A Metalloprotease Inhibitor Blocks Shedding of the 80-kD TNF Receptor and TNF Processing in T Lymphocytes

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Summary

TNF is synthesized as a 26-kD membrane-anchored precursor and is proteolytically processed at the cell surface to yield the mature secreted 17-kD polypeptide. The 80-kD tumor necrosis factor (TNF) receptor (TNFR80) is also proteolytically cleaved at the cell surface (shed), releasing a soluble ligand-binding receptor fragment. Since processing of TNF and TNFR80 occurs concurrently in activated T cells, we asked whether a common protease may be involved. Here, we present evidence that a recently described inhibitor of TNF processing N-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2'napthyl)-alanyl-L-alanine, 2-aminoethyl amide (TAPI) also blocks shedding of TNFR80, suggesting that these processes may be coordinately regulated during T cell activation. In addition, studies of murine fibroblasts transfected with human TNFR80, or a cytoplasmic deletion form of TNFR80, reveal that inhibition of TNFR80 shedding by TAPI is independent of receptor phosphorylation and does not require the receptor cytoplasmic domain.

Materials and Methods

Reagents and Cells. All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 µg/ml streptomycin and penicillin and 2 mM glutamine (complete medium).
PBMC were obtained from healthy volunteers by Ficoll-Hypaque centrifugation (21) and washed and resuspended in complete medium. Effector T cells were obtained by culturing PBMC in complete medium containing anti-CD3 (OKT3) for 3 d followed by complete medium supplemented with rIL-2 (10 ng/ml) for 7 d. These cells were used as a model for differentiated effector T cells and, when indicated, were restimulated with anti-CD3 (20 ng/ml) and PMA (10 ng/ml), as described previously (19). The L.929 (murine fibrosarcoma) cell line stably expressing human TNFRs0 (Lp80) (19) was cultured in complete medium containing 400 µg/ml G418 (GIBCO, Grand Island, NY). Ionomycin was purchased from Sigma Chemical Co. (St. Louis, MO), and PMA was from LC Laboratories (Woburn, MA).

Flow Cytometry. Cells were stained with primary mAbs anti-TNFFRs0 (M1) (20) or anti-TNF (104C) at a concentration of 10 µg/ml for 30 min in ice-cold binding buffer (HBSS supplemented with 10% newborn calf serum, 10 µg/ml human IgG, 20 mM Hepes, pH 7.2, and 0.1% sodium azide). Washed cells were incubated with PE-conjugated goat anti-murine or goat anti-rat IgG (affinity purified, Southern Biotechnology Associates, Birmingham, AL) for 30 min, washed twice, and analyzed directly with a FACScan® instrument (Becton Dickinson, Mountain View, CA). Cell viability was >90% as monitored by propidium iodide staining.

Soluble TNFRs0 and TNF ELISA. Human peripheral blood T cells (PBT) were isolated via sequential purification with isopaque, SRBC-rosetting, and G10 passage, as previously described (22). PBT were stimulated with OKT3 (10 µg/ml, solid phase) and PMA (10 ng/ml) in the presence or absence of protease inhibitors. Protease inhibitors included TAPI (compound 2 in [4]), trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane (E64); N-carboxymethyl-Phe-Leu); and α-1-antitrypsin (all from Sigma). Soluble TNFRs0 and TNF levels were quantitated from culture supernatants obtained 24 h after activation. TNFRs0 levels were evaluated with a commercial ELISA (R & D Systems, Minneapolis, MN). Human TNF levels were quantitated with antibodies derived at Immunex Corp. (Seattle, WA). Briefly, maxisorp plates (Nunc, Roskilde, Denmark) were incubated overnight with anti-TNF mAb (M1), as described previously (19). Briefly, cells were incubated with 500 pM 1~I-M1 for 1 h at 4°C. Nonspecific binding was determined by incubation in the presence of unlabeled M1. Unbound mAb was removed by four washes with cold binding buffer and bound mAb quantitated by gamma counting.

