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Mechanism of Release of Soluble Forms of Tumor Necrosis Factor/Lymphotoxin Receptors by Phorbol Myristate Acetate-Stimulated Human THP-1 Cells in vitro

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Abstract. The mechanism involved in the release of the soluble forms of 55 and 75 kDa TNF and lymphotoxin (LT) membrane receptors was studied in a continuous human monocytic cell line, THP-1, in vitro. THP-1 cells were found to spontaneously release soluble forms of both 55 and 75 kDa TNF/LT receptors. Release was up-regulated by PMA, and optimal release was achieved at 10^{-8} M PMA. Serine protease inhibitors such as PMSF,3,4 dichloroisocoumarin, Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were found to inhibit the production of both soluble TNF/LT receptors. PMSF (2 mM) also blocked receptors shedding from paraformaldehyde-fixed THP-1 cells coincubated with conditioned media from PMA-stimulated THP-1 cells. Colchicine at 1 and 10 μM stimulated the production of both soluble TNF/LT receptors, but the PMA-induced release of both soluble TNF/LT receptors was inhibited. It appears that the PMA-induced release of soluble TNF/LT receptors involves serine proteases in the extracellular space where the soluble parts of the TNF/LT receptors are cleaved directly off the cell membrane. *Journal of Immunology,* 1993, 151: 5631.

TNF-α and LT[2] are related cytokines produced by activated macrophages and lymphocytes (1–3). Two distinct TNF/LT membrane receptors of 55 and 75 kDa have been isolated, sequenced, and cloned (4–6). Both receptors are anchored on the cell membrane by a single transmembrane region. The extracellular domains share 28% homology, whereas the intracellular domains share none. The Kd values of 55 and 75 KDa receptors for TNF are approximately 0.5 and 0.1 nM, respectively (4–6). The specific function of each receptor is still under investigation. Soluble forms of both receptors of 30 to 40 kDa have been identified in urine from normal individuals and patients with chronic inflammatory diseases (7, 8) and in serum from patients with various types of cancer (9, 10). These soluble forms are the NH_2-terminal extracellular domains of these membrane receptors and have the ability to bind to and inactivate human TNF and LT in vitro and in vivo.

The role of soluble TNF/LT receptors in immunologic reactions is not clear. It has been suggested that they may have important roles in the regulation of TNF and LT activity in vivo. They may help cancer cells to evade immunosurveillance by blocking TNF and LT activity (11). However, soluble receptors were also found to stabilize TNF activity in vitro at low TNF:receptor ratios (12). Thus, these molecules may have both positive and negative regulatory effects. The mechanism by which soluble TNF/LT receptors are released from cells is still largely unknown. Immunoprecipitation studies by Lantz et al. (13) suggested that the soluble form receptor derived from the 55 kDa TNF/LT receptor is generated by proteolytic cleavage of the membrane receptor. Porteu and Nathan (14) showed FMLP can stimulate human neutrophils to release soluble TNF/LT receptors in vitro. Porteu et al. (15) also found that elastase in the azurophil granules of neutrophils could

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[2] Abbreviations used in this paper: LT, lymphotoxin; TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone.

cleave a 32-kDa fragment from the membrane-bound 75 kDa receptor. However, inhibitors of elastase could not block FMLP-induced receptor release. Thus, the role of elastase in the release of soluble TNF/LT receptors by these cells in vitro is not clear.

In this study, PMA-stimulated human THP-1 cells were used to study the mechanism of release of soluble TNF/LT receptors. The data suggest that the PMA-induced release is probably mediated by the serine proteases that cleave the NH_2-terminal extracellular portion of TNF/LT receptors off the cell membrane extracellularly.

Materials and Methods

Cell line and reagents

THP-1, a human monocytic cell line that grows in suspension, was purchased from American Type Culture Collection (Rockville, MD). These cells were passed twice a week in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Irvine Scientific, Santa Ana, CA). PMA and the various inhibitors colchicine, PMSF,3,4 dichloroisocoumarin, TLCK, and TPCK were purchased from Sigma Chemical Co. (St. Louis). Recombinant forms of the soluble 55 and 75 kDa human TNF/LT receptors were kindly provided by Synergen (Boulder, CO). Rat anti-human 75 kDa TNF receptor mAb was kindly provided by Immunex (Immunex Corp., Seattle, WA).

