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Research Article

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Inhibition of Human Colony-forming-Unit Erythroid by Tumor Necrosis Factor Requires Accessory Cells

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Abstract

Recombinant tumor necrosis factor (rTNF) inhibits erythropoiesis in vivo and in vitro. To study the mechanism of this inhibition, the effect of rTNF on highly purified human CFU-erythroid (E) (mean purity 63.5%), which were generated from peripheral blood burst-forming units-erythroid (BFU-E), was compared to its effect on unpurified human marrow CFU-E (mean purity 0.21%). Although growth of colonies from marrow CFU-E was inhibited by rTNF, no significant effect on purified BFU-E-derived CFU-E colony growth was found. Removal of accessory marrow cells by soy bean agglutinin (SBA) ablated the inhibition of marrow CFU-E colonies by rTNF. Inhibition of colony growth was then restored by adding back SBA+ cells, but not by adding T lymphocytes or adherent cells. Conditioned medium prepared from bone marrow mononuclear cells stimulated by rTNF inhibited the growth of colonies from highly purified BFU-E derived CFU-E resistant to direct inhibition by rTNF.

These findings indicate that rTNF does not directly inhibit CFU-E, but requires accessory cells to decrease erythropoiesis. These accessory cells reside in the SBA+ cell fraction, but are neither T cells nor adherent cells. Therefore, in order to produce anemia, TNF must induce release or production of a factor that directly inhibits erythroid colony growth. (*J. Clin. Invest.* 1990. 86:538–541.) Key words: erythropoiesis • tumor necrosis factor • anemia of chronic disease • cytokines

Introduction

The anemia of chronic disease (ACD)[1] may be the most common anemia other than that due to hemorrhage and iron deficiency (1). Recent attempts to elucidate the mechanism of ACD have concentrated on mediators of the inflammatory response that have been shown to inhibit erythropoiesis. These include interleukin-1 (2, 3), gamma interferon (4), and tumor necrosis factor (TNF) (5). Johnson and colleagues have recently described an animal model of ACD in which TNF-secreting tumors are implanted into nude mice (6). These mice develop a hypoferremic anemia with normal iron stores, and show preferential inhibition of erythroid progenitors. In addition, in a phase I study of recombinant human TNF (rTNF) in cancer patients, patients at all dosage levels had a decrease in hemoglobin after a month of rTNF treatment (7). In vivo or in vitro studies of marrow progenitors that are not highly purified however, cannot distinguish direct effects on erythroid progenitors from effects mediated by other cells or cell products.

A method has been reported from our laboratory by which CFU-erythroid (E) can be purified from peripheral blood burst-forming units-erythroid (BFU-E) (8). 40–90% of the cells obtained by this method are CFU-E, and have been used to determine the growth requirements of erythroid progenitors (9).

We have studied the effect of TNF on these highly purified BFU-E-derived CFU-E and have found that TNF does not inhibit purified CFU-E, but requires the presence of accessory marrow cells to exert its effect.

Methods

Blood and bone marrow were obtained from normal volunteers after informed consent. The studies were approved by the Vanderbilt University and Nashville Veteran's Affairs Medical Center Institutional Review Boards.

Recombinant human TNF alpha. rTNF with bioactivity 24×10^6 U/mg was generously provided by Dr. Abla Creasey, Cetus Corp., Emeryville, CA.

Purification of CFU-E from peripheral blood. This method has been reported in detail by Sawada et al. (8, 9). Briefly, 400 ml heparinized blood was separated over Ficoll-Hypaque (FH; 1.077 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ; Winthrop-Breon Laboratories, New York) at $400\,g$ for 25 min at 24°C. The light density mononuclear cells (LDMN) were collected in alpha MEM (Sigma Chemical Co., St. Louis, MO), washed, and resuspended in Iscove's modified Dulbecco's medium (IMDM; Sigma Chemical Co.). T cells were depleted by sheep erythrocyte rosetting, and B lymphocytes were removed by adherence to plastic culture dishes (Becton Dickinson & Co., Oxnard, CA) which were coated with affinity purified sheep anti–human IgG directed against F(ab)₂ fragments (Cappel Laboratories, Cochranville, PA) (8). Adherent cells were then depleted by overnight incubation in 75 cm² polystyrene tissue culture flasks in IMDM with 20% FCS (Hyclone Laboratories, Logan, UT) and 10% giant cell tumor conditioned medium (CM) (Gibco Laboratories, Grand Island, NY) at 37°C in a 5% CO_2 atmosphere.

