

Evidence that elastase is the TNF-R75 shedding enzyme in resting human polymorphonuclear leukocytes

Chiara Gasparini, Renzo Menegazzi, Pierluigi Patriarca, Pietro Dri*

Department of Physiology and Pathology, University of Trieste, Via A. Fleming 22, Trieste, Italy

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Abstract We previously showed that a metalloprotease and a serine protease mediate shedding of the TNF-R75 (75-kDa tumor necrosis factor receptor) in neutrophils. Here we show that elastase is the TNF-R75 solubilizing serine protease. Release of the TNF-R75 by resting cells was almost totally inhibited by the serine protease inhibitor diisopropylfluorophosphate (DFP), by two synthetic, chemically unrelated, elastase-specific inhibitors and by α 1-protease inhibitor. Release after TNF or FMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine) stimulation was blocked by DFP and a metalloprotease inhibitor used in combination. Supernatants from resting neutrophils contained a 28-kDa fragment of the receptor, compatible with that generated by elastase, whose appearance was inhibited by DFP. Upon FMLP stimulation, the release of 28-kDa and 40-kDa fragments was observed, which was inhibited by DFP and a metalloprotease inhibitor, respectively. We conclude that elastase is the TNF-R75 sheddase of resting neutrophils and that it contributes to shedding of this receptor in stimulated cells.
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Key words: Neutrophil; Human; Elastase; Shedding; TNF receptor

1. Introduction

Tumor necrosis factor (TNF) is a cytokine produced by a number of cells, including macrophages, lymphocytes and natural killer cells [1]. Based on the diverse biologic activities it exerts, TNF is generally considered a potent mediator of host response to injury, inflammation, immunity and repair [2]. Cellular responses to TNF are initiated following interaction with two receptors, namely the 55-kDa receptor (TNF-R55) and the 75-kDa receptor (TNF-R75), which are expressed on all nucleated cells. Neutrophils, the most abundant cells of the acute inflammatory reaction, express surface TNF receptors and likewise respond to the cytokine by activating several cell functions, including adherence [3,4], production of superoxide anion and hydrogen peroxide [4–7], efflux of chloride ions [8],

and solubilization of TNF receptors themselves [7,9]. Solubilization, or shedding, is an important mechanism for control of the expression of TNF receptors. In neutrophils, the solubilization of TNF receptors can be induced by agonists other than TNF, including FMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine), C5a, GM-CSF or PMA [7,9–12]. The down-regulation of TNF receptors causes functional deactivation of neutrophils to TNF and appears to be a mechanism of self-protection against excessive stimulation by the cytokine [11,13,14]. On the other hand, by competing for TNF with membrane receptors, solubilized receptors may contribute to dampening pro-inflammatory and potentially toxic effects in conditions such as rheumatoid arthritis, Crohn's disease, multiple sclerosis or other pathologies in which TNF has been shown to play an important role [15–18].

Based on experiments with protease inhibitors, it has been shown in different cell types that both metalloproteases and serine proteases are involved in the release of TNF receptors [19,20]. We have also recently shown that the TNF-induced shedding of TNF receptors from polymorphonuclear leukocytes (PMN) involves metalloproteases and serine proteases [9]. More specifically, we have shown that release of TNF-R55 was almost completely inhibited by KB8301, a hydroxamate-based metalloprotease inhibitor, while the release of TNF-R75 was only partially affected by this inhibitor. The solubilization of this latter receptor, but not TNF-R55, was also inhibited to some extent by the broad-spectrum serine protease inhibitor diisopropylfluorophosphate (DFP) [9]. In this study, we identify the DFP-sensitive protease involved in shedding of TNF-R75. Based on the results of studies with protease-specific inhibitors and by molecular analysis of the solubilized receptor fragments, it can be suggested that elastase is the TNF-R75 sheddase in resting neutrophils. In stimulated cells, both elastase and a metalloprotease contribute to the shedding of TNF-R75.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA) fraction V, FMLP, porcine pancreatic elastase type VI, BCIP (5-bromo-4-chloro-3-indolyl phosphate), NBT (nitro blue tetrazolium), protein G solubilized and cross-linked on agarose beads were obtained from Sigma (St. Louis, MO, USA); Percoll was obtained from Pharmacia (Uppsala, Sweden); streptavidin-AP conjugate was from BIOSPA (Milan, Italy). Pure, recombinant human TNF, expressed in *Escherichia coli* was obtained from Bissendorf Biochemicals (Hannover, Germany). Immunoassays for human TNF-R55 and TNF-R75 were performed using ELISA kits obtained from R&D Systems (Minneapolis, MN, USA). All solutions were made in endotoxin-free water for clinical use.