Cell stimulation and supernatant collection

THP-1 cells from 3 to 4 day cultures were pelleted by centrifugation at 400 × *g* for 10 min and resuspended to a density of 8×10^5 cells/ml in RPMI 1640 supplemented with 10% FBS in a 50 ml polypropylene centrifuge tube (Corning Glass Works, Corning, NY). One milliliter aliquots were dispensed in each well of a 24-well polystyrene plate (Corning Glass Works). One hundred times concentrated stock solutions of PMA were added to each well in a volume of 10 μl, and the plate was incubated at 37°C in a CO_2 incubator for 8 h. The supernatants were then collected for the dose-response study. In the time course study, PMA (10^{-8} M) was added to each well, and supernatants were collected at various time points after incubation at 37°C in a CO_2 incubator. In the studies with inhibitors, supernatant were collected after 6 h incubation of the cells with both PMA and the specific inhibitor. Supernatants were cleared of cells by centrifugation at 500 × *g* for 5 min and were assayed for the concentration of soluble TNF/LT receptors by ELISA, as described in the following section.

ELISA for soluble 55 and 75 kDa TNF receptors

Anti-TNF receptor sera was generated by immunization of rabbits with human recombinant 55 or 75 kDa receptors by the method of Yamamoto et al. (16). The IgG fraction of rabbit serum was purified using a protein G (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity column by the method of Ey et al. (17). The IgG fraction was then labeled with horseradish peroxidase (Sigma Chemical Co.) as described (18, 19). The specificity of the antisera used in this study has been checked. No cross-reactivity was observed when antisera were tested against each TNF receptor, and no reactivity was observed when antisera were tested against human recombinant forms of LT, TNF, IFN-γ, IL-1β, IL-2, IL-4, and IL-6. To start the ELISA, 100 μl of unlabeled IgG (5 μg/ml in 0.05 M sodium bicarbonate buffer, pH 9.5) was added to each well of a 96-well ELISA plate (Corning Glass Works) and incubated at 4°C overnight. Individual wells were washed three times with 300 μl 0.2% Tween 20 in PBS. One hundred microliter samples and recombinant TNF/LT receptor standards were then added to each well. The plates were incubated at 37°C for 3 h. The wells were then washed and 100 μl of peroxidase-labeled IgG was added. The substrate (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Pierce, Rockford, IL); hydrogen peroxide, 30% (Fisher Scientific, Fair Lawn, NJ) was prepared as instructed by the manufacturer. After 1 h incubation at 37°C with peroxidase-labeled IgG, wells were washed and 100 μl of substrate solution was added to each well. The plates were incubated at room temperature for 20 min and the results were obtained by measuring OD405 on an EAR 400 AT plate reader (SLT-Lab Instruments, Salzburg, Austria). The concentration of soluble receptors in each sample was calculated from the regression line computed by known standards for each receptor. The background absorbance was approximately 0.17 for the blank. The concentration of standards ranged from 125 pg/ml to 10 ng/ml. The highest values of the standard curve were approximately 1.3. Most of the R^2 values of the linear regression were greater than 0.99.

Assessment of TNF receptor release from paraformaldehyde-fixed THP-1 cells

THP-1 cells (approximately 10^8 cells) from 3 to 4 day cultures were pelleted as described above in six 50 ml polypropylene centrifuge tubes. Forty milliliters of PBS was added to each tube to resuspend the cells. The cells in each tube were pelleted again and resuspended in 10 ml PBS. Ten milliliters of 2% paraformaldehyde (Polysciences, Warrington, PA) in PBS was then added to each tube and the resulting suspension was incubated for 20 min at room temperature. The cells were then pelleted and washed with 40 ml of PBS for three times. The pellets were then resuspended, pooled together in 10 ml PBS, and stored at 4°C until use.