The following morning, the nonadherent cells were collected in warm MEM and underwent negative panning with CD11b/OKM*1, CD2/OKT*11 (Ortho Diagnostic Systems, Inc., Raritan, NJ), CD45/My11, and CD16/My23, as previously described (8), to remove CFU granulocyte-macrophage, neutrophils, monocytes, lymphocytes, and natural killer cells. The remaining cells (0.34% BFU-E [8]) were then

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1. *Abbreviations used in this paper:* ACD, anemia of chronic disease; BFU-E, burst-forming units-erythroid; CCM, control CM; CFU-E, colony-forming units-erythroid; CM, conditioned medium; FH, Ficoll-Hypaque; IMDM, Iscove's modified Dulbecco's medium; LDMN, light density mononuclear marrow cells; PDGF, platelet-derived growth factor; rTNF, recombinant human TNF; SBA, soybean agglutinin; TNF, tumor necrosis factor; TNFCM, TNF-stimulated CM.

cultured at a concentration of 3×10^5/ml in 0.9% methylcellulose (Fisher Scientific Co., Fair Lawn, NJ) with 30% FCS, 1% deionized HSA (American Red Cross Blood Services, Washington, DC), 10^{-4} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY), penicillin 500 U/ml, streptomycin 40 μg/ml, recombinant human insulin 10 U/ml (Eli Lilly and Co., Indianapolis, IN), recombinant human interleukin-3 50 U/ml (Genetics Institute, Cambridge, MA), and recombinant human erythropoietin 2 U/ml (AmGen Biologicals, Thousand Oaks, CA). 1-ml aliquots were plated in 12 well tissue culture plates (Linbro, Flow Laboratories, Inc., McLean, VA) at 37°C in a high humidity 5% CO_2 incubator for 7 d.

After 7 days, the cells were collected in MEM. Debris and residual adherent cells were then separated from BFU-E derived CFU-E by centrifugation through 10% BSA (Armour Pharmaceutical Co., Kankakee, IL), followed by centrifugation over FH, and adherence in plastic flasks with 20% FCS for 1 h at 37°C. This process reproducibly yields a 94% viable population of day 8 peripheral blood cells 40–90% of which are BFU-E-derived CFU-E (8, 9). The remaining cells are polymorphonuclear and basophilic leukocytes (8).

Marrow cell preparation. 6 ml of bone marrow was aspirated from the posterior iliac crest and collected into an equal volume of IMDM containing 5 U sodium heparin/ml. Marrow cells were then enriched for LDMN by separation over FH. For some studies, LDMN cells were depleted of accessory cells by incubation at a concentration of 1 $\times$ 10^8/ml with equal volumes of soybean agglutinin (SBA; Boehringer Mannheim Biochemicals, Indianapolis, IN) 2 mg/ml and density sedimentation over 5% BSA (10). The interface (LDMN SBA− cells) was collected for studies. The agglutinated accessory cells (SBA+ cells) were collected, incubated with 0.2 M D-galactose for 10 min at 25°C, and washed three times in MEM (10). The cells were then suspended in IMDM at a concentration of 1 $\times$ 10^7/ml and kept at 3°C for further studies. For some experiments, adherent cells and T lymphocytes were collected from marrow cells. LDMN cells were depleted of adherent cells by incubation in plastic flasks for 2 h with 20% FCS (11). The nonadherent cells were collected by gentle washing with MEM at 37°C, and the adherent cells were collected by vigorous washing with cold MEM and a sterile cell scraper. The adherent cells were then suspended in IMDM at a concentration of 1 $\times$ 10^7/ml and stored at 3°C. T lymphocytes were collected from LDMN marrow cells by sheep erythrocyte rosetting (11). The lymphocytes were released from rosettes by hypotonic lysis of erythrocytes, suspended in IMDM with 0.1% BSA at a concentration of 1 $\times$ 10^7/ml, and stored at 3°C.

