Tumor Necrosis Factor (TNF)-α Directly Inhibits Human Erythropoiesis In Vitro: Role of p55 and p75 TNF Receptors

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Two tumor necrosis factor receptors (TNFRs) with molecular weights of 55 kD (TNFR-p55) and 75 kD (TNFR-p75) have recently been identified and cloned. In previous studies, TNFR-p55 has been shown to exclusively mediate bidirectional effects of TNF-α on committed bone marrow granulocyte-macrophage progenitor cells, whereas both TNFR-p55 and TNFR-p75 can mediate inhibition of primitive progenitors requiring multiple cytokines to proliferate. We show here that TNF-α potently and directly inhibits the in vitro growth of committed erythroid progenitor cells in response to multiple cytokine combinations, and that TNF-α-induced inhibition of burst-forming unit-erythroid colony formation is mainly mediated through TNFR-p55, although TNFR-p75-mediated inhibition could be observed on progenitors responsive to erythropoietin alone. Moreover, at low TNF-α concentrations (2 ng/mL), TNF-α stimulates interleukin-3-dependent in vitro growth of committed granulocyte-macrophage progenitor cells, whereas it potently inhibits erythroid progenitor cell proliferation, showing that one concentration of TNF-α can simultaneously and bidirectionally modulate interleukin-3-dependent growth of committed granulocyte-macrophage (stimulation) and erythroid progenitor cells (inhibition).

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TUMOR NECROSIS FACTOR-α (TNF-α), a multifunctional cytokine shown to affect a number of different cell types, is mainly produced by activated macrophages and lymphocytes. In hematopoiesis, TNF-α acts as both a positive and negative regulator of myeloid cell proliferation and differentiation. The effects of TNF-α can be mediated either directly or indirectly by inducing other cells to produce hematopoietic growth factors. Furthermore, TNF-α can modulate the expression of c-kit as well as cell surface receptors for the colony-stimulating factors (CSFs) on both normal bone marrow (BM) cells and acute myeloid leukemia cells.

Several previous studies have demonstrated inhibitory effects of TNF-α on erythroid colony formation. However, many of these studies were performed in systems not allowing segregation of direct and indirect effects of TNF-α. Even more importantly, the interactions between TNF-α and specific hematopoietic growth factors in erythropoiesis have only been examined to a limited extent.

Two TNF receptors (TNFRs) with molecular weights of 55 kD (TNFR-p55) and 75 kD (TNFR-p75) have recently been identified and cloned. TNFR-p55, expressed on a majority of cell types, has been shown to mediate most effects of TNF-α, such as cytotoxicity, proliferation of fibroblasts, and prostaglandin synthesis. Whereas effects of TNF-α mediated through TNFR-p75 appear to be much more restricted, it has been shown that the TNFR-p75 can signal stimulation of T-cell proliferation. Also, TNF-p75 has been suggested to be involved in cytoxicity.

We have recently found a differential role of the two TNFRs in murine hematopoiesis in that the p55 TNFR exclusively mediates the effects of TNF-α on committed granulocyte-macrophage progenitor cells, whereas the p75 TNFR is essential in signaling inhibition of primitive progenitors. Similarly, on human BM progenitor cells, we have shown that the potent effect of TNF-α on granulocyte-macrophage colony-stimulating factor (GM-CSF)- or interleukin-3 (IL-3)-induced proliferation as well as the inhibition of granulocyte colony-stimulating factor (G-CSF)-stimulated colony formation is mediated exclusively through TNFR-p55. However, both TNFR-p55 and TNFR-p75 are involved in mediating growth inhibition of primitive high proliferative potential colony-forming cells. However, the relative role of the two TNFRs in erythropoiesis has not been established.

Furthermore, because TNF-α has been implicated in the pathogenesis of the anemia associated with chronic diseases, it is important to determine which of the two TNFRs mediates this effect.

Whereas TNF-α has been shown to have potent antitumor activity, its clinical use has been hampered by severe toxic side effects. The recent development of TNF-α mutants with selective activity on either TNFR-p55 or TNFR-p75 has opened the potentiality of reducing the serious side effects of TNF-α while maintaining the anticancer effect.

The present study was designed to determine the direct effects of TNF-α on erythroid colony formation in response to growth factor combinations not previously explored for interactions with TNF-α. This is of particular importance because the effects of TNF-α (stimulatory or inhibitory) on granulocyte-macrophage progenitors have been demonstrated to be strictly dependent on the specific growth factor stimulating proliferation. Using agonistic anti-TNFR antibodies, as well as TNF-α mutant proteins specific for either TNFR-p55 or TNFR-p75, we also examined the relative role of the two TNF receptors in mediating TNF-α effects on erythropoiesis.

