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Abnormalities in Long-Term Bone Culture from Mice Deficient in Tumor Necrosis Factor: Induction of Apoptosis and Cell Proliferation

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The total duration of hemopoiesis and the total cell production in long-tern bone marrow cultures from mice deficient by tumor necrosis factor are increased, while proliferation of granulocyte-macrophage precursor cells, the main cell populations in long-term bone marrow cultures, did not differ from that in wild-type mice. In bone marrow cultures from knockout mice the intensity of apoptosis remained low during 40-week culturing and was similar to that in early wild-type cultures (10 weeks). Then, the intensity of apoptosis in bone marrow cultures from knockout mice did not differ or even surpassed that in wild-type cultures. However, we observed no inhibition of hemopoiesis in bone marrow cultures from knockout mice. The absence of autocrine expression of tumor necrosis factor did not affect proliferation of precursor cells, but modulate the intensity of apoptosis. It remains unclear whether changes in apoptosis are related to intensive cell production.

Key Words: *stem hemopoietic cell; tumor necrosis factor; hemopoietic precursor cells; long-term bone marrow culture; tumor necrosis factor-deficient mice; proliferation; apoptosis*

Tumor necrosis factor (TNF) acts as a polyfunctional regulator of hemopoiesis affecting precursor cells of different maturity. TNF inhibits proliferation of hemopoietic cells [5,11] and induces apoptosis via p55 and p75 receptors [4]. TNF receptors transmit cell division signals [5,9,10]. These data suggest that the mechanisms and regulation of cell proliferation and programmed cell death are probably impaired in TNF-deficient mice ($TNF^{-/-}$). No hemopoietic abnormalities except structural changes in the spleen were found in $TNF^{-/-}$ mice [7,8]. The main quantitative characteristics of hemopoiesis are similar in knockout and wild-type mice (WT) [1]. *In vivo* effects of other cytokines probably compensate for the absence of autocrine TNF, while *in vitro* changes in hemopoiesis are more pronounced. The long-term bone marrow culture (LTBMC) from $TNF^{-/-}$ mice differs from that from WT animals by higher number of granulocyte-macrophage precursor cells (CFU-GM), more intensive cell production, and longer maintenance of hemopoiesis [2]. This is probably related to intensive proliferation of cells and/or suppression of apoptosis in hemopoietic cells due to the absence of autocrine TNF expression. For evaluation of changes in proliferation of hemopoietic precursor cells in LTBMC from $TNF^{-/-}$ mice we studied proliferation of CFU-GM from the suspension fraction (SF) of the culture. Apoptotic cells in SF were counted weekly throughout culturing.

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MATERIALS AND METHODS

The experiments were performed on 10-28-week-old male and female WT C57Bl/6 and TNF$^{-/-}$ mice (Institute of Bioorganic Chemistry, Pushchino) [8].

LTBMC were prepared as described elsewhere [3]. The femoral bone marrow was placed (without preparing single cell suspension) in 25-cm^2 plastic flasks with 10 ml complete nutrient medium containing Fischer medium, glutamine, antibiotics, 10^{-6} M hydrocortisone, and 20% serum ($^1/_3$ fetal bovine serum, FBS, and $^2/_3$ equine serum). The medium was half replaced weekly. Viable cells were visualized by trypan blue staining.

For evaluation of proliferation of CFU-GM, the cells (10^6 cells/ml) were incubated with 1 mg/ml hydroxyurea in Fisher medium containing 2% FBS at 37°C for 2 h, washed with the same medium, and cultured (10^5 cells/well) in a 24-well plate with 0.5 ml medium containing αMEM medium, 0.3% agar, 30% FBS, and 10% medium conditioned by WEHI 3B and L929 cells (2:1 ratio) used as the source of growth factors. After 7-day culturing at 37°C and 5% CO_2 colonies were counted under an inverted microscope. The number of proliferating cells was estimated by the difference between the counts of CFU-GM before and after treatment with hydroxyurea that eliminates dividing cells.

Apoptotic cells were counted weekly during medium replacement. The cells were washed with cold phosphate buffer, fixed in 4% paraformaldehyde, washed with phosphate buffer, permeabilized in 0.2% Triton X-100 in phosphate buffer, washed 2 times with cold phosphate buffer, and stained with 10^{-4} mg/ml 4',6-diamidino-2-phenylindole for visualization of cells with fragmented chromatin [6].