Preparation of conditioned medium. LDMN marrow cells were suspended in IMDM with 30% FCS at a concentration of 1 $\times$ 10^6/ml and incubated in tissue culture flasks at 37°C in a 5% CO_2 incubator with rTNF 10^{-10} M (TNF stimulated CM, TNFCM) or without rTNF (control conditioned medium, CCM). After 48 h, the suspension was collected, centrifuged at 400 g, 3°C, for 5 min to remove cells, and the supernatant was collected and stored at −20°C.

Culture of CFU-E in plasma clots. Peripheral blood or marrow CFU-E were cultured at concentrations of 10^3 peripheral blood day 8 cells/ml or 0.5–2.0 $\times$ 10^5 marrow cells/ml with IMDM, 15% FCS, 15% pooled human AB serum, 10% HSA or BSA, recombinant human erythropoietin 1 U/ml, penicillin, streptomycin, epsilon aminocaproic acid 1.5 mM, fibrinogen 1.3 mg/ml (Fibrinogen Kabi, Grade L, Kabi Diagnostica, Stockholm, Sweden), thrombin 0.2 U/ml (Parke-Davis Pharmaceuticals, Morris Plains, NJ), and with varying concentrations of rTNF. Plasma clots were plated in 48 well-tissue culture plates (Costar Data Packaging Corp., Cambridge, MA) in 0.2-ml aliquots. In some experiments with peripheral blood cells, CM 10% (vol/vol) was substituted for IMDM. In other experiments, SBA+ cells, T lymphocytes, or adherent cells were added to the plating mixture at a concentration of 0.5 $\times$ 10^5/ml. Cells were cultured for 7 d at 37°C in 5% CO_2 and were then fixed and stained with benzidine-hematoxylin as described by McLeod et al. (12). CFU-E were defined as colonies of 8–49 hemoglobinized cells (13).

Each point was studied with 3–6 replicates, and results were expressed as a percentage of control (cultures without rTNF) so that

results of different experiments might be compared. When marrow cells at different levels of enrichment were compared, each cell fraction had a separate control. Statistical comparison was by t test.

Results

The effects of rTNF on highly purified BFU-E-derived CFU-E and on LDMN marrow cells are shown in Fig. 1. The results of three experiments with purified CFU-E were combined. The purity of CFU-E ranged from 41.7 to 86.8%. No significant inhibition of colony growth from highly purified BFU-E-derived CFU-E by rTNF was found at any concentration tested. Although there was a downward trend at the highest concentration, this did not achieve statistical significance compared to control cells that were not exposed to rTNF ($P = 0.11$).

In contrast, LDMN marrow CFU-E colony growth was significantly inhibited ($P < 0.001$) relative to controls and to BFU-E-derived CFU-E colony growth at all rTNF concentrations above 10^{-12} M. The appearance of TNF-resistant marrow CFU-E did not differ from the appearance of marrow CFU-E not exposed to TNF. Fig. 1 combines the results of three experiments with marrow cells with mean CFU-E purity 0.21%.

The effect of enrichment of human marrow CFU-E on inhibition of erythroid colony growth by rTNF is shown in Fig. 2. LDMN marrow cells with 0.51±0.37% CFU-E (range 0.18–1.0% in five experiments) were further enriched by depletion of SBA+ cells and cultured with 10^{-10} M rTNF. LDMN SBA− marrow cells contained 1.05±0.4% CFU-E. Inhibition of CFU-E colony formation was abolished with removal of accessory cells by SBA. When autologous SBA+ cells were added to LDMN SBA− cells, inhibition of erythroid colony growth was restored. The addition of autologous adherent cells or T lymphocytes to LDMN SBA− cells did not restore the inhibitory effect of rTNF on CFU-E growth (Fig. 2).

To demonstrate the effect of marrow accessory cells on BFU-E-derived CFU-E in the presence of TNF, bone marrow cells were obtained 8 d after blood was collected from the same donor for methylcellulose culture of BFU-E and generation of
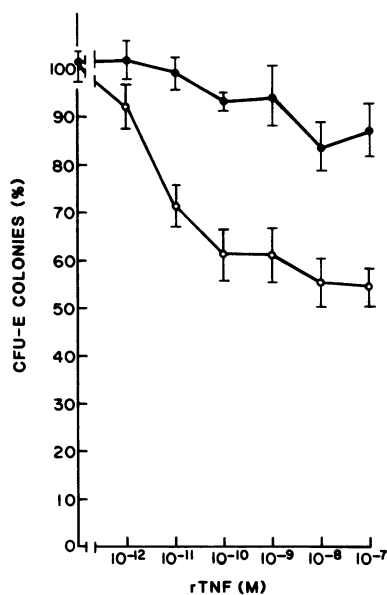