*Corresponding author. Fax: (39)-040-567862.

E-mail address: dri@units.it (P. Dri).

Abbreviations: TNF, tumor necrosis factor; TNF-R75, 75-kDa TNF receptor; TNF-R55, 55-kDa TNF receptor; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; DFP, diisopropylfluorophosphate

2.2. Protease inhibitors

The α 1-protease inhibitor was from Sigma, DFP was purchased from Acros Organics, NJ, USA; [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-methylsuccinyl]-L-3-(5,6,7,8-tetrahydro-1-naphthyl)alanine-*N*-methylamide (KB8301) [21] was kindly provided by K. Yoshino, Kanebo Ltd., Osaka, Japan; ZM200,355 (or ICI200,355) [22] was provided by Zeneca Pharmaceuticals, Cheshire, UK; L658758 [23] was from Merck, Sharp and Dohme Research Laboratory, NJ, USA.

2.3. Antibodies

M80, a rabbit anti-human TNF-R75 polyclonal antibody [24], was a generous gift of Dr. P. Scheurich (University of Stuttgart, Stuttgart, Germany); utr-1 (subclass IgG1), a mouse anti-human TNF-R75 mAb [25], was kindly provided by Dr. M. Brockhaus (Hoffman-LaRoche, Basel, Switzerland); biotinylated goat anti-mouse antibody was from BIOSPA (Milan, Italy).

2.4. Neutrophil isolation

Blood was drawn from healthy volunteers and anticoagulated with 4 mM EDTA. Neutrophils were isolated according to a single-step separation procedure as previously described [26] with an adaptation of the method to larger blood volumes, by layering 15 ml of blood over the two-step gradient (15 ml 75% isotonic Percoll, 15 ml 62% isotonic Percoll) formed in 50 ml conical test tubes. The cell population contained 95–98% neutrophils, 2–4% eosinophils, and 1–2% mononuclear cells. The entire procedure was carried out in the absence of divalent cations to avoid neutrophil aggregation and activation. Before starting each experiment, the cell suspensions and the incubation medium were supplemented with 1 mM CaCl_2 and 1 mM MgCl_2 (HBS-BSA).

2.5. Soluble receptor assay

PMN (5×10^6 /ml in HBS-BSA), prewarmed to 37°C, were incubated without and with protease inhibitors for 3 min and then for an additional 20 min with 2.5 ng/ml TNF or 10^{-7} M FMLP. After cooling on ice and centrifugation, the supernatants were collected and used for assays. Soluble receptors were measured by means of two ELISA kits specific for TNF-R55 or TNF-R75. The kits utilized 96-well microplates coated with receptor-specific murine monoclonal antibodies. TNF-R-specific polyclonal antibodies conjugated to horseradish peroxidase were used to detect receptor binding to the monoclonal antibodies. The sensitivity of the ELISAs is <3 and <1 pg/ml for TNF-R55 and TNF-R75, respectively. According to the manufacturers, in these immunoassays TNF does not show any significant cross-reactivity and only a low interference (with TNF- α at 5 ng/ml, the observed value was decreased by 10%).