THP-1 cells (8×10^5 cells/ml) were stimulated with 10^{-8}

M PMA in a T150 polystyrene tissue culture flask for 24 h at 37°C. The supernatant from this culture was collected and cleared of cells by centrifugation at 400 × *g* for 10 min. It was then preincubated with or without 2 mM PMSF for 1 h at 37°C. An aliquot of PMSF stock solution was then added to the supernatant to make the final PMSF concentration 4 mM. Five hundred microliters of supernatant were combined with 500 μl of the paraformaldehyde-fixed THP-1 cells in individual wells of a 24-well polystyrene plate. After incubating at 37°C for 3 to 4 h, the supernatants were collected and cleared of cells by centrifugation at 500 × *g* for 5 min. The concentration of soluble TNF/LT receptors was then obtained by ELISA.

Molecular mass of soluble TNF/LT receptors

THP-1 cells (8×10^5 cells/ml) in 500 ml RPMI 1640 were stimulated with 10^{-8} M PMA overnight. The supernatant was collected then cleared of cells and concentrated to 15 ml by Amicon membrane filtration system using a filter with m.w. cut at 10 kDa (Amicon, Beverly, MA). Three milliliters of the concentrated supernatant was loaded on a Sephadex G100 (Pharmacia Fine Chemicals) column (2.5 × 44 cm) equilibrated in PBS buffer. The column was eluted with PBS at flow rate of 0.5 ml/min and the eluate was collected in 3.7-ml fractions. All fractions were assayed by ELISA to determine the presence of soluble TNF/LT receptors. The peak fractions of the soluble TNF/LT receptors from six runs were pooled to obtain a final volume of approximately 240 ml each. The pooled eluates were concentrated in two steps to approximately 750 μl, first with the Amicon membrane filtration system, followed by Centriprep 10 concentrator (m.w. cut at 10 kDa) (Amicon). The soluble receptor from the 55 kDa TNF/LT receptor was further purified by running the concentrated eluate on a 10% preparative SDS-PAGE in Model 291 Prep Cell using the procedures provided by the manufacturer (Bio-Rad, Richmond, CA). Volumes of the concentrated samples containing 5 ng of soluble receptor from either the 55 or 75 kDa receptor were loaded and run on a 1.5 mm 10% acrylamide slab gel as described (20). The proteins were then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). Immunostaining was performed using the biotin-streptavidine system (Amersham, Amersham, UK) and peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA).

Results

Release of soluble 55 and 75 kDa TNF/LT receptors by PMA-stimulated THP-1 cells in vitro

THP-1 cells were cultured alone or with different concentrations of PMA for 8 h in a 24-well plate in a CO_2 incubator. Cell-free supernatants were assayed for soluble forms of both 55 and 75 kDa TNF/LT receptors by ELISA. As shown in the control columns of Figures 1*A* and *B*, THP-1 cells spontaneously released soluble forms of both receptors. However, the level of soluble TNF/LT receptors increased with increasing concentrations of PMA. The optimal effect of PMA in inducing TNF/LT receptors release was reached at 10^{-8} M. Next, 10^{-8} M PMA was used to stimulate the THP-1 cells and the time course of release was observed (Fig. 1*C* and *D*).

M.W. of soluble TNF/LT receptors in supernatants from PMA-stimulated THP-1 cells

The soluble TNF/LT receptors were partially purified by gel filtration and preparative SDS-PAGE. Partially purified samples were then subjected to Western blotting. Each soluble TNF/LT receptor showed specific single band. The soluble TNF/LT receptor derived from the 55 kDa membrane receptor corresponds with a 30-kDa band (Fig. 2*A*, lane 1), whereas that from the 75 kDa membrane receptor corresponds with a 40-kDa band (Fig. 2*B*, lane 1). To demonstrate the specificity of these bands, immunostaining was performed in the presence of excess amount of free recombinant extracellular portion of each TNF/LT receptor. Both bands disappeared in the presence of competing molecules (Fig. 2*A* and *B*, lane 2).

