Aminopeptidase N (CD13) Regulates Tumor Necrosis Factor-α-induced Apoptosis in Human Neutrophils

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Neutrophil apoptosis plays a central role in the resolution of granulocytic inflammation. We have shown previously that tumor necrosis factor-α (TNFα) enhances the rate of neutrophil apoptosis at early time points via a mechanism involving both TNF receptor (TNFR) I and TNFRII. Here we reveal a marked but consistent variation in the magnitude of the pro-apoptotic effect of TNFα in neutrophils isolated from healthy donors, and we show that inhibition of cell surface aminopeptidase N (APN) using actinonin, bestatin, or inhibitory peptides significantly enhanced the efficacy of TNFα-induced killing. Notably, an inverse correlation is shown to exist between neutrophil APN activity and the sensitivity of donor cells to TNFα-induced apoptosis. Inhibition of cell surface APN appears to interfere with the shedding of TNFR, and as a consequence results in augmented TNFα-induced apoptosis, cell polarization, and TNFα-primed, formyl-methionyl-leucyl-phenylalanine-stimulated respiratory burst. Of note, actinonin and bestatin had no effect on TNFRII expression under resting or TNFRα-stimulated conditions and did not alter CXCR1 or CXCR2 expression. These data suggest significant variation in the activity of APN/CD13 on the cell surface of neutrophils in normal individuals and reveal a novel mechanism whereby APN/CD13 regulates TNFα-induced apoptosis via inhibition of TNFR shedding. This has therapeutic relevance for driving neutrophil apoptosis in vivo.

Neutrophil apoptosis is critical to the resolution of acute inflammation and the prevention of granulocytes-mediated tissue injury (1, 2). In chronic inflammatory conditions such as acute respiratory distress syndrome, (3, 4) rheumatoid arthritis, and the systemic inflammatory response syndrome (5–9), host tissue damage is thought to arise in part from ineffective clearance of neutrophils because of delayed apoptosis or impaired phagocytic clearance. Neutrophils undergo constitutive apoptosis when aged in vitro, and this provides an ideal model to study the regulation of apoptotic thresholds in vivo. The cytokine TNFα is unique in its ability to act both as a neutrophil-priming agent and as an inducer of apoptosis. As a priming agonist TNFα is able to enhance greatly the ability of other inflammatory mediators (such as fMLP and IL-8) to induce degranulation and respiratory burst activity (10). TNFα also stimulates apoptosis in a subpopulation of neutrophils between 1 and 6 h in culture (11, 12), although it causes an overall survival response after 20 h in culture (11). TNFα therefore appears to be one of the very few natural ligands capable of inducing early neutrophil apoptosis, and an ability to enhance this effect may have important therapeutic implications in the resolution of neutrophilic inflammation.

Aminopeptidases (AP) catalyze the removal of amino acids from peptide substrates and play an important role in protein maturation, hormone regulation, and the degradation of biologically active proteins and peptides (13). One of the most widely studied AP is the 967-amino acid protein termed aminopeptidase N (APN). This peptidase is identical to the cell surface antigen CD13 (14) and displays activity toward an array of substrates, including the enkephalins, bradykinin, bacterial derived chemotactic peptides, tachykinins, and the cytokines granulocyte colony-stimulating factor, interferon-γ, IL-1β, IL-2, IL-6, and IL-8 (15, 16). Moreover, CD13 is involved in cell surface antigen processing through the “trimming” of HLA class I- or class I-bound peptides on antigen-presenting cells (17–19). AP have also been reported to play an important role in the evolution of the inflammatory response, because they are responsible for the formation of leukotriene B4 from leukotriene A4 (20). AP inhibitors, such as actinonin and bestatin, have been reported to have anti-tumor effects through augmentation of the host immune system (21–25). More relevant to the present study, bestatin has been shown to induce apoptosis through inhibition of cell surface APN (15, 26) and increase apoptosis in human leukemic cell lines by enhancing processing of caspase-3 in the presence of death-inducing ligands such as TNFα and CH11 (anti-Fas mAb) (27).

