Long-Term Self-Maintenance of Hemopoietic Precursors in Bone Marrow Culture Derived from Tumor Necrosis Factor-Deficient Mice Is Not a Result of Neoplastic Transformation

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In long-term bone marrow cultures derived from tumor necrosis factor-deficient mice the total cell production and the total duration of hemopoiesis are increased (the latter is comparable with mouse life span). Telomerase activity in cells of nonadherent fraction of long-term bone marrow cultures from tumor necrosis factor-deficient mice increases with time and peaks after 1-year culturing. Karyotyping of nonadherent and adherent cells of long-term bone marrow cultures revealed instability of nonadherent cells and hyperploidy of the stromal sublayer cells, which attested to the presence of a neoplastic transformation. However, cell differentiation is not blocked in long-term bone marrow cultures. The nonadherent fraction of long-term bone marrow cultures from tumor necrosis factor-deficient mice cannot be cultured without exogenous growth factors; in the presence of growth factors the cells proliferate, but cannot be passaged; stromal sublayer cells cannot be passaged as well. Intraperitoneal and intravenous injections of nonadherent cells to recipients with normal and radiation-attenuated immunity induced no tumor growth. Hence, peculiar dynamics of long-term bone marrow cultures from tumor necrosis factor-deficient mice cannot be explained by neoplastic transformation.

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

Tumor necrosis factor (TNF) is a pleiotropic cytokine. TNF induces necrosis of tumors transplanted to mice and exhibits selective cytotoxicity against transformed cells. Now, the participation in immune reactions and modulation of inflammation are considered to be the main function of TNF. However TNF is a multifunctional regulator of hemopoiesis: it directly and indirectly stimulates and inhibits the growth of hemopoietic cells, including early hemopoietic precursors [8,11, 12]. In TNF-deficient mice ($TNF^{-/-}$) various disturbances in inflammatory reactions and immunity are reported [9,10]. Changes in the hemopoietic system were not described, but disturbed interactions between stromal and hemopoietic cells are responsible for abnormal structure of the spleen [9] and impaired capacity cells carrying stromal microenvironment to form ectopic hemopoiesis [1]. The dynamics of long-term bone marrow culture (LBMC) is modified as well: cell production and duration of hemopoiesis are increased in comparison with cultures from wild type (WT) mice [2].

Hemopoiesis in LBMC from WT mice decreases after 16-20 weeks, while in LBMC from $TNF^{-/-}$ mice

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hemopoiesis is maintained without signs of inhibition, and the "oldest" culture was maintained for 104 weeks (this is comparable with mouse life span). This attests to modification of self-maintenance of hemopoietic cells in these cultures. This can be due to impaired regulation of proliferation of precursor cells, or abnormal function of TNF-dependent apoptosis pathways, or tumor transformation of the culture. We investigated the possibility of neoplastic transformation in LBMC from TNF$^{-/-}$ mice.

MATERIALS AND METHODS

Experiments were carried out on male and female 10-28-week-old C57Bl/6 mice (WT and TNF$^{-/-}$ [10], Institute of Bioorganic Chemistry, Pushchino). Before the experiment TNF$^{-/-}$ mice derived on a mixed sv129/C57Bl/6 genetic base were crossed 5 times with inbred C57Bl/6 strain. The mice were irradiated on a ^{137}Cs device (Institute of Blood Transfusion) at a dose power of 17 sGy/min. Chimeras were obtained after irradiation of recipients in a dose of 425 sGy (2 times with 3-h intervals). Cell growth in cultures from sublethally irradiated recipients (300 sGy) was also evaluated.

The cells from 45-week cultures (2×10^6) were injected to intact and irradiated (300 sGy) mice and the results were evaluated after 2 months. Chimeras were reconstituted with bone marrow from TNF$^{-/-}$ mice (group 1) or with 2×10^6 cells from the culture (24 weeks)+10^5 bone marrow cells from C57Bl/6 mice (group 2); the results were evaluated after 12 and 15 months, respectively.

LBMC was prepared as described previously [7]. Bone marrow from one femur was transferred (without suspending to single cells) into 25-cm^2 plastic flasks in 10 ml complete nutrient medium (CNM) consisting of Fisher medium supplemented with glutamine, antibiotics, and 10^{-6} M hydrocortisone, and containing 20% serum ($^1/_3$ fetal calf serum and $^2/_3$ equine serum). The medium was half-replaced once a week. For evaluation of the growth capacity of nonadherent cells of LBMC in the absence of stromal sublayer, 10^5 cells/ml were cultured in CNM or CNM with 10% medium conditioned with WEHI 3B cells as the source of growth factors.

Stromal sublayer was passaged (1:3) in CNM once a week. The sublayer was collected routinely with Versene (0.02% EDTA) and 0.25% trypsin.