2.6. Immunoprecipitation, SDS-PAGE, and Western blot analysis

PMN ($25\text{--}30 \times 10^6$ cells in 1 ml HBS-BSA) were incubated with protease inhibitors for 3 min at 37°C and then in the absence (60 min) or in the presence (20 min) of FMLP (10^{-7} M) or elastase (20 $\mu\text{g}/\text{ml}$). The PMN suspensions were then cooled on ice, centrifuged, and the supernatants collected. TNF receptors were also extracted from intact PMN. In these experiments, PMN (5×10^6) were centrifuged and resuspended in lysis buffer (0.5% sodium deoxycholate, 1% Triton, 0.1% SDS, 1 mM 1,10-Phenanthroline, 2.5 mM Na_4EDTA , 2 mM iodoacetic acid, 2.8 mM DFP in HBS) and incubated at 4°C for 20 min. After centrifugation at $12000 \times g$ for 5 min supernatants were collected.

Supernatants were precleared by incubation with protein G Sepharose at 4°C for 1 h under continuous rotation. After centrifugation, TNF-R75 was immunoprecipitated from the supernatants after overnight incubation at 4°C with antibody M80 (6 $\mu\text{g}/\text{condition}$) and protein G Sepharose. After washing three times with 0.4 M NaCl, 0.5% DCC, 0.5% Triton, 0.05% SDS in BSA-free HBS, the protein G Sepharose pellets were suspended in sample buffer, boiled for 3 min, and subjected to SDS-PAGE on a 10% gel under non-reducing conditions, according to the method of Laemmli [27]. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by Western blotting according to the method described by Towbin [28]. The nitrocellulose membrane was incubated overnight at 4°C with blocking buffer (25 mM Tris-HCl, 0.9% NaCl, pH 7.4 containing 5% non-fat dry milk and 0.02% Tween 20) and then with 1 $\mu\text{g}/\text{ml}$ mAb utr-1 in blocking buffer at room temperature under agitation for 45 min. From this step on, dry milk was omitted from the incubation buffer. The membrane was washed twice and incubated with a bio-

tinylated secondary antibody for 45 min. After two additional washings and incubation with streptavidin conjugated with alkaline phosphatase for 30 min, the bound alkaline phosphatase was detected by incubation with NBT and BCIP.

3. Results and discussion

3.1. A serine protease is involved in shedding of TNF-R75 from PMN

While studying the effect of a panel of protease inhibitors on TNF-induced shedding of its receptors in PMN, it was found that the release of TNF-R55 was almost completely inhibited by the hydroxamic acid-based metalloprotease inhibitor KB8301 ($90.7 \pm 9.1\%$). The release of TNF-R75 was only partially inhibited by this compound ($51.0 \pm 8.1\%$) (Fig. 1). The characteristics of the KB8301-sensitive metalloprotease responsible for the TNF-induced release of TNF-R55 and TNF-R75 have recently been defined [9]. Fig. 1 also shows that TNF-induced shedding of TNF-R55 was unaffected by DFP, a broad-spectrum serine protease inhibitor, while shedding of TNF-R75 was slightly but significantly inhibited ($17.4 \pm 5.2\%$) by this compound. KB8301 and DFP in combination almost totally suppressed release of TNF-R75, indicating that metalloproteases and serine proteases completely account for the TNF-induced shedding of this receptor. Fig. 2 shows that results similar to those obtained with TNF are observed when FMLP is used as a stimulus, indicating that a common biochemical mechanism is involved in shedding of TNF-Rs, irrespective of the inducing agent. The observation that the inhibitory effect of the two compounds in combination is larger than the sum of either compound alone suggests that a synergistic cooperation between serine proteases and metalloproteases might be involved in the stimulated shedding of TNF-R75. Cooperation between these proteases has also been suggested for the PMA-induced shedding of TNF receptors in myeloid cell lines [20]. Based on previous observations, showing that resting PMN release substantial amounts of TNF-R75 but negligible amounts of TNF-R55 [9], we tested the effect of KB8301 and DFP on the resting release of both receptors. Table 1 shows that in contrast to the release of