In light of previous findings that AP inhibitors augment apoptosis in cancer cells exposed to TNFα (27), we explored the possibility that neutrophils, which express high levels of cell surface AP (28, 29), may also demonstrate this effect. We have shown previously that the pro-apoptotic effect of TNFα requires, unusually, co-ordination of both TNFR and TNFRII (11) and that NF-κB plays a key role in regulating the cytotoxic efficacy of this cytokine (30). Here we show that a range of biological and chemical inhibitors of APN amplify TNFα-induced signaling in neutrophils, in particular that they augment TNFα-induced apoptosis, TNFα-induced priming, and respiratory burst activation (as

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3 The abbreviations used are: TNFα, tumor necrosis factor-α; TNFR, TNF receptor; fMLP, formylmethionylleucylphenylalanine; APN, aminopeptidase N; AP, aminopeptidases; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; mAb, monoclonal antibody; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; IL, interleukin; ANOVA, analysis of variance; PAF, platelet-activating factor; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay; sTNFR, soluble TNFR; pNA, p-nitroanilide; RT, reverse transcription.
indicated by an increase in FMLP-stimulated superoxide anion generation), and TNFα-induced shape change. This effect is mediated via the ability of APN inhibitors to prevent the shedding of cell surface TNFRI with no effect on TNFRII or CXCR1/2 expression or cleavage. We have further demonstrated the ability of different priming agents to affect APN activity and for this to subsequently dictate the efficacy of TNFα-stimulated apoptosis. Moreover, the marked inter-donor variation we now report for TNFα-induced neutrophil apoptosis is shown to correlate with the individual neutrophil APN activity. These data suggest that neutrophil cell surface APN/CD13 activity is a key determinant of TNFRII expression and function and that variation in APN/CD13 activity dictates the efficacy of TNFα-induced neutrophil apoptosis.

MATERIALS AND METHODS

Isolation of Human Neutrophils—Neutrophils were purified by dextran sedimentation and discontinuous plasma PercollTM gradients (31). Purified cells were resuspended at 5 × 10⁶ cells/ml in Iscove’s modified Dulbecco’s medium supplemented with 10% autologous serum and 50 units/ml streptomycin and penicillin. Neutrophils were cultured in the presence or absence of actinonin (100 μM), bestatin (100 μM), azide-free CD13 blocking antibody (8 μg/ml; WM15), or PAF (1 μM) with or without TNFα (10 ng/ml).

Assessment of Neutrophil Apoptosis in Vitro—Neutrophils were harvested at the time points indicated, cyto-centrifuged, fixed in methanol, and stained with May-Grunwald-Giemsa (Merck), and the morphology was examined by oil immersion light microscopy. Apoptotic neutrophils were defined as those with darkly stained pyknotic nuclei (300 cells counted/slide and 3 slides per experimental condition and time point) (32). Apoptosis was also assessed by FACS with FITC-labeled recombinant human annexin V and propidium iodide staining (33). In the studies examining the effect of caspase inhibition on TNFα-induced apoptosis, neutrophils were preincubated (20 min) with the broad spectrum caspase inhibitor Z-VAD-fmk (30 μM) in the presence or absence of actinonin (0.1 μM) prior to the addition of vehicle control or TNFα for 6 h. Caspase-3 activity was measured by colorimetric substrate reaction (Calbiochem) according to the manufacturer’s instructions.

Neutrophil Shape Change—Neutrophils were preincubated in the presence or absence of actinonin (0.1 mM) or CD13 antibody (8 μg/ml) for 20 min in Dulbecco’s PBS; FMLP (100 nM, positive control) or TNFα (12 ng/ml) were then added for 30 min. The incubations were stopped with an equal volume of ice-cold glutaraldehyde (final concentration 1.6% [v/v]). The effect of actinonin and CD13 blocking antibody on neutrophil polarization was assessed by FACS analysis as detailed previously (34).

Neutrophil Respiratory Burst Activity—Respiratory burst activity was assessed by measuring the generation of superoxide anion using superoxide dismutase-inhibitable reduction of cytochrome c as described previously (11).

APN Activity—Cell surface AP activity was determined as the rate of amide bond hydrolysis of alanine p-nitroanilide substrate (Sigma). Alanine-p-nitroanilide (final concentration 2 mM) was incubated with 1 × 10⁶ cells at 37 °C for 30 min. Amide bond hydrolysis was quantified by p-nitroanilide absorbance at 405 nm, corrected for spontaneous hydrolysis of the substrate, and converted to mM/min. For the APN inhibitor studies actinonin (0.1 mM), bestatin (0.1 mM), and CD13 blocking antibody (8 μg/ml) were incubated for 20 min at 37 °C prior to addition of vehicle control or TNFα. In studies utilizing PAF (1 μM), neutrophils were preincubated for 5 min prior to APN inhibitor or TNFα incubation.

