Abnormal Hemopoiesis in Long-Term Bone Marrow Culture from Tumor Necrosis Factor-Deficient Mice

M. S. Drutskaya, D. V. Kuprash, S. A. Nedospasov, I. L. Chertkov, and N. I. Drize

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We studied hemopoiesis in mice deficient by the tumor necrosis factor gene. The total number of cells in long-term bone marrow cultures from these mice 2-fold surpassed that in wild-type and tumor necrosis factor p55 receptor-deficient animals. Increased cell production was related to the absence of tumor necrosis factor expression by hemopoietic precursors. The total cell production by explanted hemopoietic cells from tumor necrosis factor-deficient mice did not depend on the genotype of irradiated stromal sublayer in long-term cell cultures from wild-type mice and animals deficient by tumor necrosis factor or p55 receptor. These results suggest that tumor necrosis factor, but not its p55 receptor, is involved in transduction of signals regulating production of cultured cells. Tumor necrosis factor probably regulates hemopoiesis in long-term bone marrow cultures by initiating apoptosis of hemopoietic cells or inhibiting cell proliferation. Increased cell production probably attests to the absence of one or both effects.

Key Words: *stem hemopoietic cell; tumor necrosis factor; hemopoietic precursors; long-term bone marrow culture; knockout mice*

Tumor necrosis factor (TNF) plays an important regulatory role in the immune response and inflammation. TNF acts as a bifunctional regulator of *in vitro* hemopoiesis. TNF can inhibit and stimulate hemopoietic precursors *in vitro* [8,13,14,16] depending on its concentration and the presence of interleukin-3 and granulocyte/macrophage and granulocyte colony-stimulating factors [14].

Previous studies of the physiological role of TNF were performed on TNF-deficient knockout mice and showed the dynamics of proliferation and differentiation of hemopoietic cells with impaired TNF-mediated signal cascade. The role of TNF in the regulation of hemopoiesis *in vivo* was studied on mice deficient by TNF receptors, but not on TNF-deficient animals.

Research Center for Hematology, Russian Academy of Medical Sciences; V. A. Engel'gardt Institute of Molecular Biology, Russian Academy of Sciences; A. N. Belozerskii Institute of Physicochemical Biology, Moscow State University, Moscow. ***Address for correspondence:*** nodrize@blood.ru. N. I. Drize

According to the structure of cytoplasmic domains, TNF receptors are divided into 2 subtypes: containing and lacking "death domain". TNF p55 (molecular weight 55 kDa) and TNF p75 receptors (molecular weight 75 kDa) belong to subtypes 1 and 2, respectively [15]. It was hypothesized that p55 receptors are involved in the inhibitory effect of TNF on cell proliferation (apoptotic signal transduction), while TNF-induced activation is realized via p75 receptors. Recent studies showed that these subtypes of TNF receptors transduce both activating and apoptotic signals [5,17]. Apart from cytotoxic activity of TNF that causes cell death, p55 receptors transduce signals activating fibroblast proliferation and prostaglandin synthesis [3,4]. TNF p55 receptors and adaptive molecules trigger cascades, which simultaneously induce opposite signals activating transcriptional factors NF-kappa-B and AP1 or programmed cell death [17]. NF-kappa-B is involved in the initiation of genes protecting cells from apoptosis. It remains unclear which factors contribute to pro-

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apoptotic or antiapoptotic effects of TNF on these cells.

The role of TNF in hemopoiesis was studied in *in vitro* experiments with recombinant TNF. In the present work, we evaluated the role of TNF in hemopoiesis on the model of long-term bone marrow culture from knockout mice.

MATERIALS AND METHODS

Experiments were performed on 6-18-week-old male and female wild-type (WT) mice and animals deficient in TNF ($TNF^{-/-}$) [2,11] or TNF p55 receptor ($p55^{-/-}$) [12]. These knockout animals were bred from mixed genetic sv/C57/Bl/6 mice and crossbred 2 with inbred C57Bl/6 mice.

