Tumor Necrosis Factor-α (TNFα) and FMLP Receptors Are Functionally Linked During FMLP-Stimulated Activation of Adherent Human Neutrophils

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Tumor necrosis factor-α (TNFα) is a potent cytokine that has diverse effects on tissues in vivo and in vitro. Upon activation, TNFα is synthesized and released by leukocytes including PMN. It has been shown, for example, that treating peripheral blood neutrophils (PMN) with lipopolysaccharide, granulocyte-macrophage colony-stimulating factor (GM-CSF), or interleukin-2 (IL-2) induces the appearance of TNFα mRNA within 30 minutes, with TNFα being released by 60 minutes. In addition to de novo synthesis, TNFα has been localized to at least two different cellular compartments in resting and activated cells. In resting cells, TNFα is found in intracellular compartments, such as the large secretory granules of mast cells and the secretory granules of Paneth cells. After endotoxin treatment in vivo, TNFα has been observed on the plasma membrane and in the secretory granules of eosinophils, neutrophils, and monocytes from bone marrow, spleen, lung, and the proximal intestine. In addition, metalloproteinases can release a bioactive TNFα from the membrane-bound, 26-kD form. Thus, TNFα from activated PMN may come from de novo synthesis or can be released from secretory granules or the plasma membrane.

Two receptors for TNFα have been described and cloned. The mechanisms by which TNFα activates PMN remain unclear. However, in HL-60 and other cells, ligation of TNFα receptors leads to rapid and transient increases in both diacylglycerol and ceramide. These second messengers are increased when PMN are activated by phorbol 12-myristate 13-acetate or FMLP, and ceramide levels also increase after activation by TNFα. For PMN activated while in suspension, TNFα increases FMLP-mediated phospholipase D activation, suggesting that there may be cross-talk between these two signaling pathways. Thus, it is possible that ligation of TNFα receptors initiates a signaling cascade that influences FMLP-mediated phospholipase D (PLD) activation in adherent PMN as well.

In addition to cross-talk between signaling pathways, there are recent studies that show a physical association between two different surface receptors. For example, resonance energy transfer was used to show that CR3 links FcRIII receptors to the cytoskeleton, supporting more indirect evidence that these receptors function together during PMN activation. In another study, it was shown that an Fab fragment of an anti-FcRIIB antibody inhibited both FMLP-stimulated PMN chemotaxis and transendothelial migration. Furthermore, Fc fragments, produced by the action of PMN elastase, inhibited FMLP-mediated chemotaxis. These results indicate that FcRIII and FMLP receptors are also functionally linked and suggest that direct communication between receptors for different ligands plays a role in PMN activation.

When PMN are plated onto fibrinogen and stimulated with having relative molecular weights of 55 and 75 kD (p55 and p75, respectively). Both of these receptors are constitutively expressed on PMN and the promyelocytic leukemia cell line, HL-60. Recently, Richter et al. showed that both p55 and p75 are required for superoxide production by adherent PMN stimulated with TNFα. However, the nature of the relationship between these two receptors was not examined. Both p55 and p75 are also required for TNFα potentiation of Fc-mediated phagocytosis. In this case, p55 is required for signaling, whereas p75 regulates the binding of TNFα to p55.