A major concern of the specificity of the rabbit anti-human 75 kDa TNF receptor polyclonal antibody was brought up by a major nonspecific band observed in the 67 kDa region in Figure 2*B*. One possible problem is that this nonspecific protein is also secreted by macrophages and is up-regulated by PMA. To validate the ELISA data, a rat anti-human 75 kDa TNF receptor mAb was used to establish another ELISA. The difference between data obtained from the polyclonal and the monoclonal ELISA was approximately 5% (data not shown). The values obtained from the monoclonal ELISA were actually higher than those from the polyclonal ELISA. These findings eliminate the possibilities that the polyclonal ELISA was picking up changes of a nonspecific protein.

Effects of serine protease inhibitors on the release of soluble TNF/LT receptors by THP-1 cells in vitro

Several serine protease inhibitors were tested for their effects on the release of soluble TNF/LT receptors. In general, a dose-dependent inhibition of soluble TNF/LT receptors release could be demonstrated. As shown in Table I, the PMA-induced release of soluble receptor of the 55 and 75 kDa TNF/LT receptors decreased as the dose of inhibitors increased. 3,4 Dichloroisocoumarin failed to inhibit the spontaneous release of both soluble TNF/LT receptors, but showed significant inhibition on the PMA-induced release (for 55 kDa, $p < 0.001$ for both concentrations; for 75 kDa, $p < 0.02$ at 0.01 mM and