Long-term bone marrow culture was obtained by the method of Dexter [1]. Femoral bone marrow (not suspended to single cells) was cultured in 25-cm^2 plastic flasks containing 10 ml Fischer's medium, glutamine, antibiotics, 10^{-6} M hydrocortisone, and 20% serum (1/3 fetal bovine serum and 2/3 horse serum). Half of the medium was replaced once a week. To obtain stromal sublayers of adherent cells, the bone marrow was cultured for 3 weeks until confluence. The cultures were irradiated in a dose of 40 Gy on a ^{137}Cs IPK device (3.75 Gy/min). The concentrations of granulocyte/macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) were routinely measured in agar and methylcellulose, respectively, using conditioned media from WEHI-3B and L929 cells and erythropoietin. The number of colonies was estimated on days 7 and 14, respectively.

The results were analyzed by Student's *t* test.

RESULTS

Cell production in cultures from WT, $TNF^{-/-}$, and $p55^{-/-}$ mice was similar over the first 6 weeks of culturing (Fig. 1). On weeks 5-8, cell count continuously increased in $TNF^{-/-}$ cultures, while in WT cultures cell production was attenuated and remained unchanged from the 16th week of culturing (Fig. 1). In bone marrow cultures from $TNF^{-/-}$ mice, hemopoietic processes were intensive to the 30th week. After 30-week culturing, the total cell count in bone marrow cultures from WT, $p55^{-/-}$, and $TNF^{-/-}$ mice were 61.7×10^6, 86.2×10^6, and 166.5×10^6, respectively (the initial cellularity of implanted bone marrow was $12\text{-}15\times10^6$ cells). A 2-fold increase in the count of cultured cells from $TNF^{-/-}$ mice was probably associated with TNF-induced apoptosis or inhibition of cell proliferation. Cell production insignificantly increases in the presence of TNF and blockade of signal transduction via p55 receptors. Previous studies showed that TNF modulates the hemopoietic microenvironment by inhibiting proliferation of adipocytes [10] or changing adhesive properties of endotheliocytes [7] in long-term bone marrow cultures. The physiological role of TNF in *in vitro* hemopoiesis is probably realized via its effects on the stromal microenvironment and hemopoietic cells.

To evaluate the role of TNF expression by stromal or hemopoietic cells, 2×10^6 bone marrow cells from WT, $TNF^{-/-}$, and $p55^{-/-}$ mice (suspended to single cells) were explanted into irradiated stromal sublayers containing cells of the same genotypes in various combinations. Independently on the genotype of sublayers, cell production 2-fold increased after the 2nd explantation of $TNF^{-/-}$ bone marrow cells (Fig. 2, *a*). The absence of TNF expression by hemopoietic cells considerably promoted cell production. Normal expression of TNF by stromal cells did not decrease the total cellularity of the bone marrow cultures. Therefore, TNF expression by hemopoietic cells is the major factor stimulating cell proliferation or preventing cell death. TNF regulates hemopoietic precursors by the autocrine mechanism. It should be emphasized that cytokines/ligands of the TNF family exist in transmembrane (anchored) or soluble forms. Previous studies showed that TNF exists in the soluble form. The role of membrane-bound biologically active TNF [6] and its contribution to the regulation of hemopoiesis remain unclear. The count of WT and $p55^{-/-}$ cells cultured on stromal sublayers containing no TNF did not significantly increase compared to that on TNF-expressing sublayers. A considerable decrease in the cellularity of cultures from $p55^{-/-}$ mice suggested the ability of TNF to stimulate hemopoietic cell growth

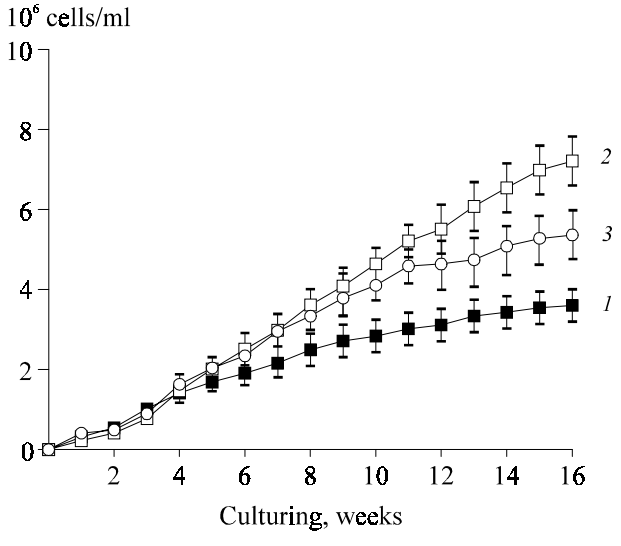

Fig. 1. Total cell count in long-term bone marrow cultures from wild-type mice (*1*) and animals deficient by tumor necrosis factor (*2*) or p55 receptor (*3*).