APN RT-PCR—Total RNA was isolated using RNeasy column (Qiagen, Crawley, UK). RNA (2 μg) was transcribed into cDNA using oligo(dT) primers (Invitrogen) and 50 units of reverse transcriptase (Stratagene, Cedar Creek, TX). PCR amplification was performed using specific primer sets for CD13 (sense, 5’-ACG CCA CCT CTA CCA TCA TC; antisense, 5’-AGC ACC ACC TCC TTG TTC TC, 304-bp product). For control reactions, a specific primer set for β-actin (sense, 5’-GGG CGC CCC AGG CAC CA; antisense, 3’-CTT AA TAT GTC AGC CAG CAC GAT TTTC, 548-bp product) was used. PCR (28 cycles) was performed using 2 units of AmpliTaq DNA polymerase (Bioline, London, UK). PCR products were analyzed by agarose gel electrophoresis and imaged with ethidium bromide under UV light.

Measurement of TNFRI/II and CXCR1/2 Cell Surface Expression—Neutrophils (1 × 10⁶ cells per condition) were preincubated with actinonin or bestatin (both at 0.1 mM) for 30 min at 37 °C in PBS. For TNFRI and TNFRII expression, this incubation was followed by a 30- (TNFRI) or 60-min (TNFRII) exposure to TNFα (10 ng/ml). In the case of CXCR1 and -2 studies, the preincubation with AP inhibitors was followed by 1 h of incubation with either IL-8 (500 ng/ml) or TNFα (50 ng/ml) in the presence or absence of bestatin. The cells were centrifuged at 256 × g for 5 min at 4 °C and then washed with ice-cold PBS. The cells were then incubated with optimal concentrations of FITC-conjugated anti-TNFRI, TNFRII, CXCR1, or anti-CXCR2 antibodies (R & D Systems, Abingdon, UK) for 40 min at 4 °C. The cells were washed with cold PBS and resuspended for analysis on a FACS (BD Biosciences). CellQuest software was used for gating and analysis of cell population.

Soluble TNFRII/II ELISA—Cell supernatants were obtained from the above series of experiments by analyzing the cell surface expression of TNFRII and centrifuged at 1000 × g for 10 min at 4 °C and stored at −80 °C until analysis. ELISA for sTNFRI and sTNFRII were performed according to the manufacturer’s instructions. With the exception of the substrate, to enable continuous analysis during development, p-nitrophenyl phosphate (Sigma) at 1 mg/ml in diethanolamine buffer, pH 9.8, was added, and the plates were read at 405 nm in an automated plate reader (3550; Bio-Rad). Results were analyzed using a standard curve generated in parallel with each assay (Microplate Manager software; Bio-Rad). The lower level of sensitivity of the sTNFRII assay was 10 pg/ml and for the sTNFRII assay was 15 pg/ml.

Caspase-3 Activity Assay—Isolated neutrophils (2 × 10⁶) were preincubated in the presence or absence of actinonin (0.1 mM) and Z-VAD-fmk (30 μM) for 20 min prior to TNFα stimulation for 4 h. Neutrophils were pelleted and washed with ice-cold PBS and lysed with the buffer provided by the assay manufacturer. The assay was completed according to the manufacturer’s instructions, and the substrate cleavage was analyzed at 405 nm in an automated plate reader (3550; Bio-Rad).

Statistical Analysis—All data represent the mean (± S.E.) of n separate experiments unless otherwise stated. Differences between groups were assessed using the one-way analysis of variance (ANOVA) and post hoc analysis with Tukey’s multiple comparisons and Student’s t test using Graph-pad Prism software. A p value of <0.05 was considered significant.

RESULTS

We and others have reported previously on the unique property of TNFα to induce neutrophil apoptosis at early time points (11, 35, 36). This early (6 h) pro-apoptotic effect of TNFα (10 ng/ml) is confirmed in this study, but examination of the magnitude of the response in neutrophils isolated from 12 different donors identified a very broad effect (mean 18.5% and range 6.8–34.3%) (Fig. 1A). Neutrophil isolations were regularly analyzed for basal shape change and FMLP-induced superoxide anion generation to ensure minimal cell priming because this is recognized to affect the cells ability to respond to TNFα (11). Moreover,
the extent of the TNFα response for an individual was determined in three donors spanning the response range and was found to be highly reproducible (Fig. 1B).

The AP inhibitors, actinonin, and bestatin have been shown to inhibit a wide variety of cytosolic, secretory, and cell surface APs, including APN (37, 38). Sekine et al. (15) have shown previously that AP inhibitors, such as bestatin, induce apoptosis of human leukemic cell lines in a concentration-dependent manner as well as inhibiting cell growth. Their findings pointed to the involvement of an intracellular AP because blocking CD13 on the surface of leukemic cells with an inhibitory monoclonal antibody had no effect on apoptosis. Culturing isolated human neutrophils for 6 h with the AP inhibitors actinonin, bestatin, or the blocking antibody had no effect on the basal rate of neutrophil apoptosis at any inhibitor concentration used (Fig. 2A).