Figure 1. Effect of rTNF on the growth of blood BFU-E-derived CFU-E and bone marrow CFU-E. Growth of highly purified peripheral blood BFU-E-derived CFU-E colonies from three experiments (63.5±22.5% CFU-E) (*closed circles*) and marrow CFU-E colonies from three experiments (0.21±0.07% CFU-E) (*open circles*). Data from each experiment was normalized to CFU-E colony growth without rTNF. Results are expressed as mean±SE.
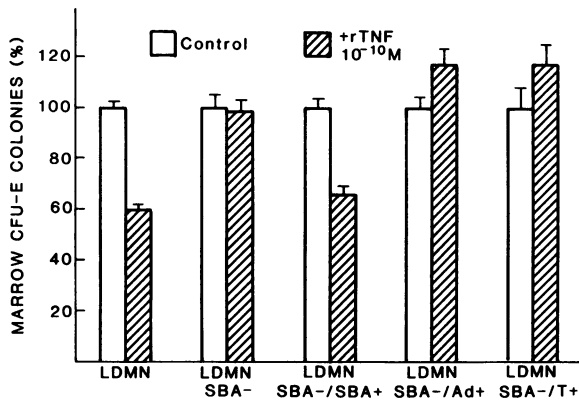
Figure 2. Effect of accessory cell removal by SBA on inhibition of marrow CFU-E colony growth by 10^{-10} M rTNF. LDMN, light density mononuclear cells at 0.5–2.0 × 105 cells/ml; LDMN SBA−, SBA+ cell depleted LDMN cells at 0.5–2.0 × 105 cells/ml; LDMN SBA−/SBA+, LDMN SBA− cells with 0.5 × 10^5 SBA+ cells/ml; LDMN SBA−/Ad+, LDMN SBA− cells with 0.5 × 10^5 adherent cells/ml; LDMN SBA−/T+, LDMN SBA− cells with 0.5 × 10^5 T lymphocytes/ml. Data from each cell fraction was normalized to CFU-E colony growth without rTNF. Five experiments are combined. Results are expressed as mean±SE.
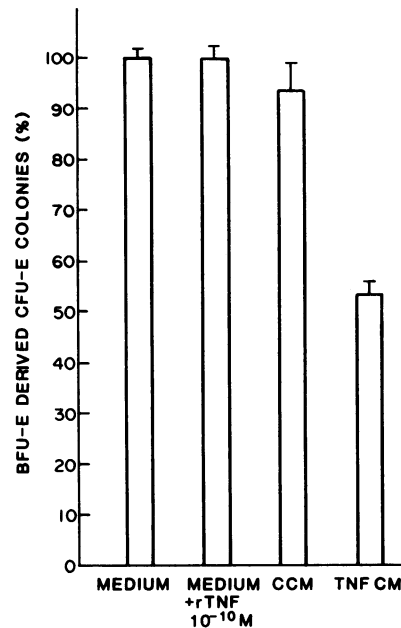

Figure 3. Effect of CM on highly purified BFU-E derived CFU-E colony growth. Inhibitory effect of TNFCM is compared to the effect of CCM and to the growth of colonies from BFU-E-derived CFU-E cultured without CM (control). Mean percent of CFU-E present in three experiments was 45.1±10.1%. Data from each experiment were normalized to CFU-E growth without CM. Results expressed as mean±SE.

CFU-E. Autologous T lymphocytes, adherent cells, and SBA+ cells were cultured with blood BFU-E-derived CFU-E with and without 10^{-10} M rTNF in plasma clots. Growth of CFU-E was significantly inhibited by rTNF in the presence of SBA+ cells but not in the presence of adherent cells or T lymphocytes (Table I). Inhibition of BFU-E-derived CFU-E colony growth by rTNF required the presence of 50–100 SBA+ cells for each peripheral blood day 8 cell (data not shown).