RESULTS

In LTBMC from WT mice hemopoiesis was exhausted by the 20th week of culturing. The intensity of cell production in LTBMC from TNF$^{-/-}$ mice decreased, but persisted even after 1-year culturing (Fig. 1, *a*). The ratio of viable cells in LTBMC from TNF$^{-/-}$ and WT mice was 95-99%. The dynamics of apoptosis was different in cultured cells from TNF$^{-/-}$ and WT mice (Fig. 1, *b*). The degree of apoptosis in cultured cells from both mouse strains was low over the first 10 weeks of culturing (1-4%). Then the intensity of apoptosis in cultured cells from WT mice increased to 60%, which correlated with inhibition of cell production and rapid exhaustion of hemopoiesis. The intensity of apoptosis in cultured cells from TNF$^{-/-}$ mice changed insignificantly over 40-week culturing. In these cultures cell production was $1\text{-}7\times10^5$ cells/ml. In cultured cells from TNF$^{-/-}$ mice the intensity of apoptosis progressively increased after 30-40 weeks in culture and even surpassed that in WT cultures. However, these changes did not abolish hemopoiesis. The cell count in culture remained practically unchanged from the 30th to 60th week of culturing, while the number of apoptotic cells progressively increased.

The production of CFU-GM and colony-forming cells in the spleen (CFU-S) in LTBMC from TNF$^{-/-}$ mice was observed throughout the entire period of culturing. After culturing for 42, 55, and 58 weeks the count of CFU-GM was 359±28, 177±8, and 164±11 per 10^5 SF cells, respectively. The number of CFU-S was 5±2, 29±7, and 16±3 per 10^5 SF cells by the 39th, 51st, and 54th weeks of culturing, respectively. CFU-S formed granulocyte, megakaryocyte, erythroid, undifferentiated, and mixed colonies. Thus, cultured precursor cells retain their differentiation potency and

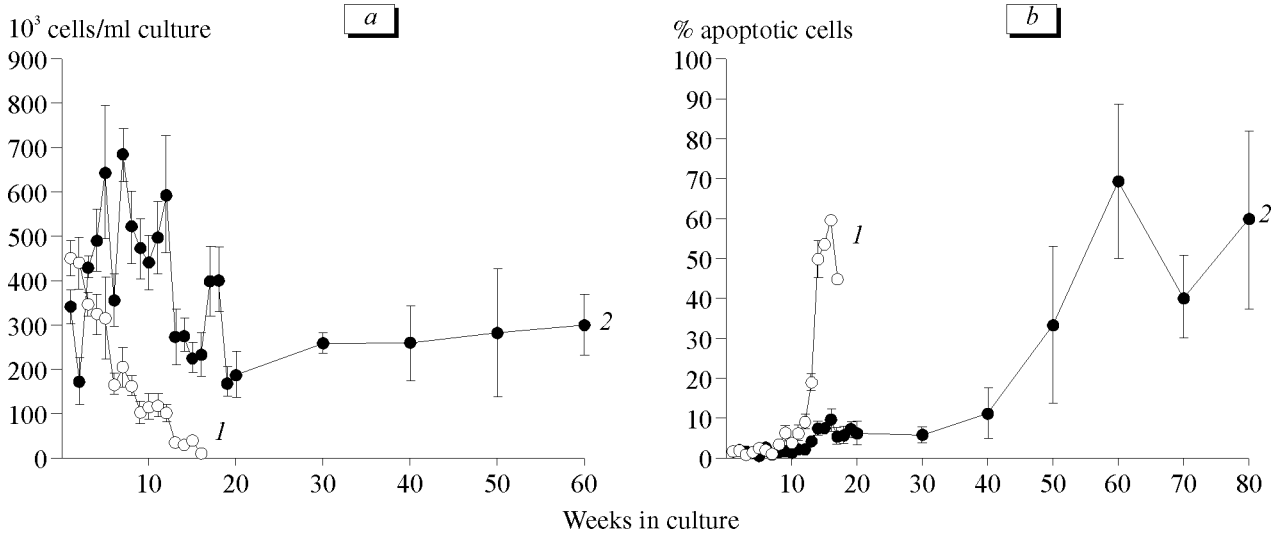

Fig. 1. Cell production (*a*) and apoptosis (*b*) in long-term bone marrow culture from wild-type (*1*) and tumor necrosis factor-deficient mice (*2*).