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FMLP, the release of lactoferrin-containing specific granules coincides with the release of H₂O₂. However, before this coordinated release of H₂O₂ and lactoferrin occurs, there is a lag period of about 45 minutes, during which PMN begin to adhere and spread on the fibrinogen-coated surface. Because ligation of β₂-integrins causes TNFα release by leukocytes, we hypothesized that FMLP binding to its receptor, in combination with adhesion to fibrinogen, might stimulate the release of TNFα. We predicted that this TNFα was a critical part of the FMLP activation pathway in adherent PMN and that blocking its activity would block H₂O₂ and lactoferrin release. Although it is currently unclear whether TNFα from PMN actually contributes to their activation, exogenous TNFα, like FMLP, can directly stimulate the release of lactoferrin and H₂O₂ from adherent PMN. To document a functional association between FMLP and TNFα receptors during FMLP-activation of adherent PMN, we used antibodies against either TNFα or TNFα receptors. We now report that antibodies recognizing TNFα, or a combination of antibodies recognizing p55 and p75, inhibited TNFα release only if FMLP-stimulated cells (30 v 45 minutes) and because ligation of β₂-integrins is known to influence TNFα production, we speculated that TNFα might be synthesized and released after stimulation with FMLP. We found that PMN plated onto fibrinogen and stimulated with FMLP (10⁻⁸ mol/L) released significant (P < .05) amounts of TNFα by 45 minutes (Fig 2), reaching maximal levels by 90 minutes. In contrast, control cells at time = 0 incubated in wells containing fibrinogen for as long as 60 minutes did not release detectable levels of TNFα (Fig 2).

To determine whether the release of TNFα was contributing to PMN activation in our assay system, we evaluated the effect of anti-TNFα antibodies on H₂O₂ and lactoferrin release by PMN plated onto fibrinogen and activated with FMLP. Consistent with a role for TNFα in FMLP-mediated activation, rabbit anti-TNFα inhibited both H₂O₂ and lactoferrin release (Fig 3). The simultaneous addition of recombinant human TNFα (1,000 U/mL) with anti-TNFα overcame the inhibition (Fig 4, ■), indicating that the antibody effect was specific for TNFα. In contrast, control IgG and antibodies specific for HLA-A,B,C, IL-8, and CD14 hastened the release of TNFα (Fig 3). This result was somewhat different than what we observed for FMLP where we did not see significant inhibition (P < .05) until 45 minutes (Fig 1). Because the lag period was shorter for TNFα-stimulated cells than for FMLP-stimulated cells (30 v 45 minutes) and because ligation of β₂-integrins is known to influence TNFα production, we speculated that TNFα might be synthesized and released after stimulation with FMLP. We found that PMN plated onto fibrinogen and stimulated with FMLP (10⁻⁸ mol/L) released significant (P < .05) amounts of TNFα by 45 minutes (Fig 2), reaching maximal levels by 90 minutes. In contrast, control cells at time = 0 incubated in wells containing fibrinogen for as long as 60 minutes did not release detectable levels of TNFα (Fig 2).
FMLP-mediated H$_2$O$_2$ release by adherent PMN. The addition of either anti-p55 or anti-p75 alone to our assay system resulted in a small but significant ($P < .01$) reduction in H$_2$O$_2$ release by 120 minutes (Table 1). However, the combination of both antibodies reduced H$_2$O$_2$ release to control levels. Neither murine nor rat IgG inhibited H$_2$O$_2$ release (data not shown). The combination of anti-p55 and anti-p75 antibodies had no effect on H$_2$O$_2$ release stimulated by phorbol 12-myristate 13-acetate (PMA; Table 1), which activates PMN by bypassing the membrane and directly activating protein kinase C.

The TNF$\alpha$ released during FMLP-induced PMN activation could come from three potential sources. First, it could come from a pre-existing intracellular pool because intracellular stores of TNF$\alpha$ have been reported in unstimulated PMN. Second, TNF$\alpha$ bound to the plasma membrane could be released by proteolysis. Finally, TNF$\alpha$ could be derived through de novo synthesis. To determine if the TNF$\alpha$ affecting FMLP-stimulated activation of adherent PMN is newly synthesized, we treated cells with 10 mol/L actinomycin D or cycloheximide before conducting our assays. Under these conditions, either agent reduced H$_2$O$_2$ and lactoferrin release to control levels (Fig 6), i.e., values from treated cells were not statistically different from control values. The addition of TNF$\alpha$ to PMN treated with either actinomycin D or cycloheximide returned H$_2$O$_2$ and lactoferrin levels to those seen with FMLP stimulation (data not shown). These data indicate that the TNF$\alpha$ released during activation of FMLP-
Effect of various antibodies on FMLP-stimulated H$_2$O$_2$ release by adherent PMN. PMN were added to wells of a 24-well plate coated with fibrinogen and containing FMLP (10$^{-7}$ mol/L) alone or FMLP plus anti-TNF$\alpha$ antibodies in the presence or absence of TNF$\alpha$ (1,000 U/mL). Some wells contained either buffer alone (no treatment) or FMLP plus rabbit IgG, horse anti-HLA-A,B,C, rabbit anti-CD14, or rabbit antihuman IL-8 antibodies, as indicated. After 120 minutes of incubation at 37°C, the supernatant fractions were assayed for H$_2$O$_2$. Each value represents the mean $\pm$ SEM from two to three experiments performed in triplicate. *Values are significantly different from FMLP alone, $P < .01$.