However, a combination of the apoptotic agent TNFα and actinonin (0.1 mM) resulted in a significant augmentation of TNFα-induced killing effect at 6 h from 16 ± 2 to 30 ± 2% (p < 0.05, n = 6) (Fig. 2A). The AP inhibitor bestatin also augmented TNFα killing to an identical extent (p < 0.01, n = 3) (Fig. 2A). To support these data we repeated the experiments using an azide-free CD13 blocking antibody (WM15) (Fig. 2A). These experiments revealed that blocking the CD13 active site also significantly increased TNFα-stimulated neutrophil apoptosis (28.7 ± 1.8%, p < 0.05, n = 3). In order to confirm augmented TNFα-induced neutrophil apoptosis, annexin V staining was assayed by FACS analysis. Fig. 2B is a representative quartet that shows no effect of actinonin on basal annexin V staining (and hence apoptosis) at 6 h, whereas TNFα-induced apoptosis increased from 15 to 32% after 6 h of culture with actinonin.

An AP activity assay was devised to determine the efficacy and optimal concentration of AP inhibitors required to inhibit whole cell surface APN. A concentration response for actinonin gave an IC50 of 3.1 μM with a maximal effective concentration of 0.1 mM; bestatin gave an IC50 of 10 μM and a maximal effective concentration of 1 mM, although 0.1 mM was used throughout this study to avoid cell toxicity. Cell surface AP activity was significantly inhibited by all three agents, actinonin (18.8 ± 2.9 μM/min/10^6 cells, p < 0.001), bestatin (12.2 ± 3.1 μM/min/10^6 cells, p < 0.001), and CD13 blocking antibody (31.0 ± 3.1 μM/min/10^6 cells, p < 0.001) when compared with control (53.5 ± 7.8 μM/min/10^6 cells, p < 0.001, n = 6) (Fig. 2C). TNFα did not affect AP activity either in control or AP inhibitor-treated cells. The protease inhibitors leupeptin (50 μM), aprotinin (0.3 μM), and pepstatin (50 μM) alone or in combination with actinonin did not alter the rate of alanine-pNa hydrolysis supporting the specificity of the APN inhibition assay (data not shown).
Any agent that affects the priming or activation state of human neutrophils may influence (in a specific or nonspecific manner) cell survival or apoptosis pathways. To investigate whether actinonin or bestatin exerted such effects, we assessed neutrophil superoxide anion generation and shape change responses because these are highly sensitive markers of neutrophil priming and activation status (34). Neutrophils undergo characteristic shape change (polarization) when challenged with chemotactic agents under nongradient conditions. Using the light scattering properties of primed and activated neutrophils compared with unprimed cells, actinonin and bestatin were shown to have no discernible effect on neutrophil shape change compared with control cells (Fig. 3A). However, TNFα resulted in a characteristic concentration-dependent shape change response with the maximal effect at 10 ng/ml, and more importantly, the blocking antibody for CD13 induced a significant leftward shift in the TNFα concentration-response curve. Actinonin also enhanced TNFα-stimulated shape change, but to a lesser degree (Fig. 3A).

Its well known that priming of neutrophils with TNFα and subsequent stimulation by the prototypic bacterial formyl peptide fMLP triggers an intense activation of the NADPH oxidase. Superoxide anion generation was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome c (11) in the presence or absence of the AP inhibitors or CD13 blocking antibody. Again, the AP inhibitors/blocking antibody alone or in combination with TNFα or fMLP had no effect on superoxide anion generation compared with the relevant controls (Fig. 3B). However, actinonin/blocking antibody caused a significant increase in the TNFα- and fMLP-stimulated superoxide anion response.

Z-VAD-fmk, used at concentrations of 30 μM, which is considered to be specific for caspase inhibition (39), caused a near complete loss of the pro-apoptotic effect of TNFα at 6 h. In addition, the augmented apoptosis response observed in the presence of actinonin was also inhibited by Z-VAD-fmk (Fig. 4A). Moreover, Z-VAD-fmk inhibited whole cell caspase-3 activity stimulated by TNFα also and that augmented by actinonin (4 h) to levels below that of control (Fig. 4B). Actinonin alone did not stimulate caspase-3 activity. Sekine et al. (15) have suggested previously that AP inhibitors could induce apoptosis through processing of caspase-3 to its active form. In neutrophils, however, AP inhibitors alone had no effect on basal apoptosis and failed to activate caspase-3 (data not shown). A robust activation of caspase-3 was observed with TNFα, in keeping with the annexin V and morphology data, and was enhanced further in the presence of AP inhibitors. These data indicate that the augmentation of TNFα-induced neutrophil apoptosis by AP inhibitors is a caspase-dependent process, but the inhibitors alone have no effect on basal caspase-3 processing.