LDMN marrow cells were incubated for 48 h with rTNF 10^{-10} M to produce TNFCM, while CCM was prepared by incubating LDMN marrow cells 48 h without rTNF. Highly purified BFU-E-derived CFU-E were then cultured with 10% CM to determine the effect on erythropoiesis. The results of three experiments are combined in Fig. 3. The mean purity of CFU-E was 45.1±10.1%. CFU-E were significantly inhibited ($P < 0.001$) by TNFCM but not by CCM ($P = 0.22$).

Discussion

Erythropoiesis in vitro (5) and in vivo (6, 7) is inhibited by TNF. In order to more completely understand this inhibition,

Table I. Effect of Marrow Accessory Cells on Inhibition of Blood BFU-E-derived CFU-E Colony Growth by rTNF

Addition to BFU-E-derived CFU-E	Percent control
rTNF 10^{-10} M	95.1±4.9%
rTNF 10^{-10} M + T lymphocytes	103.0±4.0%
rTNF 10^{-10} M + adherent cells	82.7±4.2%*
rTNF 10^{-10} M + SBA+ cells	72.9±3.4%‡

BFU-E derived CFU-E were cultured at 10^3 cells/ml, and marrow accessory cells were added at 0.5 × 10^5/ml. Data from three experiments are combined. Results are expressed as mean±SE.
* $P > 0.05$ compared to addition of rTNF only.
‡ $P = 0.01$ compared to addition of rTNF only.

we have studied the effect of rTNF on CFU-E generated from peripheral blood BFU-E. These CFU-E are sufficiently pure to allow us to distinguish a direct inhibitory effect on erythroid colony growth from an indirect effect. Our results indicate that inhibition of CFU-E growth by rTNF is not a direct inhibitory effect, but requires accessory cells.

Inhibition of erythroid colony growth by rTNF could be mediated by a soluble factor or might require direct cell–cell interaction. To address this question, cell-free TNFCM was prepared from LDMN cells stimulated by 10^{-10} M rTNF. This concentration of rTNF was chosen because it represents a point at which inhibition of erythroid colony growth from LDMN marrow cells has reached a plateau. TNFCM inhibited colony growth from BFU-E-derived CFU-E resistant to direct inhibition by rTNF. Since the TNFCM cultures contained rTNF at a final concentration of 10^{-11} M, we cannot rule out the possibility that TNF participates in inhibition of erythropoiesis in cooperation with some other factor that acts directly on CFU-E or possibly by sensitizing CFU-E to the effect of some other factor induced by TNF.

TNF stimulates the release of a large number of potential modulators of hematopoiesis, such as IL-1, IL-6, granulocyte-macrophage colony-stimulating factor, platelet-derived growth factor (PDGF), transforming growth factor-B, prostaglandins, and leukotrienes (14). It may also stimulate the release of gamma interferon indirectly via IL-1 (15). These factors may stimulate (PDGF [16]) or inhibit erythropoiesis (IL-1 [3], transforming growth factor-B [17]). PDGF and granulocyte-macrophage colony-stimulating factor, however, do not effect the growth of BFU-E-derived CFU-E (9), so the net effect of TNF is inhibitory.

Enrichment of marrow for CFU-E by removal of SBA+ cells ablated inhibition of erythroid colony growth by rTNF. This indicates that the cell population which releases the inhibitor of erythropoiesis resides in the SBA+ marrow cell fraction. We confirmed this finding by adding SBA+ cells to the LDMN SBA− marrow cells and restoring inhibition of CFU-E growth by rTNF. BFU-E-derived CFU-E resistant to inhibition by

rTNF also showed inhibition of colony formation in the presence of autologous SBA+ marrow cells. The SBA+ cell fraction includes the majority of human marrow accessory cells, including T cells, B cells, monocytes (10), and stromal elements, including endothelial cells (18). Experiments in which autologous marrow cells were mixed with either LDMN SBA− marrow cells or BFU-E-derived CFU-E indicate that it is neither adherent cells (monocytes) nor T lymphocytes that mediate this effect.

In conclusion, we have provided evidence indicating that TNF does inhibit erythropoiesis, but does not exert a direct inhibitory effect on CFU-E. TNF leads to the release of a soluble factor which in turn inhibits the growth of CFU-E in vitro. The cell(s) and factor(s) that mediate inhibition of CFU-E require further studies for precise identification. While many potential mediators of ACD have been described, the means by which they exert their inhibitory effects, and the final common pathway of ACD, remain questions for continuing investigation.

Acknowledgments

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