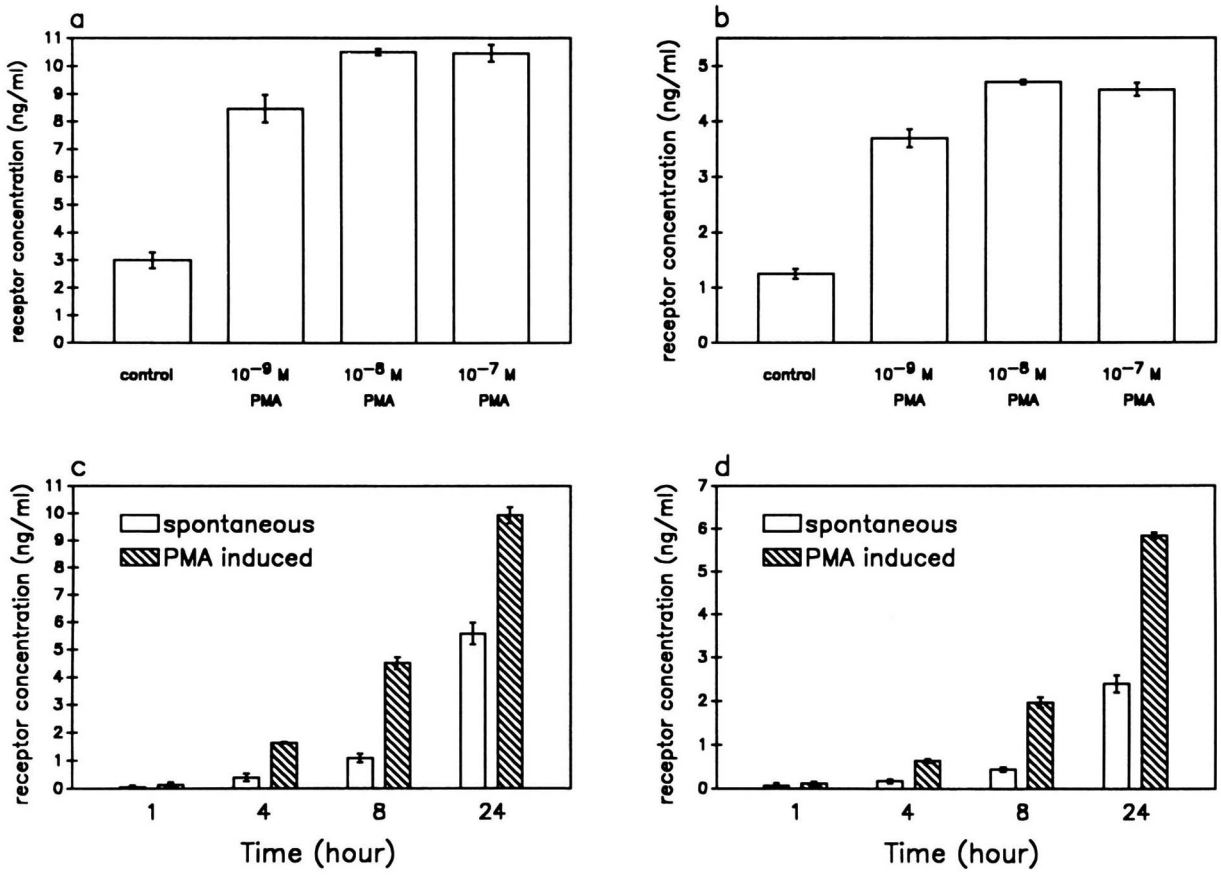

FIGURE 1. Dose response and time course of PMA-stimulated release of soluble TNF receptors. THP-1 cells were cultured with different concentrations of PMA for 8 h as described in *Materials and Methods*. The concentration of the soluble receptors was measured by ELISA (*A*, 55 kDa; *B*, 75 kDa). The time course was done with PMA at 10^{-8} M (*C*, 55 kDa; *D*, 75 kDa).

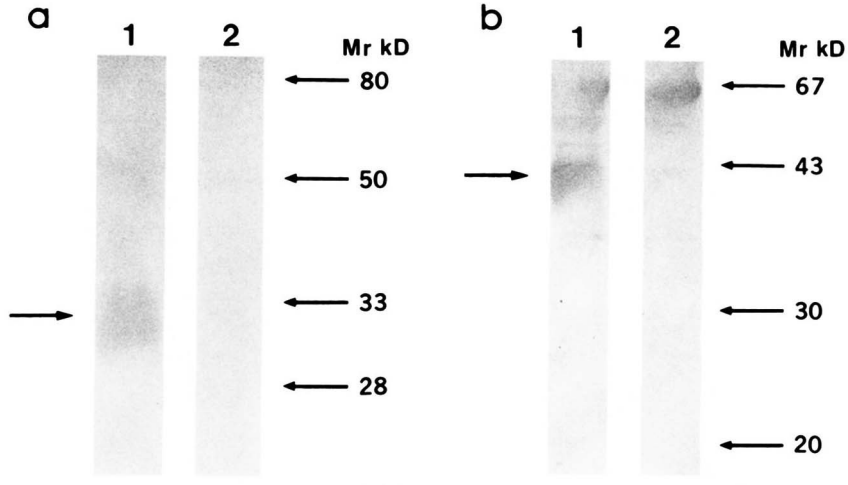

FIGURE 2. M.w. of the soluble TNF/LT receptors. Soluble TNF/LT receptors were partially purified from the supernatant collected from PMA-stimulated THP-1 cells by gel filtration and preparative SDS-PAGE, as described in *Materials and Methods*. The partially purified samples were subjected to Western blotting. *A*: soluble form receptor of 55 kDa TNF/LT receptor. *B*: soluble form receptor of 75 kDa TNF/LT receptor. Lane 1, samples alone: Lane 2, 100-fold excess of free recombinant soluble TNF/LT receptors coincubated with the anti-TNF/LT receptor antibodies.

$p < 0.01$ at 0.10 mM). TPCK at 0.1 mM generated a pattern similar to that of 3,4 dichloroisocoumarin. TPCK at 1 mM appeared to shut off the release of soluble TNF/LT receptors independent of PMA stimulation. However, the effect was secondary to the cytotoxicity of TPCK that killed all the cells at 1 mM. TLCK (0.01 and 0.1 mM) showed the

Table I
Effect of serine protease inhibitors on the release of soluble TNF receptors