Dri et al. (40) demonstrated TNFα induced shedding of both the TNFRI and -RII and that a membrane-bound, nonmatrix metalloprotease was involved in this process. The possibility that actinonin or bestatin may exert their effects on apoptosis through inhibition of a membrane-bound AP such as APN/CD13 and a novel interaction with TNFRI or TNFRII therefore warranted investigation. If AP was
involved in shedding or internalization of TNFRI or TNFRII then the presence of an AP inhibitor or blocking mAb may maintain receptor integrity at the neutrophil cell surface and thereby enhance TNF receptor signaling. Analysis of TNFRI cell surface expression using flow cytometry, following incubation with actinonin alone, showed a small but significant increase in receptor expression compared with control ($p < 0.01$, $n = 6$) (Fig. 5A). In contrast, TNFRII cell surface expression was not affected by actinonin (data not shown). Neutrophils incubated with 10 ng/ml TNFα for 30 min showed significant loss of both TNFRI and TNFRII compared with untreated neutrophils; however, this was not influenced by the presence of actinonin (Fig. 5A). These experiments were also repeated following 20 min of stimulation with TNFα, where TNFRI loss is less complete. Nevertheless, actinonin still did not influence TNFRI surface expression (control 12.1 ± 1.0 (mean fluorescence), TNFα 7.9 ± 0.9, actinonin 14.6 ± 1.0, TNFα/actinonin 9.2 ± 1.6; $n = 5$). However, fMLP (an agonist that induces TNFRI shedding only)-induced TNFRI shedding was completely inhibited by actinonin (Fig. 5A, inset).

To assess further the potential effects of APN on TNF shedding, soluble TNFRI/II was assayed in parallel using the supernatants generated in the TNFRI/II surface expression studies. Although actinonin did not affect the quantity of sTNFRI under control conditions (actinonin, 39.3 ± 7.5 pg/ml; control, 46.9 ± 8.5 pg/ml) (Fig. 5B), the APN inhibitor caused a marked reduction in the TNFα-stimulated increase in sTNFRI (TNFα, 135.5 ± 14.1 pg/ml; TNFα + actinonin, 76.5 ± 12 pg/ml; $p < 0.001$; $n = 6$). fMLP-stimulated shedding of TNFRI was also complemented by analysis of sTNFRI, indicating the ability of actinonin to inhibit TNFRI shedding (data not shown). The levels of sTNFRII under control or TNFα-stimulated conditions were unaffected by incubation with actinonin (Fig. 5C). These data, together with the capacity for AP inhibitors and blocking antibodies to enhance an array of TNFα-mediated neutrophil responses, suggest that these compounds enhance the efficacy of TNFα-mediated apoptosis through modulation of TNFRI expression.

We have shown previously that the pro-apoptotic effect of TNFα requires co-ligation of both TNFRI and TNFRII (11), with the TNFRII being an essential facilitator of TNFRI-mediated apoptosis. To investigate the involvement of TNFRII and TNFRII in mediating the augmentation of TNFα-induced apoptosis by actinonin, a similar series of experiments were conducted using neutralizing mAbs that selectively block TNFRI and TNFRII (11). Preincubation of neutrophils with either TNFRI mAb or in combination with TNFRII mAb completely abolished TNFα and TNFα plus actinonin-induced apoptosis (Fig. 6). However, although the TNFRII mAb alone completely blocked TNFα-induced apoptosis, it caused only a partial inhibition of TNFα/actinonin augmented apoptotic response. These data indicate that the actinonin augmentation of TNFα-induced apoptosis is mediated by TNFRI rather than TNFRII. This concurs with the selective effect of APN inhibitors on TNFRI expression and shedding (Fig. 5).

Previous reports by Bhattacharya et al. (41, 42) indicate the involvement of APs in the proteolytic cleavage of IL-8R induced by serum-
activated LPS. Bestatin was reported to inhibit serum-activated LPS-induced loss of IL-8 binding to neutrophils. However, Khandaker et al. (43) reported that bestatin had no effect on LPS- or TNF-α/H9251-induced loss of CXCR1 and CXCR2, although bestatin may influence IL-8-induced internalization of CXCR1 and CXCR2. In view of these data we considered that one plausible explanation for augmented TNF-α killing following APN inhibition might be because of a decrease in CXCR1/2 receptor expression and thereby a reduction in IL-8-induced neutrophil survival. However, Fig. 7 shows that neutrophil cell surface expression of CXCR1 and CXCR2 was unaffected by the presence of bestatin either in the presence or absence of TNF-α or IL-8, which alone caused characteristic receptor shedding (Fig. 7, A and B) (43). Therefore, we did not observe altered CXCR expression following APN inhibition, suggesting that APs are not major players in the maintenance of CXCR1/2 expression on the surface of neutrophils under these conditions.

