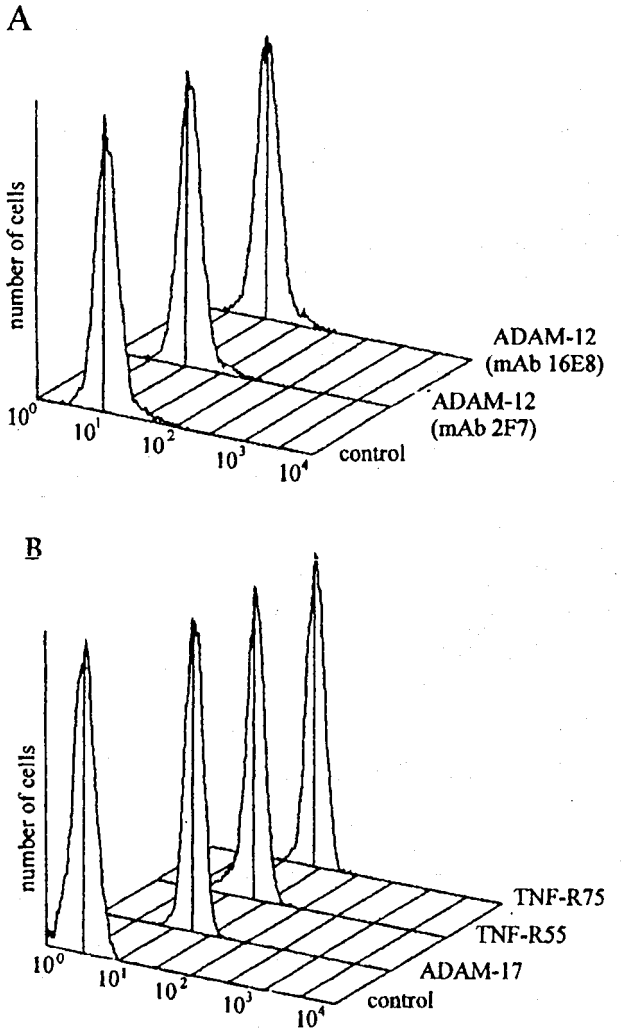

FIGURE 6. Expression of ADAM-12 (*A*) and ADAM-17 (TACE), TNF-R55, and TNF-R75 (*B*) on PMN. PMN (5×10^6/ml in HBS-BSA) were cooled on ice immediately after isolation and then divided into several aliquots. After incubation without (control) and with the appropriate mAb, Ab binding to PMN was measured as described in *Materials and Methods*. The fluorescence intensity in log scale is reported in the abscissa. The results of one experiment representative of three similar ones are reported.

proteinases is ADAM-17. Initially identified as the TACE (28, 60), it now appears to possess a more relaxed specificity and is probably involved in the release of a number of cytokines, growth factors and the corresponding receptors initially synthesized as membrane-anchored proteins. For example, it has been recently shown that cultured cells homozygous for a targeted mutation in ADAM-17 were markedly deficient not only in the release of TNF but also of TGF-α, L-selectin, and TNF-R75 (44). The presence of ADAM-17 in PMN has been demonstrated by Northern and Western blotting (28) with an expression comparable to that of TNF receptors, as shown in this paper (Fig. 6). Altogether, these observations can be taken as a plausible circumstantial evidence in favor of this metalloproteinase as the TNF receptor sheddase of stimulated PMN. If this conclusion is correct, then the recently published crystal structure of the catalytic domain of ADAM-17 cocrystallized with a hydroxamic acid inhibitor (61) may provide useful hints to explain the two apparently incongruous results obtained with the metalloproteinase inhibitors, i.e., the inability of zinc to restore the activity inhibited by KB8301, and the lack of effect of EDTA (Table I). Compound 3, the hydroxamic acid inhibitor used in the above-mentioned study, has been shown to interact with the catalytic site of ADAM-17 not only by chelation of Zn^{2+} with the carbonyl and hydroxyl oxygens of the hydroxamic acid moiety but also by forming hydrogen bonds and by inserting an isobutyl residue into an hydrophobic pocket contained in the active-site cleft. KB8301, the inhibitor used in the present study, is very similar to compound 3 and is therefore expected to interact with the catalytic site with high affinity, thus making it impossible its displacement by Zn^{2+}. This possibility is substantiated by the observation that in the literature there are no examples that we are aware, of a reversal by zinc of ADAMs activity after inhibition by hydroxamic acid compounds.

The lack of effect of EDTA (Table I), which has a pKa_3 of 6.2 (62) and, therefore, is expected to be highly polar at physiological pH, may be the consequence of its inability to efficiently chelate the zinc contained in a catalytic site with hydrophobic characteristics.

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