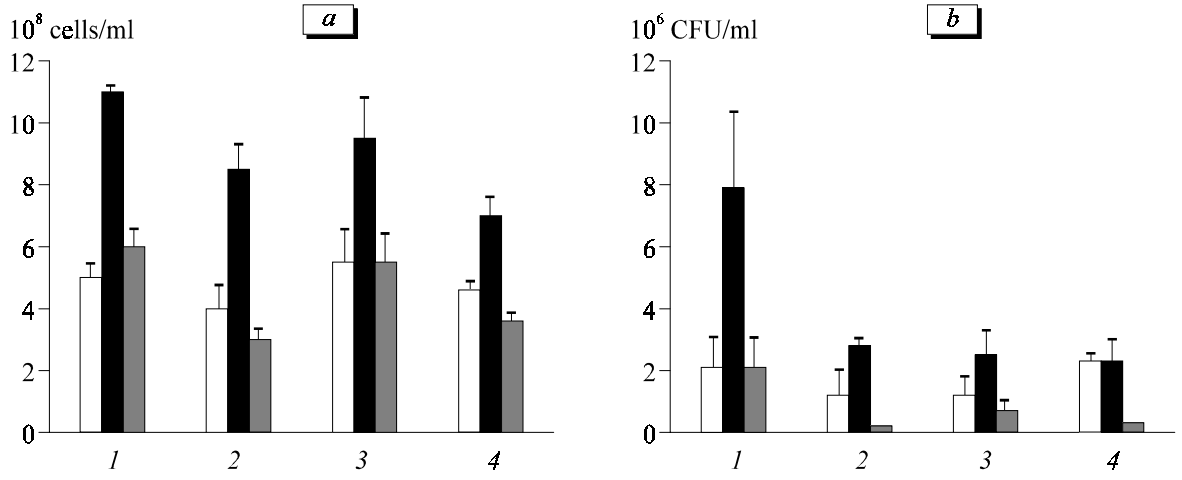

Fig. 2. Total counts of cultured cells (*a*) and CFU (*b*) after 19-week culturing on stromal sublayers of adherent cells (SAC) with various genotypes: initial culture (*1*), wild-type SAC (*2*), SAC from TNF-deficient mice ($TNF^{-/-}$, *3*), and SAC from p55-deficient mice ($p55^{-/-}$, *4*). Wild-type (light bars), $TNF^{-/-}$ (dark bars), and $p55^{-/-}$ (shaded bars) cell cultures.

via p55 receptors. Thus, TNF produces a pleiotropic effect on hemopoietic cells. TNF-induced stimulation or inhibition of hemopoiesis was previously demonstrated in *in vitro* experiments on mouse and human WT cells, cultured in the presence of various concentrations of TNF [8,14,16]. We studied the effects of TNF on various hemopoietic cells. After 19-week culturing on various sublayers, the content of colony-forming units in $TNF^{-/-}$ cells (CFU-C) 4-fold surpassed that in WT and $p55^{-/-}$ cultures (Fig. 2, *b*). Thus, transduction of TNF signals via p55 receptors does not modulate colony formation. Unlike mature cells, the count of precursors did not significantly increase after culturing on various sublayers. Therefore, TNF can both inhibit and stimulate apoptosis of cells more differentiated than CFU-C. Since suppression of mature hemopoietic precursors is realized via p55 receptors [9], these receptors probably transduce TNF signals stimulating CFU-C growth. The total number of precursors in $p55^{-/-}$ cultures significantly decreased when the sublayer expressed TNF (Fig. 2, *b*).

Our results indicate that cultured bone marrow cells from $TNF^{-/-}$ mice stimulate total cell production by increasing the lifetime of immature hemopoietic precursors (intensive hemopoiesis is observed for more than 30 weeks) and preventing death of cells more differentiated than CFU-C. *In vitro* expansion of hemopoietic precursors can be used in clinical practice (e.g., genotherapy and transplantation of umbilical blood cells) for modulation of the TNF-receptor system.

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