Results and Discussion

TAPI Inhibits TNF Release and TNFRs0 Shedding in Activated T Lymphocytes. Resting effector T cells (activated 7 d earlier with anti-CD3) do not secrete detectable levels of TNF and do not express any surface TNF detectable by flow cytometry (Fig. 1 A). However, within 2 h of reactivation, using a combination of phorbol ester (PMA) and calcium ionophore, abundant surface TNF was present. Consistent with previous findings (4), surface TNF expression is greatly enhanced when effector T cells are activated in the presence of TAPI, reflecting inhibition of TNF processing.

In contrast to TNF, resting effector T cells constitutively express TNFRs0 on their surfaces. However, reactivation of these cells with PMA/calcium ionophore or PMA/OKT3 triggers rapid downregulation of TNFRs0 (Fig. 1, B and C [20]). In the presence of TAPI, activation-induced loss of surface TNFRs0 was significantly inhibited (Fig. 1, B and C). A time-course analysis of effector T cells activated with phorbol ester and anti-CD3 (PMA/OKT3) confirmed that TAPI inhibits downregulation of TNFRs0 (Fig. 1 D). However, TNFRs0 surface expression diminishes even in the presence of TAPI, albeit more slowly and incompletely than cells not treated with TAPI.

Previous studies have established that downregulation of TNFRs0 in activated effector T cells occurs by shedding, releasing a soluble fragment of the receptor from the cell surface (20). To determine if the inhibition of TNFRs0 downregulation seen in the presence of TAPI is due to blockade of shedding, soluble TNFRs0 production was quantitated by ELISA. As shown in Fig. 2 A, TAPI caused a dose-dependent reduction in the amount of soluble TNFRs0 detectable in culture supernatants from activated T cells. In the presence of 200 µM TAPI, shedding of TNFRs0 was inhibited by ~80%. The concentration of TAPI necessary for half-maximal inhibition of TNFRs0 shedding is between 25 and 50 µM, which is comparable to the concentration required for half-maximal inhibition of TNF release (~50 µM) by these same cells (4). In addition, examination of various protease inhibitors showed that those which do not block TNF release by activated T cells also fail to prevent TNFRs0 shedding (Fig. 2 B), further supporting the hypothesis that a similar protease mediates both of these processes.

Pulse-chase Analysis of TNF and TNFRs0 Processing in Activated T Cells. To investigate the effect of TAPI on synthesis and processing of TNF and TNFRs0 by activated effector T cells in more detail, a pulse-chase labeling experiment was performed (Fig. 3). To study TNF processing, effector T cells activated for 1 h with PMA/OKT3 were pulse-labeled for 30 min with 35S-S-Cys/Met in the presence or absence of TAPI, then chased with unlabeled Cys/Met. As expected, the 26-kD TNF propeptide was immunoprecipitated only from cell lysates, whereas the 17-kD secreted form of TNF was detected only in the supernatant (Fig. 3 A). The rapid loss of 26-kD cell-associated TNF during the chase
period and concomitant accumulation of the 17-kD form in the supernatant, but not intracellularly, indicates that the TNF propeptide is processed to the 17-kD form primarily at the cell surface. In contrast, when the pulse-chase is performed in the presence of TAPI (Fig. 3 B), cell-associated-26-kD

**Figure 1.** Enhanced expression of surface TNF and inhibition of TNFR$_{80}$ downregulation in activated effector T cells. PBT cells activated with OKT3 and cultured in IL-2 for 7 d were reactivated with a combination of PMA and ionomycin (A and B) or PMA/OKT3 (C) in the presence or absence of TAPI (200 μM). After 2 h, cells were harvested from culture and incubated on ice with either anti-TNF antisera (A), or anti-TNFR$_{80}$ mAb (B and C) for 30 min, then stained with PE-conjugated goat anti-mouse or anti-rat IgG for 30 min, as described in Materials and Methods. (D) PBT cells cultured as described above and reactivated with a combination of PMA and OKT3 in the presence (●) or absence (○) of TAPI (200 μM) were harvested from culture at various times and incubated with anti-TNF$_{80}$ mAb, then stained with PE-conjugated goat anti-rat IgG for analysis by flow cytometry.

**Figure 2.** Inhibition of secreted TNF and soluble TNFR$_{80}$ production by TAPI. Human PBTC were stimulated with plate-bound OKT3 and PMA in the presence of (A) various concentrations of TAPI or (B) other protease inhibitors (50 μM); α-antitrypsin was at 1 μg/ml. Supernatants were harvested and the levels of TNF and soluble TNFR$_{80}$ determined by ELISA. □ TNF, ■ TNFR$_{80}$.