Fig 5. Effect of incubation time on anti-TNF$\alpha$ inhibition of H$_2$O$_2$ release by adherent PMN stimulated with FMLP. PMN were added to wells of 24-well Primaria plates coated with fibrinogen and containing FMLP (10$^{-7}$ mol/L; time = 0 minutes). At the indicated times, anti-TNF$\alpha$ antibody was added to wells containing cells. Samples were removed from plates after 90 minutes (maximal H$_2$O$_2$ release) and assayed for H$_2$O$_2$. Each bar represents the mean $\pm$ SEM of triplicate determinations representative of two experiments. *Values are significantly different from FMLP alone, $P < .001$.

stimulated PMN is newly synthesized and is most likely involved in the activation cascade.

DISCUSSION

Some agonists, such as GM-CSF and TNF$\alpha$, do not stimulate functional responses by PMN in suspension, but prime them for a heightened response to a subsequent stimulus. On the other hand, the same stimuli activate PMN adherent to extracellular matrix proteins. Nathan showed that TNF$\alpha$ stimulates adherent PMN to release H$_2$O$_2$. We confirmed and extended these findings by showing that TNF$\alpha$ activated adherent PMN to release both H$_2$O$_2$ and lactoferrin with kinetics similar to those of FMLP, but found that TNF$\alpha$ activated PMN with a shorter lag period than FMLP.

TNF$\alpha$, released in nanogram quantities from endothelium and monocytes during inflammation, acts as an agonist for other cells in vivo and in vitro. In addition, TNF$\alpha$ has been implicated as an autocrine factor in HL-60 cells, lymphocytes, and macrophages when cells are stimulated with an agonist other than TNF$\alpha$. We found that the release of TNF$\alpha$ by FMLP-activated PMN correlated with the release of both H$_2$O$_2$ and lactoferrin. Other reports have documented that TNF$\alpha$ mRNA is produced by PMN within 30 to 60 minutes after stimulation with LPS or IL-2. Thus, it is plausible that the TNF$\alpha$ released by PMN stimulated with FMLP and adherent to fibrinogen serves as a secondary stimulus to activate H$_2$O$_2$ and lactoferrin release. However, these results do not preclude the possibility that TNF$\alpha$ may be playing a role other than that of an autocrine factor. We showed that anti-TNF$\alpha$ antibodies significantly inhibited, but did not completely block, the release of both H$_2$O$_2$ and lactoferrin from FMLP-stimulated PMN adherent to fibrinogen. These results suggest that additional signal transduction pathways leading to oxidant and lactoferrin release are activated by FMLP. We showed that blocking TNF$\alpha$ and TNF$\alpha$ receptors with antibodies and blocking protein synthesis inhibited H$_2$O$_2$ and lactoferrin release stimulated by FMLP. These data support our hypothesis that TNF$\alpha$ release and ligation of TNF$\alpha$ receptors are important for FMLP-stimulated oxidant release from adherent PMN.