MATERIALS AND METHODS

Hematopoietic growth factors and antibodies. Purified recombinant human (rHu) stem cell factor (SCF) was generously supplied by Dr Ian K. McNiece (Amgen Inc, Thousand Oaks, CA). rHuIL-3 was generously provided by Dr Steven Gillis (Immunex Corp, Seattle, WA). rHuIL-9 was a gift from Genetics Institute (Cambridge, MA). rHu-erythropoietin (Epo) was purchased from Cilag AG (Schaffhausen, Switzerland). rHuTNF-α, TNF-α mutant pro-
teins, and anti-TNFR antibodies were a kind gift from Drs. W. Lesslauer and H. Loetscher (Hoffmann-LaRoche, Basel, Switzerland). HuTNF-α mutants specific for TNFR-p55 and TNFR-p75 have been prepared by site-directed mutagenesis.6,7 Solid-phase binding studies have shown that the Thr18→Thr20 TNF-α mutant protein binds with wild-type affinity to TNFR-p55 and does not bind at all to TNFR-p75, whereas the Asn13→Arg14 TNF-α mutant protein exclusively binds to TNFR-p75, although with a 5- to 10-fold lower affinity than wild-type TNF-α.6,7 Both mutant proteins were titrated and used at optimal concentrations as previously described.8 Rabbit anti-human TNFR-p75 polyclonal antibodies with TNF-α agonistic activity (patTNFR-p75) and a monoclonal antibody (MoAb) against the TNFR-p55 with TNF-α agonistic activity (hrr-p75) were raised and prepared as previously described.9,9 Htr-9 and patTNFR-p75 were initially titrated as described elsewhere10 and thereafter used at optimal concentrations of 10 μg/mL (hrr-9) and 2 μg/mL (patTNFR-p75). Unless otherwise indicated, all growth factors were used at predetermined optimal concentrations: rHuIL-3 (20 ng/mL), rHuSCF (50 ng/mL), rHuIL-9 (100 IU/mL), and Epo (5 IU/mL).

**Cell separation.** Human BM cells were obtained by iliac crest aspiration from normal adult volunteers with informed consent and the approval of the Ethics Committee of The Norwegian Radiation Hospital. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Positive selection of CD34+ cells was performed according to a previously described method.11 Briefly, BM mononuclear cells were rosetted with Dynabeads M-450 directly coated with the CD34 MoAb BI-3C5 (Product No. 1.10; Dynal, Oslo, Norway) for 45 minutes at 4°C on an apparatus that provided tilting and gentle rotation. The bead to total cell ratio was 1:1. Rosetted cells were attracted to a samarium cobalt magnet and nonrosetting cells were removed by pipetting and washed seven times. Detachment of beads from positively selected cells was performed by incubation with anti-Fab antibody (DETACHaBEAD; Dynal) at a concentration of 35 mg/mL for 1 hour at room temperature. Isolated cells, free of beads, were washed and counted. The purity of CD34+ cells isolated by this method was reproducibly greater than 90% as determined by flow cytometric analysis.

To enrich and separate committed erythroid (burst-forming unit-erythroid [BFU-E]) and granulocyte-macrophage (colony-forming unit–granulocyte-macrophage [CFU-GM]) progenitors, we took advantage of the differential expression of CD45 isoforms previously described on these cells.12 Because all BFU-E are CD45RO+ and the majority of CFU-GM are CD45RO-, CD34+CD45RO+ cells are enriched in committed erythroid progenitors, whereas committed granulocyte-macrophage, but not erythroid, progenitors reside in the CD34+CD45RO− subpopulation. In addition, we wanted to deplete our test cell populations in primitive progenitors, which was obtained by selecting progenitors expressing CD38.35-37 To isolate cells with the CD34+CD38+CD45RO− or CD34+CD38−CD45RO+ phenotype by cell sorting, positively selected CD34+ cells with a mean purity (n = 3) of 94% as assessed by flow cytometry were stained with anti-CD45RO (UCHL-1; Becton Dickinson, San Jose, CA) directly conjugated to phycoerythrin (PE) and with anti-CD38 (IB6; Immunotech, Marseilles, France) directly conjugated to fluorescein isothiocyanate (FITC). FITC-conjugated matched FITC- and PE-conjugated irrelevant mouse MoAbs served as controls. Cell sorting was performed on an Epics Elite cell sorter (Coulter Electronics, Hialeah, FL). A sort gate within a dual-parameter cytogram of forward light scatter against 90° side scatter was drawn. A second amorphous gate was drawn on two-color cytograms, and sort equations were set to positively sort cells satisfying both gates.