	Spontaneous (ng/ml)		PMA-Induced (ng/ml)	
	55 kDa	75 kDa	55 kDa	75 kDa
Control	1.26 ± 0.23	1.22 ± 0.20	5.56 ± 0.32	4.60 ± 0.45
PMSF (mM)				
0.25	1.03 ± 0.04	1.10 ± 0.09	4.64 ± 0.74	4.36 ± 0.61
1.00	0.94 ± 0.12	1.04 ± 0.12	3.88 ± 0.36	3.64 ± 0.44
4.00	0.51 ± 0.24	0.65 ± 0.21	0.78 ± 0.69	0.87 ± 0.56
3,4 Dichloroisocoumarin (mM)				
0.01	1.20 ± 0.04	1.24 ± 0.08	4.16 ± 0.52	3.79 ± 0.36
0.10	1.33 ± 0.06	1.39 ± 0.04	3.92 ± 0.23	3.56 ± 0.18
TLCK (mM)				
0.01	0.01 ± 0.02	0.04 ± 0.02	0.14 ± 0.03	0.22 ± 0.02
0.10	0.01 ± 0.01	0.04 ± 0.01	0.08 ± 0.04	0.15 ± 0.02
TPCK (mM)				
0.10	1.11 ± 0.21	1.10 ± 0.20	3.13 ± 0.15	2.59 ± 0.12
1.00	0.00 ± 0.00	0.06 ± 0.01	0.00 ± 0.00	0.05 ± 0.02

same strong inhibitory effects without changing the survival rate of cells (99.7 and 99.8%, respectively vs 99.7% of control) measured by trypan blue exclusion method. PMSF showed moderate inhibitory effects on the release of both receptors. But the stronger effect observed for 4 mM might be secondary to lower survival rate of cells (76.9 vs 99.7% of control).

Effects of PMSF on the shedding of soluble TNF/LT receptors from the paraformaldehyde-fixed THP-1 cells by conditioned media

To further study whether the soluble TNF/LT receptors can be shed directly from the cell surface by proteases that can be inhibited by PMSF, THP-1 cells were fixed with paraformaldehyde and coincubated with conditioned media from 24 h PMA-stimulated THP-1 cells for 3 to 4 h. The amount of soluble TNF/LT receptors in the presence of PMSF is significantly lower than that of control and that in the presence of ethanol (Fig. 3*A* and *B*, $p < 0.001$ for both receptors). PMSF alone can slow down the degradation of soluble TNF/LT receptors when incubated with the conditioned media in the absence of paraformaldehyde-fixed cells (data not shown).

Effect of colchicine on the release of soluble TNF/LT receptors

Colchicine, an inhibitor of microtubule formation, stimulated the release of both soluble receptors (Fig. 4). The strength of induction appeared to be inversely related to dose. There was no cooperative effect between colchicine and PMA. The levels of soluble receptors were between the levels achieved with the individual agents used independently (Fig. 4). It seems that the inducing effect of PMA is antagonized by the colchicine.

Discussion

The soluble forms of membrane receptors have been identified for cytokine receptors IL-1R (21), IL-2R (22), IL-4R (23), and TNFR (7–10); receptors on lymphoid cells CD14 (24), CD23 (25), CD16 (26, 27), lymphocytes homing receptors (28), and CD27 (29); and receptors on nonlymphoid cells DAF (30) and GHR (31). A number of these soluble receptors have been identified in biologic fluids and they are still capable of specific ligand binding. The biologic significance of these soluble form receptors is under active investigation. It has been reported that alternative mRNA splicing is probably involved in producing a soluble IL-4R in mouse T cell line in vitro (23). Proteolysis and shedding were proposed as mechanisms involved in the release of several other soluble receptors (21, 22, 24–28).

Soluble form receptor derived from the 55 kDa TNF/LT receptor was identified by Gatanaga et al. (9) in the sera of cancer patients with various forms of cancer. This soluble form of 30 kDa has the ability to inhibit human TNF and LT activity both in vitro and in vivo (11). Elevated serum levels of soluble TNF/LT receptors in patients with solid tumors have also been reported by Aderka et al. (10). The ability of these receptors to inhibit TNF and LT bioactivity and the presence of the soluble TNF/LT receptors in these patients led these investigators to the hypothesis that soluble TNF/LT receptors may be immunosuppressive and allow cancer cells to evade host anti-tumor mechanisms. However, it was also proposed by Aderka et al. (12) that the soluble receptors at low receptor: TNF ratios could stabilize TNF molecules in vitro. It is clear that additional research is necessary to define the roles of these molecules in vivo.