For karyotyping, nonadherent cells of LBMC were treated with colchicine (40 μg/ml, 10 μl/ml suspension) for 1 h, fixed, processed routinely, and stained with G-specific dye [5].

Telomerase activity in cells from LBMC suspension was evaluated by a method based on combination of TRAP analysis with PCR and enzyme immunoassay. Standard Boehringer Mannheim kits were used [6].

TNF$^{-/-}$ cells in peripheral blood of chimeric mice were identified by PCR as described previously [10].

RESULTS

The absence of replicative aging (immortalization), attenuation of apoptosis induction, blockade of cell differentiation, self-sufficiency in proliferative signals, genetic instability, resistance to growth-suppressing signals, changes in cytoskeleton/locomotion, and stimulation of neoangiogenesis are the most important characteristics of neoplastic cells [3]. The possibility of neoplastic transformation in culture was tested by a number of parameters.

Cell production in LBMC from TNF$^{-/-}$ and WT mice was virtually the same during the first 15 weeks, after which hemopoiesis in WT LBMC died (Fig. 1), while cell production in TNF$^{-/-}$ LBMC decreased, but persisted even after 104 weeks. This long-term maintenance of precursor cells in culture is similar to immortalization.

A special study was devoted to induction of apoptosis in LBMC [4]. Termination of hemopoiesis in LBMC from WT mice after 12-16-week culturing correlated with increased level of apoptosis in the nonadherent fraction. The level of apoptosis in LBMC from TNF$^{-/-}$ mice increased only after 40-60 weeks and after 90 weeks it surpassed the level critical for WT LBMC. However, this did not lead to termination of hemopoiesis. Hence, apoptosis induction in characteristic of LBMC from TNF$^{-/-}$ was not observed.

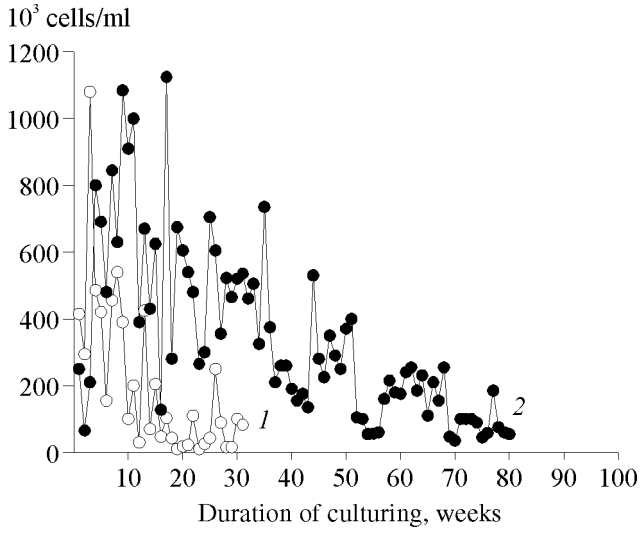

Fig. 1. Cell count in long-term bone marrow culture from wild type mice (*1*) and in culture from tumor necrosis factor-deficient mice (*2*).

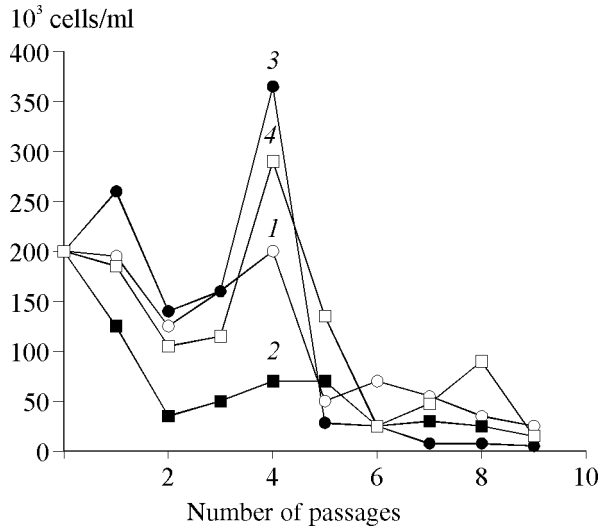

Fig. 2. Growth dynamics of nonadherent cell from long-term bone marrow culture without stromal sublayer in the presence of growth factors 91-B1 (*1*), 91-TNF (*2*), 80-B1 (*3*), and 80-TNF (*4*).

Morphological study of nonadherent cells showed the presence of myeloid cells of different maturity in TNF$^{-/-}$ culture, similarly to WT cultures. Hence, cell differentiation was not blocked at the level of blasts in this culture.