**Colony assay.** CD34+ cells were plated in a volume of 1 mL Iscove's modified Dulbecco's medium (IMDM; GIBCO, Paisley, UK) containing 20% fetal calf serum (FCS; Sera-lab, Sussex, UK), 1.2% methylcellulose (Methocel 4,000 mPa·s; Fluka AG, Buchs, Switzerland), 5 × 10−4 mol/L 2-mercaptoethanol, 300 mg/L glutamine, 66 mg/L penicillin, 100 mg/L streptomycin, and recombinant human growth factors as indicated. After 2 weeks of incubation at 37°C and 5% CO2 in air, colonies (>40 cells) derived from CFU-GM and BFU-E were assessed according to established criteria.26,27 Cultures were scored for BFU-E colony growth after 14 days of incubation at 37°C and 5% CO2 in air. Results are presented as the mean number of colonies per 2 × 103 cells from at least four independent experiments with triplicate determinations; error bars show the SEM. *No colony formation.

**Single-cell proliferation assay.** Test cells were seeded in Terasaki plates (Nunc, Kamstrup, Denmark) at a concentration of 1 cell per well (300 cells per group) in 20 μL IMDM containing 20% FCS (Sera-lab), L-glutamine, penicillin, and streptomycin. Wells were scored for erythroid colony formation (>40 cells) after 2 weeks of incubation at 37°C and 5% CO2 in air. To verify the erythroid composition of colonies induced by SCF + Epo, morphologic examination of randomly picked colonies was performed on May-Grünwald-Giemsa-stained cytocentrifuge cell preparations.

**Statistical analysis.** All results were expressed as the mean ± SEM of data obtained from three or more separate experiments. The statistical significance of differences between group means was determined using the Student’s t-test.

**RESULTS**

**Effects of TNF-α on erythroid colony formation by human BM CD34+ cells.** We and others have previously shown that TNF-α potently and directly can enhance granulocyte-macrophage colony formation of human CD34+ progenitors stimulated by IL-3 or GM-CSF, whereas TNF-α inhibits the proliferative actions of other growth factors such as G-CSF, SCF, and CSF-1.5,15,20 TNF-α has been shown to both inhibit and stimulate erythroid colony growth depending on the growth factors used, as well as the concentration of TNF-α itself.5,11,13 In the present study, TNF-α invariably inhibited BFU-E colony formation of CD34+ BM cells induced by multiple cytokine combinations (Fig 1). In agreement with previous reports,11,13 BFU-E colony growth induced by Epo...
alone or Epo in combination with IL-3 was completely inhibited by TNF-α. In addition, and not previously shown, TNF-α inhibited BFU-E colony formation induced by SCF + Epo, IL-3 + SCF + Epo, and IL-9 + Epo by 76%, 70%, and 89%, respectively (Fig 1).

TNF-α inhibited BFU-E colony formation in a concentration-dependent fashion. Fifty percent inhibition of Epo-stimulated colony growth occurred at a TNF-α concentration of 0.02 ng/mL, whereas complete inhibition of erythroid colony-forming ability was observed at 2 ng/mL (Fig 2A). In comparison, 50% inhibition of SCF + Epo-induced BFU-E colony growth was observed at a TNF-α concentration of 2 to 20 ng/mL, with a maximum inhibition of 73% occurring at 200 ng/mL (Fig 2B). Finally, 50% inhibition of SCF-induced granulocyte-macrophage cluster formation was observed at a TNF-α concentration of 0.2 to 2 ng/mL (Fig 2C).

Whereas several previous studies have suggested that TNF-α can directly modulate the growth of granulocyte-macrophage progenitor cells,12,13,15 Means et al17,18 recently reported that TNF-α-induced growth inhibition of colonies derived from human erythroid colony-forming units (CFU-E) was indirectly mediated through production of β-interferon from accessory cells. In contrast, we found that TNF-α inhibited the formation of SCF + Epo-induced erythroid colonies derived from individually plated CD34+ progenitors in a concentration-dependent fashion, suggesting a direct action of TNF-α on more primitive erythroid progenitors (Fig 3).