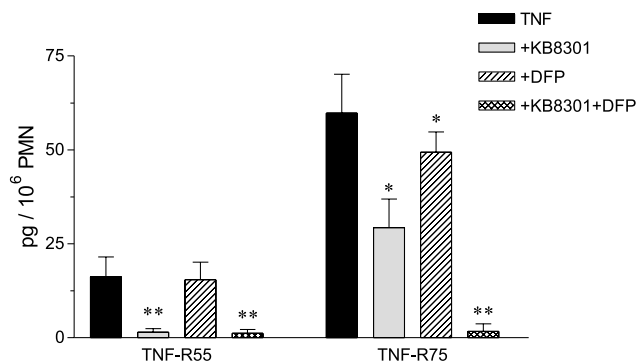


Fig. 1. Effect of the metalloprotease inhibitor KB8301 and the serine protease inhibitor DFP on the TNF-induced shedding of TNF receptors in human PMN. PMN (5×10^6 /ml in HBS-BSA) were pre-incubated at 37°C for 3 min with or without 5 $\mu\text{g}/\text{ml}$ KB8301 and/or 1 mM DFP and then for 20 min with 2.5 ng/ml TNF. After cooling and centrifugation, the solubilized receptors were measured in the supernatants as described in Section 2. The results are means of four experiments. Bars indicate standard deviations. * and **, statistical differences compared with TNF-treated cells ($P < 0.05$ and $P < 0.01$, respectively).

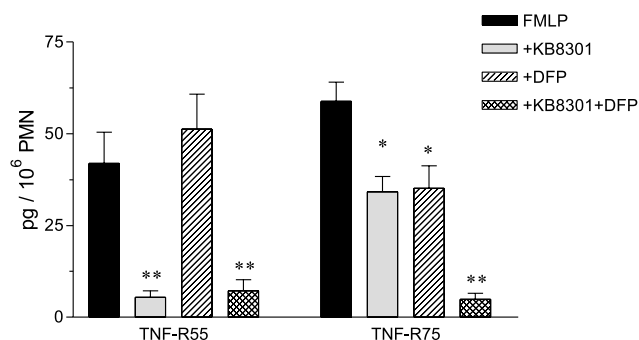


Fig. 2. Effect of the metalloprotease inhibitor KB8301 and the serine protease inhibitor DFP on the FMLP-induced shedding of TNF receptors in human PMN. PMN (5×10^6 /ml in HBS-BSA) were pre-incubated at 37°C for 3 min with or without $5 \mu\text{g}/\text{ml}$ KB8301 and/or 1 mM DFP and then for 20 min with 5×10^{-8} M FMLP. After cooling and centrifugation, the solubilized receptors were measured in the supernatants as described in Section 2. The results are means of three experiments. Bars indicate standard deviations. * and **, statistical differences compared with FMLP-treated cells ($P < 0.05$ and $P < 0.01$, respectively).

TNF-R55 (strong inhibition by KB8301, insensitivity to DFP), release of TNF-R75 was unaffected by KB8301 and almost totally inhibited by DFP. Thus, a serine protease appears to be responsible for the resting release of TNF-R75.

3.2. Elastase is the TNF-R75-releasing serine protease

It has been shown that azurophil granule-enriched fractions from either resting or FMLP-activated PMN were potent down-regulators of TNF receptors [29]. The azurophil granule-associated TNF-receptor releasing activity responsible for this down-modulation, affecting almost exclusively TNF-R75, was tentatively identified as elastase on the basis of the fact that it was inhibited by DFP and elastase inhibitors and was reproduced by exogenously added purified elastase [29].

Curiously, however, the elastase-dependent TNF-R75 cleaving activity of azurophil granules was considered distinct from the sheddase operative in intact stimulated PMN and regarded as an additional mechanism for the control of cellular responses to TNF at sites of inflammation, secondary to azurophilic granule exocytosis [29]. This latter observation might appear paradoxical if one takes into consideration that human PMN contain abundant amounts of elastase [30,31]. Moreover, this protease is expressed in an active form on the cell surface and its expression is increased in stimulated cells ([32–35] and our data not shown). We therefore hypothesized that elastase is the serine protease responsible for TNF-R75 shed-