TNF accumulates, but no secreted 17-kD TNF is detected, demonstrating that TAPI is acting at the cell surface to inhibit TNF processing. Accumulation of the 26-kD cell-associated polypeptide is followed by a gradual decrease in the density of its autoradiographic signal after 20 min, which probably reflects a decrease in specific activity as a consequence of continued protein synthesis during the chase and indicates that TAPI is not inhibiting protein synthesis.

Activated effector T cells rapidly downregulate TNFR$_{80}$ by proteolytic processing of the mature 80-kD protein at the cell surface, releasing a soluble 40-kD fragment of the extracellular domain (Fig. 3 C) (20). In the presence of TAPI, however, cell-associated 80-kD TNFR polypeptide accumulates and no soluble 40-kD fragment is detected in the supernatant, even after 80 min, confirming that proteolytic processing of TNFR$_{80}$ is inhibited (Fig. 3 D). In spite of this, processing of the 70-kD TNFR$_{80}$ precursor protein to the
Figure 3. Inhibition of TNF and TNF-R<sub>60</sub> processing in activated T lymphocytes by TAPI. Effector T cells incubated with PMA/anti-CD3 for 60 min were pulse-labeled for 30 min with <sup>35</sup>S-Cys/Met in the absence (A) or presence (B) of TAPI (200 μM), then chased with complete medium ± TAPI. At various times, an aliquot of cells was centrifuged, and TNF immunoprecipitated from the cell-free supernatants or from cell lysates, using rabbit anti-TNF antisera, was resolved by SDS-PAGE (12% gel) and autoradiography (8-d exposure). Processed TNF lacks two met residues, accounting for the lower autoradiographic band density relative to the 26-kD form. Effector T cells were pulse-labeled for 30 min with <sup>35</sup>S-Cys/Met in the absence (C) or presence (D) of TAPI (200 μM), then chased with complete medium containing PMA/anti-CD3 + TAPI. At various times, an aliquot of cells was centrifuged to prepare cell lysates and cell-free supernatants for immunoprecipitation, using anti-TNF-R<sub>60</sub> mAb (M1). TNF-R<sub>60</sub> was resolved by SDS-PAGE (10% gel) and autoradiography of the dried gel (10-d exposure).

80-kD form continues in the presence of TAPI, demonstrating that maturation of TNF-R<sub>60</sub> is not inhibited. Collectively, these findings clearly show that TAPI inhibits shedding of TNF-R<sub>60</sub> and suggest that the initial inhibition of down-regulation seen by flow cytometric analysis is also due to inhibition of shedding. The incongruity between surface staining and measurement of soluble receptor levels at later time points indicates that, when shedding is blocked, TNF-R<sub>60</sub> may be downregulated by an alternate process. In this regard, mutations in TNF-R<sub>60</sub>, which inhibit proteolytic cleavage, do not block downregulation in response to PMA, indicating that multiple mechanisms for downregulating TNFR are operative (23). Furthermore, we have observed that TAPI has no effect on expression of CD4 which is downregulated by internalization after T cell activation (24), but TAPI partially inhibits downregulation of TNF-R<sub>60</sub>, which is also subject to proteolytic cleavage (25) (data not shown) on neutrophils.

**TAPI Inhibits Shedding of TNF-R<sub>60</sub> in L929-TNF-R<sub>60</sub>-stable Transfectants.** To further investigate the mechanism of inhibition of TNF-R<sub>60</sub> shedding in a more defined cellular system, mouse L929 cells stably transfected with human TNF-R<sub>60</sub> (Lp80) were activated with phorbol ester (PMA, 100 ng/ml) for 30 min in the presence or absence of TAPI, and surface TNF-R<sub>60</sub> levels were detected by a radioimmunoassay, using anti-TNF-R<sub>60</sub> mAb. As shown in Table 1, Lp80 shed ~73% of their receptors after stimulation with PMA, whereas, in the presence of TAPI, they shed only ~27% of their TNF-R<sub>60</sub> (63% inhibition). Thus, the protease which regulates shedding of human TNF-R<sub>60</sub> expressed in mouse cells is also sensitive to inhibition by TAPI.
Table 1. Shedding of TNFR<sub>60</sub> by Lp80 and Lp80Δcyt