Differential effects of TNF-α on IL-3-stimulated granulocyte-macrophage and erythroid progenitors. Because it has been shown that low, but not high, concentrations of TNF-α can potently enhance GM-CSF- or IL-3-induced granulocyte-macrophage colony formation1,15, we next examined the effects of TNF-α on the simultaneous development of CFU-GM and BFU-E colonies in cultures supplemented with IL-3 + Epo. As shown in Fig 4, IL-3 + Epo promoted the growth of 51 ± 5 BFU-E and 17 ± 2 CFU-GM colonies per 2 × 10^5 CD34+ cells plated. Interestingly, when adding TNF-α at 2 ng/mL, a 2.7-fold increase in the number of CFU-GM colonies was observed, whereas BFU-E colony formation was completely inhibited (Fig 4). At higher concentration of TNF-α (200 ng/mL), both CFU-GM and BFU-E colony formation was inhibited. Thus, at low concentrations, TNF-α has the ability of promoting the growth of granulocyte-macrophage colonies while concomitantly suppressing the growth of erythroid colonies.

To investigate whether the bifunctional effect of TNF-α on the growth of CFU-GM and BFU-E progenitors might be explained by TNF-α preferentially directing primitive multipotent or bipotent progenitors towards granulocyte-macrophage rather than erythroid development or, alternatively, by a differential effect of TNF-α on committed granulocyte-macrophage and erythroid progenitors, we next examined the effect of TNF-α on colony formation of CD34+CD38-CD45RO+ and CD34+CD38-CD45RO- cells. Whereas both these cell populations, lacking CD38 expression, are depleted in primitive progenitors,13,15 we found, in agreement with a previous study,19 that virtually all BFU-E (99%) resided in the CD45RO+ subset. In contrast, both
Fig 3. Effect of TNF-α on erythroid colony formation of CD34+ BM cells in single-cell assay. CD34+ cells were isolated and plated at 1 cell per well as described in the Materials and Methods in the presence of predetermined optimal concentrations of SCF + Epo and different concentrations of TNF-α as indicated. Erythroid colonies (≥40 cells) were assessed (Materials and Methods) and counted after 14 days of incubation at 37°C and 5% CO₂ in air. In some experiments, individual colonies were picked to determine that all colonies formed were in fact erythroid. Each group consisted of 300 wells. Results are presented as the mean number of positive wells in four separate experiments; error bars show the SEM.

Subpopulations contained CFU-GM, although a majority (60%) were CD45RO-. As shown in Fig 5A, IL-3 + Epo induced the formation of 17 ± 1 CFU-GM colonies and 1.5 ± 1 BFU-E colonies of 2,000 CD34+CD38+CD45RO- cells plated. Interestingly, CFU-GM colony formation of CD34+CD38+CD45RO- cells increased about fourfold by adding TNF-α at 2 ng/mL. Thus, the depletion in primitive progenitors and the removal of progenitors with erythroid potential from the test cell population did not impair the ability of TNF-α to enhance IL-3-stimulated CFU-GM colony formation. Using CD34+CD38+CD45RO- cells, IL-3 + Epo stimulated the growth of 23 ± 1 CFU-GM and 114 ± 13 BFU-E colonies of 2,000 cells plated (Fig 5B). The supplementation of TNF-α at 2 ng/mL almost completely blocked BFU-E colony formation, whereas CFU-GM colony numbers increased 2.5-fold. Thus, enriching progenitors capable of erythroid colony formation did not further enhance the ability of TNF-α to stimulate CFU-GM colony formation.

Role of p55 and p75 TNFRs in TNF-α-induced inhibition of erythropoiesis. In a previous study, we have shown that
TNF-α has been demonstrated to signal both inhibition and stimulation of hematopoietic progenitor cells. Specifically, TNF-α has been shown to potently inhibit in vitro erythropoiesis stimulated by Epo alone or in combination with IL-3. We demonstrate here for the first time that TNF-α also potently inhibits BFU-E colony formation stimulated by both SCF + Epo and IL-9 + Epo. Of particular interest is the finding that TNF-α inhibits SCF-stimulated erythroid colony formation, because SCF has been shown to be maybe the most potent stimulator of erythropoiesis. In a similar fashion, previous studies have suggested that TNF-α-induced inhibition of erythroid progenitors recruited by SCF might be used to reverse the anemia observed after TNF-α administration and in chronic diseases. In another fashion, previous studies have shown that transforming growth factor-β–induced inhibition of progenitor cell growth can be abrogated either by increasing the concentration of stimulatory growth factors or by supplementing additional growth factors. Alternatively, it is possible that the presumably more primitive subset of progenitors recruited by SCF + Epo is less sensitive to TNF-α than the subset of those responding to Epo alone.