ding from resting PMN and also for the DFP-inhibitable fraction of the receptor released from TNF or FMLP-stimulated PMN. We tested this possibility using the macromolecular physiological inhibitor of elastase, $\alpha 1$ -protease inhibitor, and two chemically unrelated elastase-specific synthetic inhibitors. One of them, compound ZM200,355, belongs to a family of trifluoromethylketone derivatives [22,36] and has been shown to be highly selective for neutrophil elastase with very little or no inhibitory activity, even at relatively high concentrations, against other types of serine proteases (e.g. cathepsins), cysteine proteases or MMPs [37]. The other, compound L-658758, belongs to a family of cephalosporin-based β lactam inhibitors shown to be excellent inhibitors of leukocyte elastase with no significant activity against serine proteinases such as cathepsin G and trypsin [23,38].

Both compounds were used at concentrations reported to inhibit elastase in isolated neutrophils by more than 80% [36,39]. Fig. 3 shows that ZM200,355 and L-658758 com-

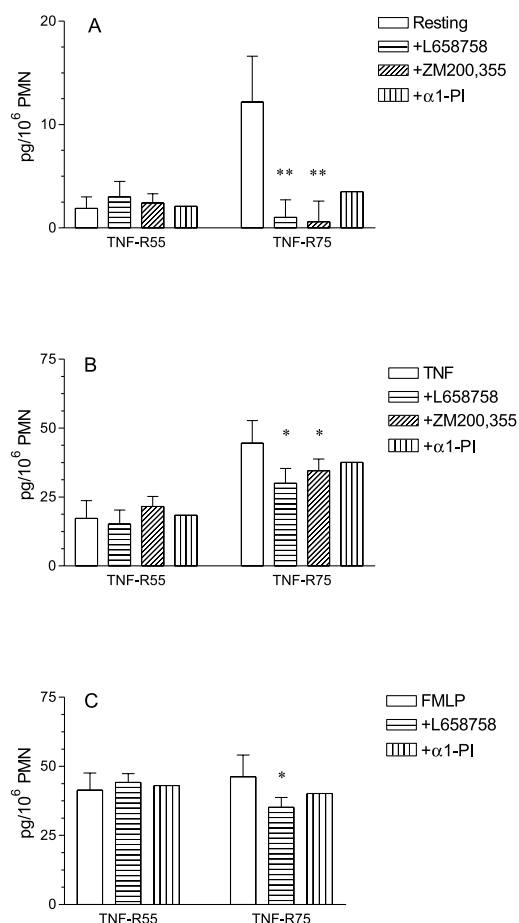


Fig. 3. Effect of the elastase-specific synthetic inhibitors L658758 and ZM200,355 and the physiologic elastase inhibitor $\alpha 1$ -PI on the release of TNF receptors from resting (A) and from TNF- (B) or FMLP- (C) treated PMN. PMN (5×10^6 /ml in HBS-BSA) were pre-incubated at 37°C for 3 min with or without $25 \mu\text{g}/\text{ml}$ L658758, $20 \mu\text{M}$ ZM200,355 or $20 \mu\text{g}/\text{ml}$ $\alpha 1$ -PI, and then for 20 min without (resting) and with $2.5 \text{ ng}/\text{ml}$ TNF or 5×10^{-8} M FMLP. After cooling and centrifugation, the solubilized receptors were measured in the supernatants as described in Section 2. The results are the means of three to four experiments with L658758 and ZM200,355 and of two experiments with $\alpha 1$ -PI. Bars indicate standard deviations. * and **, statistical differences compared with cells in the absence of inhibitors ($P < 0.05$ and $P < 0.01$, respectively).

Table 1

Effect of KB8301 and DFP on the release of TNF receptors from resting human PMN^a

	TNF-R55 (pg/10 ⁶ cells) ^b	TNF-R75 (pg/10 ⁶ cells) ^b
Resting	2.26 ± 0.7	16.90 ± 5.9
+5 μM KB8301	0.26 ± 0.2*	14.91 ± 4.4
+1 mM DFP	1.56 ± 0.6	0.22 ± 0.7*

* $P < 0.01$ vs. resting PMN.