APN activity in human neutrophils has been measured previously in different donors and classified as high, medium, or low activity (44). We confirmed this range of APN activity in donor cells (Fig. 8A; 32–78 mM/min/10⁶ cells) and showed the repeatability of AP activity within an individual donor (Fig. 8B).

One of the major observations of this study is the striking inverse correlation revealed between neutrophil APN activity and the pro-apoptotic efficacy of TNFα (ρ = −0.683, p < 0.001; n = 16) (Fig. 8C). Hence, subjects with low APN activity (<40 µM/min/10⁶ cells) all displayed high TNFα-stimulated apoptosis (>18% base line-corrected), whereas high APN activity (>70 µM/min/10⁶ cells) dictated low TNFα-stimulated apoptosis (<10% base line-corrected). A 2.5-fold difference in APN activity equated to a 6-fold difference in TNFα-stimulated apoptosis. Therefore, individual donor AP activity appears to be a major determinant of the extent of neutrophil apoptosis induced by TNFα.

The CD13 gene is mapped to chromosome 15 (q25-q26) (45), and the coding sequence spans 20 exons (46). The gene and promoter regions have been studied extensively, and a number of highly polymorphic regions have been identified, although the functional importance of these polymorphic sites has yet to be determined (47). Myeloid progenitor cells, differentiating granulocytes, and monocytes possess an additional extended promoter region containing the elements necessary for the expression of APN (48, 49). This suggests that APN expression in neutrophils is highly regulated. Semi-quantitative RT-PCR analysis for
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CD13 using primers designed to encompass the coded region exons 18–20 identified a correlation between CD13 activity and CD13 gene expression. Fig. 9A identifies two sample donors with high and low APN activity who have corresponding differences in CD13 expression as determined by semi-quantitative RT-PCR (Fig. 9A). Densitometry analysis identified a 1.5–2-fold difference in CD13 gene expression (Fig. 9B). These data suggest that one of the principal determinants of neutrophil APN activity may be the relative abundance of mRNA.

Early TNFα killing is influenced by alterations in the priming/activation state of neutrophils. Hence, we have shown previously that priming of human neutrophils with agents such as PAF prior to exposure to TNFα abolishes TNFα-induced neutrophil apoptosis (11). This is confirmed in this study (Fig. 10A), where the extent of TNFα-induced apoptosis (20.7 ± 2.6%) was significantly reduced following prior stimulation with PAF (1 mM) (6.0 ± 1.6%, p < 0.05) to a level comparable with that of control cells (6.4 ± 1.1%). In addition we now show that PAF significantly increases APN activity, that this increase is blocked by actinonin (Fig. 10B), and that actinonin restores the capacity of PAF-treated cells to undergo apoptosis in response to TNFα (Fig. 10C).

DISCUSSION

TNFα is one of the few natural ligands capable of inducing neutrophil apoptosis. This together with the central role that apoptosis plays in the resolution of granulocytic inflammation has resulted in much interest in the molecular mechanism underlying this effect. This study attempts to address the following: (a) whether important natural variation exists in an individual's neutrophil response to TNFα, and (b) if so, the underlying mechanism for such an effect. We now describe for the first time a marked and consistent variation in the magnitude of the pro-apoptotic effect of TNFα in healthy donor neutrophils, and we propose that this relates to the extent of cell surface APN/CD13 activity. Hence, inhibition of cell surface APN (CD13) enhances the efficacy of TNFα-induced killing, augmentation of APN/CD13 activity by PAF blocks TNFα killing, and an inverse correlation is shown to exist between CD13 activity and the sensitivity of donor cells to TNFα-induced apoptosis. It is highly unlikely that the effects of the aminopeptidase inhibitors were mediated by nonspecific or toxic influences because identical effects were seen with two structurally diverse compounds and a cell-impermeable inhibitory peptide. The inhibitors had no effect on basal shape change, respiratory burst activity, or viability.