In this study, soluble TNF/LT receptor release by a human macrophagelike cell line THP-1 was examined. These

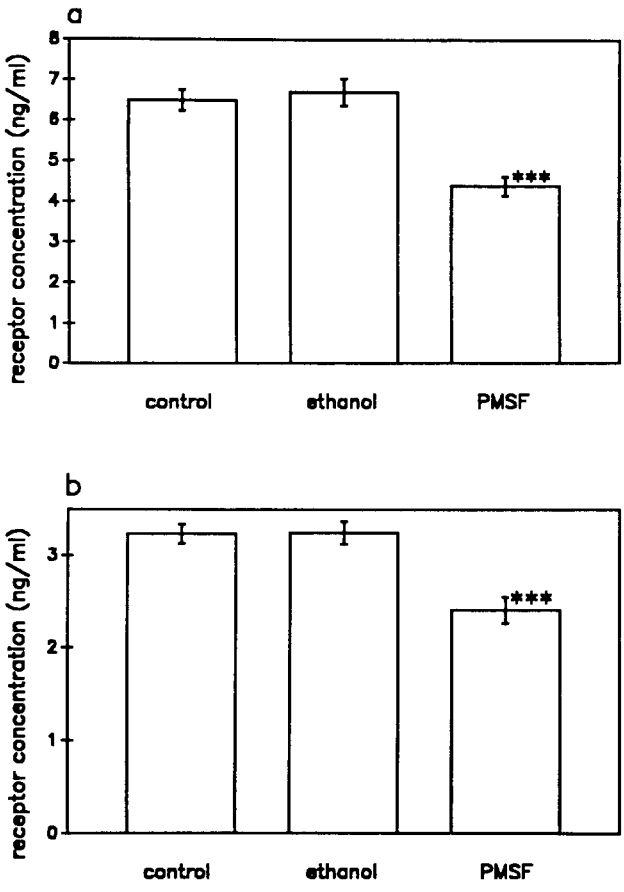

FIGURE 3. PMSF inhibits the shedding of soluble TNF receptors from paraformaldehyde-fixed THP-1 cells. The conditioned medium from PMA-stimulated THP-1 cells was preincubated with PMSF (2 mM) and ethanol (2%) for 1 h at 37°C. The paraformaldehyde-fixed THP-1 cells were then coincubated with the conditioned medium in the presence of ethanol (2%) or PMSF (2 mM) for 4 h at 37°C. The amount of the soluble receptors were assayed by ELISA. *A*: the concentration of soluble form of 55 kDa TNF/LT receptors. Control, 6.49 ± 0.26 ng/ml; ethanol, 6.69 ± 0.34 ng/ml; PMSF, 4.38 ± 0.24 ng/ml. *B*: the concentration of soluble form of 75 kDa TNF/LT receptor. Control, 3.23 ± 0.11 ng/ml; ethanol, 3.25 ± 0.12 ng/ml; PMSF, 2.41 ± 0.14 ng/ml. ***$p < 0.001$.