^aPMN (5×10^6 /ml) after temperature equilibration for 10 min at 37°C were incubated for 20 min under constant agitation. After cooling at 0°C , cells were centrifuged at $400 \times g$ for 7 min, and the supernatants used for soluble receptors assay, as described in Section 2.

^bThe results are the means $\pm$ S.D. of five experiments.

pletely inhibit the release of TNF-R75 from resting cells and, partially, the release from stimulated cells, without affecting the release of TNF-R55, as expected. α 1-Protease inhibitor also inhibited the release of the receptor, although its effect was less pronounced than that induced by the synthetic inhibitors. This latter finding would not be unexpected if indeed a membrane-bound elastase is involved in shedding. In fact, it has been shown that, contrary to soluble elastase, membrane-associated elastase is less effectively inhibited by macromolecular inhibitors such as α 1-protease inhibitor [32]. Taken altogether, these results suggest that elastase, in a membrane-associated form, is involved in the cleavage of TNF-R75. The membrane localization of the enzyme is additionally supported by the recent demonstration that supernatants from PMN stimulated with FMLP are devoid of TNF receptor-solubilizing activity [9].

3.3. Evidence for metalloprotease and elastase involvement in shedding of TNF-R75 by molecular analysis of the solubilized fragments of the receptor

It has previously been shown that cleavage of TNF-R75 by elastase generates a fragment of approximately 32 kDa, whereas a fragment of about 42 kDa is formed after cleavage by the metalloprotease [29]. To further confirm the involvement of a metalloprotease and elastase in shedding of TNF-R75, we determined whether fragments of the receptor with a molecular weight compatible with those generated by the two proteases were present in supernatants from resting and stimulated PMN.

Fig. 4 shows that supernatants from resting PMN after incubation for 60 min exhibited a band of about 28 kDa (lane 1). An incubation time of 60 min, instead of 20 min (the incubation time used in other experiments), was chosen with resting PMN since after 20 min of incubation no bands

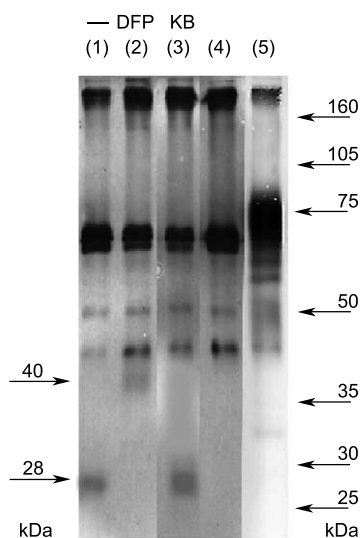


Fig. 4. Western blot analysis of TNF-R75 fragments released by resting PMN. PMN ($25\text{--}30 \times 10^6/\text{ml}$ in HBS-BSA) were incubated in the absence (lane 1) or presence of 1 mM DFP or of 7.5 μM KB8301 (lane 3) for 60 min at 37°C. After cooling on ice and centrifugation, the supernatants were collected and soluble TNF-R75 fragments, after immunoprecipitation, were analyzed by immunoblotting as described in Section 2. Lane 4: mock immunoprecipitate carried out with the incubation medium. Lane 5: immunoprecipitate from a whole-cell lysate where the 75-kDa broad band of the intact receptor is apparent.