|            | Control      | TAPI         |
|------------|--------------|--------------|
| TNFR<sub>60</sub> expression |             |              |
| (125I-M1 bound, cpm) |            |              |
| Lp80       |              |              |
| - PMA      | 21,909 ± 212 | 20,466 ± 297 |
| + PMA      | 5,872 ± 206 (73) | 14,973 ± 417 (27) |
| Lp80Δcyt   |              |              |
| - PMA      | 20,807 ± 1,012 | 21,572 ± 861 |
| + PMA      | 12,415 ± 624 (40) | 17,762 ± 118 (18) |

Cells (1.5 x 10<sup>5</sup>/well in 24-well plates) were preincubated with media only (control), and, then, TAPI (20 μM) for 90 min at 37°C, before activation with PMA (100 ng/ml) for 30 min. Specific 125I-M1 binding was determined by incubating cells with 125I-M1 (500 pM) for 1 h at 4°C. The results shown are means ± SEM of two separate experiments. Nonspecific binding cpm determined in the presence of 200-fold excess unlabeled M1 were 1,743 ± 106 for Lp80, and 2,289 ± 52 for Lp80Δcyt. Numbers in parentheses represent percent loss of specific 125I-M1 binding compared to unactivated cells.

Previously, we have shown that TNFR<sub>60</sub> is constitutively phosphorylated and that phosphorylation is a late processing event which precedes proteolytic cleavage of TNFR<sub>60</sub> (19). In addition, constitutive phosphorylation and shedding of TNFR<sub>60</sub> were blocked by the protein kinase inhibitor staurosporine, suggesting a link between receptor phosphorylation and proteolysis. As observed in Fig. 3 D, the gradual increase in apparent molecular mass of T cell–associated TNFR<sub>60</sub> in the presence of TAPI suggested that phosphorylation of TNFR<sub>60</sub> continues, even though shedding is blocked. However, to directly examine whether TAPI affects phosphorylation of TNFR<sub>60</sub>, Lp80 cells were labeled with [32P]-orthophosphate in the presence or absence of TAPI before treatment with PMA. Cell extracts were then subjected to immunoprecipitation using M1. As shown in Fig. 4, TAPI did not inhibit constitutive phosphorylation of TNFR<sub>60</sub>, but did block activation-induced loss of phosphorylated receptor. To confirm that inhibition of TNFR<sub>60</sub> shedding by TAPI is independent of receptor phosphorylation, L929 cells expressing a mutant form of the receptor lacking the entire cytoplasmic domain (Lp80Δcyt) were tested for activation-induced loss of TNFR<sub>60</sub> in the presence or absence of TAPI. Truncated TNFR<sub>60</sub> expressed by these cells is not phosphorylated but is shed in response to PMA, albeit more slowly and incompletely compared to cells expressing the full-length receptor (19). As seen in Table 1, Lp80Δcyt shed ~40% of their TNFR<sub>60</sub>, but, in the presence of TAPI only, ~18% was shed (55% inhibition), clearly demonstrating that inhibition of TNFR<sub>60</sub> shedding by TAPI does not involve the receptor cytoplasmic domain, and, therefore, is independent of receptor phosphorylation.

In conclusion, the data presented here show that a cell-surface metalloprotease is involved in shedding of TNFR<sub>60</sub> by activated T lymphocytes. Furthermore, the observation that TNF processing and TNFR<sub>60</sub> shedding occur concomitantly and are similarly sensitive to inhibition by TAPI strongly suggests that these processes are coordinately regulated during T cell activation. Notably, the protease cleavage site in TNF (Ala2-Va1214) is also present in the extracellular domain of TNFR<sub>60</sub> (Ala213-Va1214) at a site consistent with the observed molecular weight of the shed receptor fragment (26). Thus, metalloprotease inhibitors such as TAPI may offer protection from the deleterious systemic effects of TNF at two levels simultaneously: first, by preventing the release of soluble TNF, and second, by blocking accumulation of shed TNFR; soluble TNF has been shown to stabilize TNF activity in vitro (10). It will be of interest to determine whether other members of the TNF ligand family or the TNFR family are also subject to coordinated proteolytic processing.

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