CD13 is a multifunctional membrane peptidase, originally proposed to cause N-terminal cleavage of neutral amino acids from peptide mediators resulting in activation or inactivation. More recent analysis suggests that CD13 may also function as a receptor and surface molecule participating in adhesion or signal transduction (50). Natural substrates for CD13 include vasoactive peptides and neuropeptides, including Leu- and Met-enkephalins and the chemokine IL-8 (51). Of note, bradykinin and substance P are also both natural inhibitors of APN activity (52), and this together with our demonstration that PAF enhances APN activity suggest that this molecule is subject to intense and bi-directional regulation. CD13 is expressed on stem cells and during most developmental stages of myeloid cells and is generally considered as a myelomonocytic marker (51). It is highly expressed on human neutrophils and has been found to be expressed to even higher levels during the early stages of constitutive apoptosis (53). CD13 expression has also
been found to correlate with the growth and activation of acute myeloblastic and lymphoblastic leukemia cells (45, 54). Single site polymorphisms and splice variants of CD13 have also been associated with acute myeloid leukemia (47). Incubation of leukemic cell lines with the APN inhibitors, actinonin or bestatin, inhibits cell proliferation and stimulates apoptosis via a caspase-dependent pathway (56, 57). More importantly, however, this induction of apoptosis was direct and hence may not relate mechanistically to the effect we describe in neutrophils, which clearly depends on ligation of a natural death receptor. Moreover, we have been unable to show direct activation of caspase-3 using the AP inhibitors and can mimic the inhibitor effect with an anti-CD13 mAb.

Our panel of 16 donors displayed a marked and consistent variation in CD13 activity (44), which appeared to correspond with CD13 gene expression. We show that the variation seen with CD13 activity is closely associated with the extent of TNFα-induced neutrophil apoptosis in any one donor. Hence, the lower the activity of CD13, the greater the response to TNFα and vice versa. The variation in CD13 activity in our group of normal donors was 2.5-fold; it would therefore be possible to speculate that the extreme levels of APN/CD13 activity reported in myeloid leukemia may be a factor rendering these cells resistant to TNFα-induced apoptosis.

Actinonin and bestatin strongly reduced APN activity in cultured human neutrophils, although the APN-blocking mAb WM15 appeared less effective. This suggests that actinonin and bestatin inhibit cell surface peptidases in addition to CD13. However, the ability of CD13 mAb to inhibit APN activity has been reported to vary between cell types, which may reflect the presence (and variable expression) of multiple CD13 isoforms. Five CD13 species with different glycosylation patterns have been identified on U937 cells, and this may affect the epitopes recognized by the mAb (58). This study incorporated both inhibitors (actinonin and bestatin) and blocking antibody (WM15) in the TNFα death assay and produced consistent and repeatable results. We have attempted to transfect small interfering RNA into neutrophils, but despite achieving a 2-fold reduction in CD13 mRNA levels, we have not been able to influence CD13 cell surface expression or activity. We interpret this as indicating that CD13 protein is stable and not rapidly turned over.

The caspase-dependent cell death of APN inhibitors in leukemic cells leads us to investigate this pathway in human neutrophils. We have shown previously that TNFα stimulates apoptosis via a caspase-dependent mechanism (39), and by using the broad spectrum caspase inhibitor Z-VAD-fmk at a concentration previously validated to induce maximal caspase-3 inhibition in human neutrophils (39), we could inhibit not only TNFα-stimulated cell death but also the augmented cell death response induced by actinonin. This again suggested that actinonin interacted directly with the TNFα signaling pathway. By neutralizing TNFRI and TNFRII independently, we were able to show that this interaction involved principally TNFRI rather than TNFRII. We have shown previously that the apoptotic effect of TNFα observed in neutrophils is critically dependent on engagement of both TNFRI and TNFRII (11). TNFRII has been shown to reduce the TNFα concentration required for cell killing by a mechanism not requiring intracellular signaling (59). However, the rapid kinetics of TNFRII association and dissociation suggests it may be involved in ligand passing, in which TNFα bound to TNFRII is passed over to TNFRI to enhance TNFRII signaling (60). Our study suggests that APN inhibitors facilitate TNFRII signaling to the extent that TNFRII (which is normally required for efficient TNFα-mediated killing) becomes partially redundant.

Inhibition of APN also augmented other TNFα receptor-driven responses such as priming and activation. A concentration-response curve for TNFα-stimulated neutrophil shape change demonstrated that actinonin and the CD13 blocking antibody enhanced both the potency and efficacy of the TNFα response. Superoxide anion generation provides a further marker of priming. Again, actinonin was shown to enhance the extent of the TNFα primed, fMLP-activated superoxide anion response. The mechanism responsible for this may be 2-fold. First, fMLP itself is a known substrate for CD13; therefore, inhibition of CD13 may prolong the activation signal enhancing superoxide anion generation. Second, enhanced TNFRI signaling either through a change in receptor number or affinity or ligand availability may increase neutrophil priming leading to increased fMLP-activated superoxide anion generation. Of note, blood neutrophils from patients with tuberculosis display enhanced surface expression of TNFRI with no change in TNFRII, and this results in an increased capacity for fMLP-activated superoxide anion generation (61).