The effects of TNF-α can be mediated directly on target cells or indirectly by stimulating accessory cells to cytokine production. Whereas we and others have found direct effects of TNF-α on committed granulocyte-macrophage BM progenitor cells, recent studies by Means et al have suggested that TNF-α–induced inhibition of erythroid colony growth was indirect through stimulation of β-inter-
feron production from accessory cells. In contrast, the results of single-cell cloning experiments presented here suggest that TNF-α--induced inhibition of erythroid colony formation can be directly mediated on the progenitor cells. The divergent findings in the studies of Means et al. and in our study could be due to studying different erythroid progenitors, because our results were obtained assessing TNF-α--induced modulation of BFU-E colony formation stimulated by multiple cytokine combinations, whereas Means et al. evaluated TNF-α--induced inhibition of colony formation derived from more mature CFU-E progenitors stimulated by Epo alone. It is possible that TNFR expression and function varies among these two progenitor cell types. Alternatively, TNFR expression might differ on progenitors of the same type according to the conditions under which they have been generated.

It is well established that TNF-α can inhibit or stimulate the proliferation of hematopoietic progenitor cells, depending on the growth factor(s) it interacts with as well as the concentration of TNF-α in culture. The potent TNF-α--induced enhancement of CFU-GM colony growth and inhibition of erythroid colony formation stimulated by IL-3 + Epo is of particular interest, because it demonstrates that one concentration of TNF-α can simultaneously and bidirectionally modulate IL-3--dependent growth of granulocyte-macrophage (stimulation) and erythroid colonies (inhibition). The present study suggests that this can be explained by a differential effect of TNF-α on committed granulocyte-macrophage and erythroid progenitors and not by TNF-α switching more primitive multipotent or bipotent progenitors from erythroid towards granulocyte-macrophage development. This theory was supported by TNF-α retaining its ability to enhance IL-3--induced CFU-GM colony growth when the test cell population was depleted in primitive and erythroid progenitors, as well as by the fact that TNF-α--induced potentiation of IL-3--dependent CFU-GM colony formation was not further enhanced using a test cell population highly enriched in progenitors with erythroid potential.

Recent studies from our laboratory suggest that both TNFR-p55 and TNFR-p75 are involved in mediating TNF-α--induced inhibition of primitive hematopoietic progenitor cells requiring multiple cytokines to proliferate. In contrast, the TNFR-p55 exclusively mediates stimulatory effects on more mature GM-CSF-- or IL-3--responsive granulocyte-macrophage progenitor cells, as well as potent inhibition of G-CSF--induced proliferation. The present study extends these findings to show that TNF-α--induced inhibition of BFU-E colony formation in response to multiple cytokine combinations is mediated predominantly through TNFR-p55. However, whereas signaling through TNFR-p75 had no effect on the proliferation of committed granulocyte-macrophage progenitor cells, we show here that this receptor type can mediate inhibition of committed erythroid progenitor cell growth. Although a small inhibition of SCF + Epo--induced BFU-E colony formation was observed, the TNFR-p75--mediated inhibition was more obvious on progenitors responding to Epo alone, indicating a predominant effect on more mature stages of erythroid progenitors. These findings might have several important clinical implications. First, TNFR-p55--specific mutant TNF-α has been shown to have less proinflammatory (toxic) effects than wild-type TNF-α, but as potent cytotoxic (antitumor) effects, and is thus being explored for the possible use as an antitumor agent. However, the present results suggest that the TNF-α mutant specific for TNFR-p55 is as potent as wild-type TNF-α in inhibiting erythropoiesis, and might therefore induce severe anemia. In contrast, administration of TNF-α mutants with selectivity for TNFR-p75 might be associated with little or no anemia. Second, antagonistic antibodies to TNFR-p55 could potentially be used to reverse the anemia observed in chronic diseases.

It has been shown that some TNFR-p55--mediated responses are much more efficiently elicited by wild-type TNF-α than TNFR-p55--selective TNF-α mutants, suggesting an accessory role of TNFR-p75 in TNFR-p55--signaled events. It is of interest that the data presented here, as well as in previous studies, suggest that this does not appear to be the case for TNFR-p55--mediated regulation of human hematopoietic progenitor cell growth.

In conclusion, we have demonstrated that TNF-α potently and directly inhibits the in vitro growth of committed erythroid progenitor cells in response to multiple cytokine combinations, and that TNF-α--induced inhibition of BFU-E colony formation is mainly mediated through TNFR-p55, although TNFR-p75--mediated inhibition could be observed on progenitors responsive to Epo alone. Ongoing studies on TNF receptor knock-out mice will help to further elucidate the role of TNF-α and its receptors in erythropoiesis.

ACKNOWLEDGMENT

The authors are grateful to Ole P. Veiby for the cell sorting, to Dr Erlend B. Smeland for support during these studies, and to Cecile Okkenhaug and Eli Lien for expert technical assistance.

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