Nonadherent cells cultured without feeder and growth factors died. Addition of 10% medium conditioned by WEHI 3B cells and containing granulocyte- and granulocyte-macrophage colony-stimulating factors and interleukin-3 induced division of nonadherent cells: their number increased and the cells differentiated into granulocytes (Fig. 2). For evaluation of proliferative potential of the stromal sublayer of TNF$^{-/-}$ LBMC, the sublayers were passaged 3 times. The cells formed a monolayer to the next passage, but after 4 passages cell growth was terminated. Hence, stromal and nonadherent cells are not self-sufficient in proliferative signals.

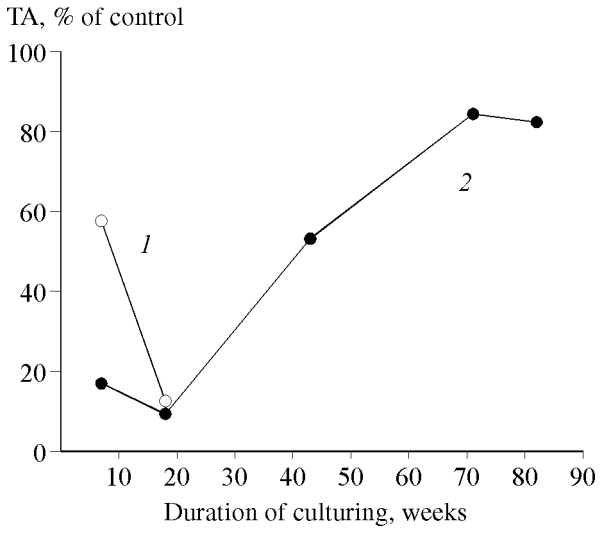

Fig. 3. Telomerase activity (TA) in cells of long-term bone marrow culture from wild type (*1*) and tumor necrosis factor-deficient (*2*) mice. Control: TA activity of K562 cell strain.

Karyotyping showed that hyperploidy is characteristic of stromal cells (14 of 14 mitoses) and aneuploidy is typical of nonadherent cells. However, cells with normal chromosome set were always present in the culture (40 of 77 mitoses), aberrations were not monoclonal (up to 3 genotypes were present in one culture simultaneously), and abnormalities differed in different cultures. Hence, we detected no signs of replacement of normal cells with transformed cells, and the genome instability was most likely a result, but not a cause of long-term culturing.

No tumor growth was observed after intravenous and intraperitoneal transplantation of nonadherent cells from TNF$^{-/-}$ LBMC to intact, irradiated (300 sGy), and lethally irradiated mice (chimeras, group 2). PCR analysis of peripheral blood 2 months after transplantation detected cells with TNF$^{-/-}$ genotype in intact mice, but not in mice irradiated in a dose of 300 sGy or in group 2 chimeras. The number of peripheral blood leukocytes was virtually the same in intact and sublethally irradiated mice, but differential blood count in mice receiving cells from the culture was changed (Table 1).

No shifts towards blast and young elements was observed in sublethally irradiated mice with TNF$^{-/-}$ cells in the blood (Table 1), which indicates the absence of neoplastic transformation in mice receiving cells from the nonadherent fraction of TNF$^{-/-}$ LBMC.

High telomerase activity is characteristic of cell lines and is believed to be responsible for switching off "cell clock". Though telomerase activity in WT nonadherent cells was initially higher than in TNF$^{-/-}$ cells, it decreased by week 20, *i. e.* by the moment of hemopoiesis "exhaustion", while in TNF$^{-/-}$ LBMC it increased (Fig. 3). We can speak about direct correlation between the absence of autocrine expression of TNF and increased of telomerase activity in long-term culture cells.

Hence, the duration of LBMC maintenance, genetic instability, and increased telomerase activity can be considered as evidence of neoplastic transformation. But the retained possibility of apoptosis induction, incapacity to tumor formation, the absence of differentiation block and capacity to self-maintenance do not permit us to make a conclusion on tumor transformation in the TNF$^{-/-}$ LBMC. Presumably, changes in the TNF$^{-/-}$ LBMC hemopoiesis are caused by another mechanism of hemopoiesis regulation in the absence of TNF, which will be the object of further investigation.

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TABLE 1. Hemograms in Different Mouse Groups (% of Control)

Cell types	Intact mice	Irradiation in a dose of 300 sGy	Chimeras	
			group 1	group 2
Myelocytes	0.0	0.0	0.0	0.0
Young	66.7	66.7	66.7	180.0
Neutrophils	60.0	35.7	73.8	205.0
Segmented	158.8	161.5	204.9	177.2
Eosinophils	124.3	108.1	262.2	247.3
Basophils	0.0	0.0	0.0	0.0
Monocytes	170.6	173.8	82.6	64.8
Lymphocytes	85.9	88.0	89.1	81.7

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