The cellular source of the TNF$\alpha$ released from PMN was not defined in the present report. Three possibilities exist for
the origin of TNFα from PMN. First, it may have been derived from a pool of plasma membrane-associated TNFα molecules liberated by proteolysis during stimulation. It has been reported that TNFα exists as a transmembrane protein,10 the extracellular domain of which can become soluble through the action of a metalloproteinase.41 Secondly, TNFα may exist in an intracellular granular pool. Indeed, TNFα has been localized to cytoplasmic granules of normal mast cells and Paneth cells.59 Finally, TNFα may be transcribed and/or translated after PMN stimulation, as suggested by the data in Fig 6. We found that either actinomycin D or cycloheximide inhibited both H2O2 and lactoferrin release and that the addition of TNFα reversed the inhibition, suggesting that PMN synthesize TNFα after FMLP stimulation. Beil et al42 localized TNFα to the Golgi complex of PMN from colonic biopsies of patients with Crohn’s disease, suggesting that TNFα is synthesized by PMN during inflammation. In addition to translation, others have shown that PMN increase transcription by 30 minutes after stimulation with FMLP, GM-CSF, or TNFα.43

Although it is clear that exogenous TNFα stimulates adherent PMN, the roles that each of the TNFα receptors play in functional activation are not well established. It is known that PMN express both p55 and p75 TNFα receptors12,14,44 and our data show that both receptors play a role in FMLP activation of PMN adherent to fibrinogen. Specifically, we found that anti-p55 and anti-p75 each moderately inhibited oxidant release, but the combination of both TNFα receptor antibodies was required to obtain maximal inhibition of H2O2 release. The specificity of TNFα receptors in this pathway is indicated by the observation that antibodies against HLA-A,B,C, IL-8, and CD14, as well as IgG, had no significant effect on PMN functional responses. In addition, anti-TNFα receptor antibodies had no effect on PMN adhesion and spreading (data not shown), suggesting that the inhibition occurred at the level of transmembrane signaling. This is supported by data showing that anti-TNFα receptor antibodies failed to block H2O2 release stimulated by phorbol ester (Table 1), which bypasses receptor signaling. Our findings are consistent with those of others showing that ligation of both TNFα receptors is required for PMN functional responses. For example, Richter et al47 showed that both TNFα receptors are involved in TNFα-mediated superoxide release by PMN adherent to fibrinogen. It was also shown that, even though the priming effect of TNFα on Fe-mediated phagocytosis by PMN signals through p55, p75 is required for TNFα binding to p55.4

Similar results have been obtained with other cells. Adding either anti-p55 or anti-p75 antibodies to human endothelial cells inhibited TNFα-stimulated IL-6 release by 41% and 42%, respectively, but the combination of both antibodies was required for more complete inhibition.15 Similarly, either p55 or p75 anti-TNFα receptor antibody partially blocked the binding of HL-60 cells to endothelial cell cultures, but a combination of both antibodies was required for complete inhibition.15 In another study, it was shown that tissue factor production by human umbilical vein endothelial cells requires both p55 and p75.45 Finally, it was shown that induction of apoptosis in PC60 (rat/mouse T-cell hybridoma) cells requires both TNFα receptors.46 Thus, it appears that in many functional responses signaling through both TNFα receptors is required.

Several studies have shown that receptors for different ligands may also be functionally linked. For example, it was shown that activating PMN Fc receptors increased the association of CR3 (CD11b/CD18) with cortical microfilaments in PMN,24 suggesting that CR3 physically interacts with Fc receptors. These results confirm and extend previous studies showing that anti-Mac-1 (CD11b) antibody inhibited IgG-stimulated PMN phagocytosis.47 It was also reported that PMN chemotaxis to FMLP is inhibited by Fc fragments of IgG,25,26 suggesting that FcRIII and FMLP receptors are also functionally associated. Our data show that antibodies against the p55 and p75 TNFα receptors inhibited H2O2 release stimulated by FMLP. These results, supporting the hypothesis that TNFα and FMLP receptors are functionally linked, perhaps through TNFα release, which mediates autocrine activation of adherent PMN. Future efforts will be directed toward determining the specific roles that p55 and p75 TNFα receptors play in FMLP activation of adherent PMN.
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