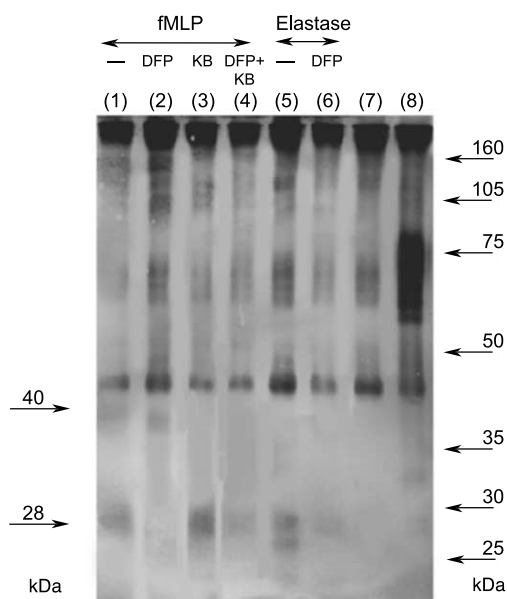


Fig. 5. Western blot analysis of TNF-R75 fragments released by PMN after FMLP stimulation or after elastase treatment. PMN ($25\text{--}30 \times 10^6/\text{ml}$ in HBS-BSA) after preincubation for 3 min with 1 mM DFP (lanes 2 and 6), 7.5 μM KB8301 (lane 3), both inhibitors (lane 4) or without addition (lanes 1 and 5) were incubated with 10^{-7} M FMLP (lanes 1–4) or 20 $\mu\text{g}/\text{ml}$ elastase (lanes 5 and 6) for 20 min at 37°C. After cooling on ice and centrifugation, the supernatants were collected and soluble TNF-R75 fragments, after immunoprecipitation, were analyzed by immunoblotting as described in Section 2. Lane 7: mock immunoprecipitate carried out with the incubation medium. Lane 8: immunoprecipitate from a whole-cell lysate where the 75-kDa broad band of the intact receptor is apparent.

were detected in the region encompassing the molecular weights of the soluble fragments of TNF-R75 (data not shown). In the supernatants from PMN incubated with DFP, the 28-kDa band was not detected, but rather a new band of about 40 kDa became visible (lane 2). The 28-kDa band was unaffected by PMN incubation with the metalloprotease inhibitor KB8301 (lane 3). Thus, resting PMN release in a DFP-inhibitable manner a fragment of the TNF-R75 with a molecular weight (28 kDa) compatible with the molecular weight of the fragment generated by elastase [29]. The 40-kDa band observed in the supernatants from DFP-treated PMN may represent the result of some degree of metalloprotease activity that becomes apparent after elastase inhibition at incubation times longer than 20 min, even in resting PMN.

After incubation with FMLP (Fig. 5), two bands of 28 and 40 kDa were observed (lane 1), although the latter band was not evident in all experiments. DFP (lane 2), KB8301 (lane 3), or in combination (lane 4) prevented the appearance of the 28-kDa band, the 40-kDa band or both bands, respectively. Fig. 5 also shows that in the supernatants from PMN incubated with exogenously added elastase, two bands of similar molecular weight were present: one 28-kDa band that may be the counterpart of the DFP-inhibitable band observed in supernatants from resting and FMLP-treated PMN and the other having a mass of 26.5 kDa. This latter band may be the product of receptor cleavage at an additional site by porcine pancreatic elastase (the enzyme used in these experiments), which has been shown to slightly differ in amino acid specificity from human leukocyte elastase [40].

In conclusion, the results of this study demonstrate that elastase, in addition to the previously described metalloprotease [9], is involved in shedding of TNF-R75 from human PMN. In particular, a membrane-bound form of the enzyme appears to participate in this process and to entirely account for receptor release in resting PMN. In stimulated PMN, a considerable fraction of receptor release is still accounted for by elastase, even though in quantitative terms most is due to the activity of a metalloprotease. Neutrophil elastase therefore appears to play a two-fold role in shedding of TNF-R-75. It acts in an autocrine manner in intact resting or stimulated PMN, as shown in the present report, and, after its release, acts in a paracrine manner, both on PMN and on other cell types present at inflammatory sites, as previously suggested [29]. Only a limited number of cell functions have been demonstrated to be mediated by TNF-R75. In PMN, in particular, this receptor has been demonstrated to cooperate with TNF-R55, the signaling receptor, for optimal responses to TNF ([26] and references therein). Therefore, the shedding activity of elastase may be regarded, on the one hand, as a mechanism wherein TNF-binding proteins in the form of soluble receptors are made available that subsequently participate in the modulation of the pathophysiologic effects of TNF and, on the other, as a means to dampen down TNF-R55-mediated responses of PMN to TNF, by decreasing expression of TNF-R75.

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