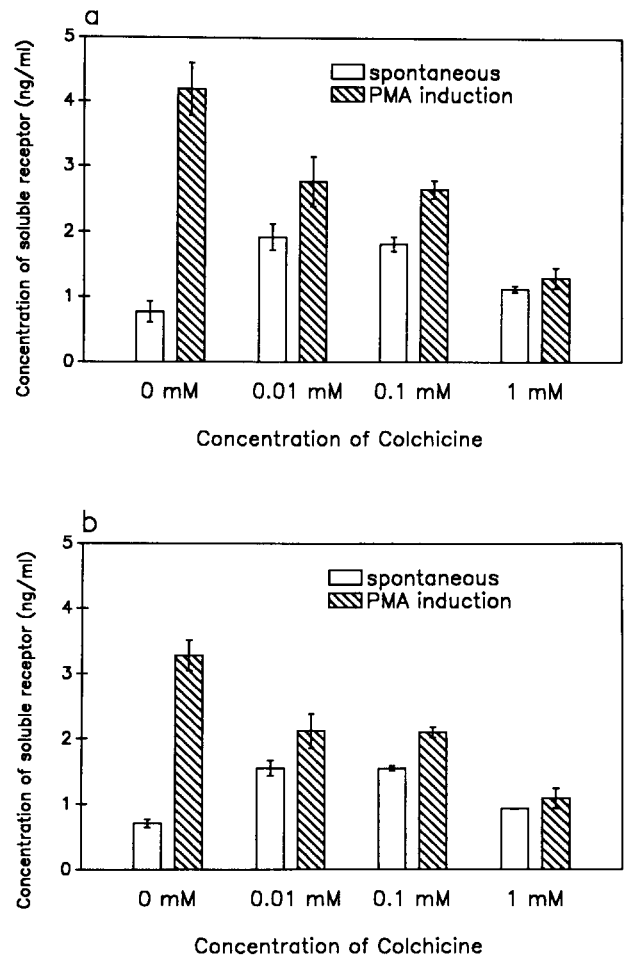

FIGURE 4. Colchicine stimulates the release of soluble TNF/LT receptors and inhibits the inducing effects of PMA. THP-1 cells were resuspended to 1 × 10^6 cells/ml in fresh medium and incubated with colchicine and/or PMA (10^{-8} M) for 8 h. The blank box shows effect of the colchicine on the spontaneous release. The hatched box shows effect of the colchicine on PMA-induced release. *A*: soluble form of 55 kD TNF/LT receptor. *B*: soluble form of 75 kDa TNF/LT receptor.

cells were stimulated with PMA, a protein kinase C activator, which was shown to stimulate the release of soluble forms of CD14 from human peripheral blood monocytes (24), homing receptors from mouse lymphocytes (28), and TNF/LT receptors from various human macrophage cell lines (15, 32, 33). The soluble TNF/LT receptors are not products of degradation because 1) they produce specific bands by Western blotting and 2) supernatants collected from frozen and thawed THP-1 cells destroys more than 70% of the soluble TNF/LT receptors preexisting in cell-free supernatants within 1 h of coincubation (data not shown). Although both TNF receptors showed almost the same response to PMA (Fig. 1), it cannot be concluded that the effect of PMA is nonspecific. It was shown that PMA did not stimulate the release of CD11c, MHC class I molecules, and CD64 (24). PMA-induced release of both soluble TNF/LT receptors is inhibited by all the serine proteases used in this study in a dose-dependent pattern. Spontaneous release is less sensitive to the inhibitory effect of serine proteases. This difference is best exemplified by 3,4 dichloroisocoumarin, which does not inhibit spontaneous release, whereas maintaining fairly strong inhibition of PMA-induced release. One possible explanation for this discrepancy is that PMA induction recruits serine proteases different from those responsible for the spontaneous release to increase the rate of soluble TNF/LT receptors production. These newly recruited serine proteases are relatively more sensitive to 3,4 dichloroisocoumarin and TPCK. The location where serine proteases work is most likely outside

the cell because PMSF significantly inhibits the production of soluble TNF/LT receptors from paraformaldehyde-fixed THP-1 cells. The ability of colchicine to counteract the inducing effect of the PMA also implies that PMA is recruiting serine proteases from inside the cells, possibly those stored in microtubule-associated vesicles. The speculated explanation for colchicine-induced receptor release is colchicine acting to inhibit internalization of TNF/LT receptors for turnover by lysosomes. The end result would be more TNF/LT receptors remaining on the plasma membrane for serine proteases to cut.

Serine proteases were found to be involved in the enzymatic cleavage and shedding of IL-1R from a human B cell line (21) and of CD14 from human monocytes in vitro (24). The release of the soluble CD16-II from human NK cells (34) and folate receptor from human nasopharyngeal carcinoma cells (35) was attributed to metalloproteases. Receptor shedding by proteases may represent a new mechanism in the turnover of membrane receptors and in the regulation of cell responsiveness to a ligand.

This report demonstrates that serine protease is responsible for both spontaneous and PMA-induced shedding of soluble forms of both TNF/LT receptors from the THP-1 cells in vitro. It supports the idea that the soluble receptor derived from the 55 kDa TNF/LT receptor is generated by extracellular proteolytic cleavage, as proposed by Lantz et al. (13). It further shows that the soluble form of the 75 kDa TNF/LT receptor is probably produced in the same manner.

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