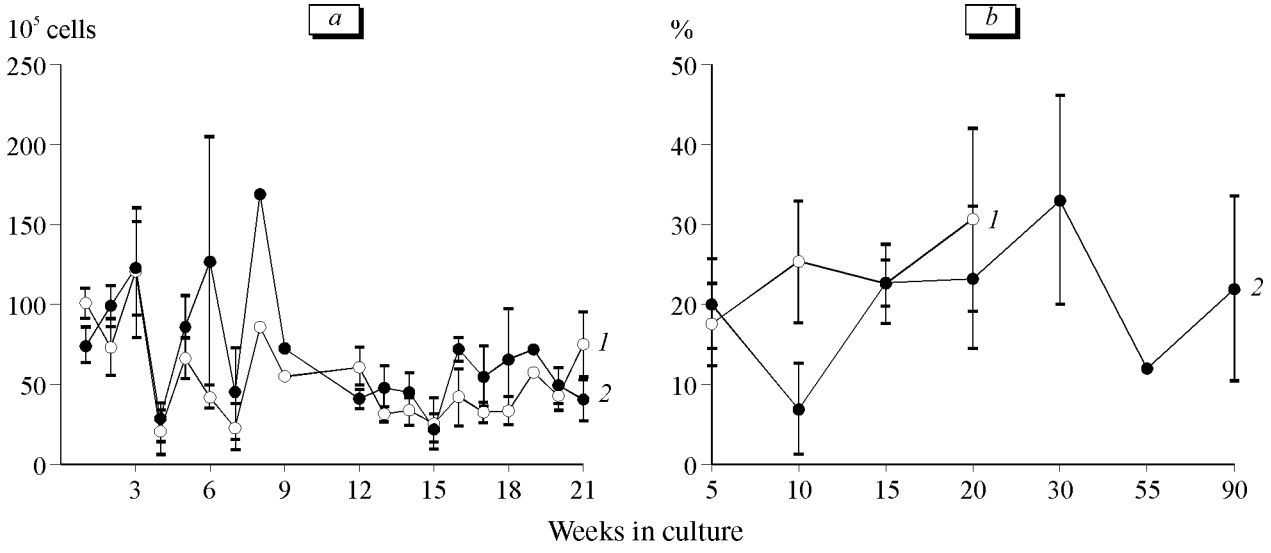

Fig. 2. Count (*a*) and percentage of proliferating CFU-GM (*b*) in long-term bone marrow culture from wild-type (*1*) and tumor necrosis factor-deficient mice (*2*).

display normal activity in the corresponding microenvironment.

The count of precursor cells in SF and their proliferative activity were comparable in early WT and TNF$^{-/-}$ cultures (Fig. 2). It can be hypothesized that stable production of cells and relatively normal number of precursor cells against the background of intensive apoptosis are related to activation of proliferation in LTBMC from TNF$^{-/-}$ mice after 30 weeks in culture. However, the percentage of dividing cells did not increase during culturing (Fig. 2, *b*). These data indicate that the absence of TNF that suppresses proliferation and induces apoptosis is not accompanied by activation of proliferation and inhibition of apoptosis in cells.

Previous studies showed that apoptosis in hemopoietic cells is triggered by the interaction of TNF with its receptors carrying the death domain and binding of Fas with its ligand [12]. Apoptosis can be induced via some indirect mechanisms (decreased content of growth factors in the culture or changes in adhesive interactions). Low content of interleukin-3 and granulocyte or granulocyte-macrophage growth factor induces apoptosis in precursor cells, in particular in CFU-GM [12]. The count of CFU-GM and the intensity of cell production in LTBMC from TNF$^{-/-}$ mice did not decrease, hence induction of apoptosis cannot be explained by low expression of growth factors. Probably, this process is associated with changes in adhesive interactions between stromal and hemopoietic cells in LTBMC. It cannot be excluded that the interaction between Fas and its ligand plays a role in the induction of apoptosis in LTBMC from TNF$^{-/-}$ mice. However, this process can stimulate apoptosis in LTBMC, but it cannot prolong hemopoiesis. Long-term maintenance of hemopoiesis is probably associated with prolonged lifetime of TNF$^{-/-}$ hemopoietic precursor cells in LTBMC. The mechanisms responsible for prolonged hemopoiesis in the culture without autocrine expression of TNF will help to understand the regulation of hemopoiesis and differentiation at the genetic level.

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