TNFα induces the rapid shedding and internalization of TNFRI and shedding of TNFR1 from human peripheral blood neutrophils (62, 63). Our results confirm the rapid down-modulation of TNFRI (Fig. 5) and TNFRII (data not shown) and reveal that inhibition of APN significantly increases basal membrane expression of TNFRII (but not TNFRII) in resting neutrophils. Likewise, we confirm a rapid increase in sTNFRI and sTNFRII in response to TNFα, and we show that APN inhibition significantly reduces TNFα-stimulated shedding of TNFRI. Shedding of TNFRII was not affected by inhibition of APN. Our data therefore demonstrate that actinonin appears to increase basal surface expression of TNFRII and inhibit ligand-induced shedding of the receptor. However, there appears to be a discrepancy between the FACS analysis (Fig. 5A) and ELISA data (Fig. 5B) for cell surface TNFRII and sTNFRII, respectively. The most likely explanation for this relates to the dominant role of TNFRII internalization over shedding following TNFα stimulation. Hence, the vast majority of the loss of TNFRI following TNFα treatment relates to receptor internalization, and this would serve to mask any effect on shedding afforded by inhibition of CD13 activity. In contrast fMLP (an agonist that induces TNFRII shedding only)-stimulated TNFRII shedding is completely inhibited by actinonin.

The proteases involved in TNFR shedding belong to the metalloprotease-disintegrin (ADAM) family, a group of zinc metalloproteases, including ADAM9, -10, -17, and -19. ADAM17 (TNFα-converting enzyme) has been identified as a sheddase for cytokines and growth factor receptors, as well as adhesion molecules (64, 65). ADAM17 has been reported previously to possess TNFR1 sheddase activity, catalyzing the cleavage of the ectodomains (66). Interestingly, Cui et al. (67) identified a novel functional AP known as aminopeptidase regulator of TNFRII shedding (ARTS-1) that binds to the extracellular domain of TNFRII and facilitates the shedding of TNFRI. Although ARTS-1 possesses no sheddase activity itself, these authors hypothesized that ARTS-1 forms a complex with TNFRI that facilitates the activity of TNFα-converting enzyme. In preliminary studies we have shown that neutrophils express ARTS-1 at the mRNA level and demonstrated ARTS-1 activity using isoleucine-pNa as a substrate (APN has a very low affinity for iso-Leu). By using this assay, we have been able to demonstrate that none of the APN inhibitors used in this study inhibit iso-Leu-pNa hydrolysis, and we suggest that ARTS-1 is not involved in the APN augmentation of TNFα-induced apoptosis.

We have reported previously the capacity of PAF to inhibit the pro-apoptotic effect of TNFα in human neutrophils (11). In the absence of any significant change of TNFRI or TNFRII expression or shedding, we considered that PAF may influence receptor coupling and/or ligand binding.
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availability. This study investigated this phenomenon by analyzing the effect of PAF on APN activity. We observed a 2-fold increase in APN activity, which was associated with an abrogation of the pro-apoptotic effect of TNFα. Both these effects were completely reversed by actinonin. The rapid increase in APN activity seen with PAF may reflect enhanced trafficking of the protease to the cell surface, although this remains unclear. However, PAF has also been reported to inhibit neutrophil apoptosis via activation of the Ras/Raf/MAPK/ERK and phosphatidylinositol 3-kinase/Akt pathways (68). Our studies would suggest that AP inhibitors can overcome this “cytoprotective” effect of PAF. As such, these agents may be valuable therapeutic agents as they enhance both TNFα-mediated neutrophil apoptosis and reverse the pro-survival effect of other inflammatory mediators. These effects would both be beneficial in driving the resolution of granulocytic inflammation. Longer term stimulation with PAF may also increase CD13 message and expression. Hence, the extended promoter region of CD13 found in myeloid cells contains binding sites for the transcription factor ETS-1 (23).

In summary, we have demonstrated a consistent and repeatable interdonor variation in the extent of TNFα-induced neutrophil apoptosis, which correlates inversely with cell surface APN activity. Manipulation of APN activity by incubation of neutrophils with specific inhibitors or blocking antibody or by using a priming agent such as PAF determined the capacity of TNFα to induce apoptosis. The capacity of APN inhibitors to augment TNFα-induced apoptosis is shown to be TNFRI- and caspase-dependent and relate to their ability to influence TNFRI shedding. These findings have important implications for our understanding of individual variation in myeloid responses to TNFα and reveal a novel function for APN/CD13.

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