Stromal Cell Strains Derived from Suspension Fraction of Bone Marrow Cultures of Tumor Necrosis Factor-Deficient Mice

I. N. Nifontova, Yu. V. Ol'shanskaya, and N. I. Drize

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Culturing of suspension fraction of a long-term bone marrow culture derived from tumor necrosis factor (TNF)-deficient mice for 75 days produced cells forming adherent cell strains. The cells of all strains expressed RNA of various stromal differentiating markers in various combinations. The cells of many strains simultaneously expressed genes encoding products characteristic of different differentiation lineages. The derived strains maintained hemopoiesis for 10 weeks. RNA-analysis of gene expression by cells of these strains showed that they express a set of various growth factors and cytokines. It was hypothesized that suspension fraction of long-term bone marrow culture derived from TNF-deficient mice includes immature stromal precursor cells, which were never detected in long-term bone marrow culture derived from wild-type mice.

Key Words: *long-term bone marrow culture; TNF-deficient mice; stromal precursor cells; hemopoiesis; stromal lineages*

Recent studies showed that in addition to a variety of control functions in immune and hemopoietic systems [7], the tumor necrosis factor (TNF) participates in splenic morphogenesis [8]. The functional *in vitro* tests showed that interaction between stromal and hemopoietic cells is disturbed in knockout mice: stromal precursor cells of mice deficient for TNF (TNF-/- mice) practically do not tolerate the microenvironment during ectopic bone marrow transplantation [1].

When culturing the bone marrow cells from TNF-/- mice, some differences from the wild-type (WT) cultures were observed. In WT cultures, hemopoiesis faded away in 20-35 weeks. By contrast, in the long-term bone marrow culture (LTBMC) the TNF-/- cells with hemopoietic markers were found on culturing week 180. This long-term hemopoiesis is related neither to the changes in proliferation or apoptosis of the precursor cells [2], nor to the appearance of neoplastic transformation in LTBMC TNF-/- cells [3]. It is known that after long-term culturing, non-hemopoietic cells appear in the suspension fraction (SF) of LTBMC TNF-/- cells, which under certain conditions can generate adherent and actively proliferating progeny. The present paper examines the properties of these cell strains.

MATERIALS AND METHODS

The experiments were carried out on male and female 10-28-week mice of C57Bl/6DT and TNF-/- strains [8]. The animals were taken from the vivarium of Institute of Bioorganic Chemistry (Pushchino).

LTBMC was obtained by the method of Dexter [6] as described elsewhere [2].

For isolation of adherent cell strains, the cells from SF of LTBMC TNF-/- of different maturity were placed into 96- or 24-well plates (500-1000 or 100,000 cells per well, respectively). In the first case, the sublayer was formed by irradiated peritoneal macropha-

Hematological Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** ndrize@yandex.ru. N. I. Drize

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ges (IPK setup on ^{137}Cs, exposure time 10 min, irradiation intensity 435 cGy/min). The cells from the wells, which continuously produced progeny, were passaged several times. The resulting strains were cloned. To this end, the cells were placed in 96-well plate (one cell in a well), where the sublayer was formed by irradiated cells of the same strain (1250 cells per well). Initially, the strains were obtained and cloned on the medium used for LTBMC. We used the medium for osteoblastic differentiation [11] containing DMEM + 10% FCS, x1 ITS (insulin-transferrin-sodium selenite), 10 mM β-sodium glycerophosphate, 0.05 mM 2-phosphate ascorbic acid, 100 nM dexamethasone, 1 mM sodium pyruvate, 2 mM glutamine, and the antibiotics.

Before immunochemical staining for extracellular matrix proteins, confluent cell monolayers were grown on coverslips. Immunochemical analysis for other markers was carried out in cell suspension. The cells were fixed in 4% paraformaldehyde. We used rat antibodies against mouse CD45, CD44, PECAM-1, Sca-1, and Mac-3 (BD Pharmingen) and rabbit antibodies against human collagen types I, II, and IV (Imtek). Goat antirat Ig conjugated with FITC (BD Pharmingen) and goat antirabbit antibodies conjugated with phycoerythrin (Sigma) were used as the second stage antibodies.

For karyotyping, colchicine solution (40 μg/ml) was added for 1 h to the cells (10 μl per 1 ml suspension). Fixation, preparation of the samples, and G-differential staining were performed by the routine methods [9].

To analyze gene expression, the cells were grown to confluency, the monolayer was scraped, and RNA was isolated as described elsewhere [5]. After producing the poly-T-primer, the first strand of DNA was elongated with reverse transcriptase (M-MLV, Promega). The presence of the product of the examined gene was tested by PCR with specific primers (Table 1).

Capability of the conditioned media of the cell strains to maintain the growth of granulocyte-macrophagal colony-forming cells in semiliquid agar was assessed as described elsewhere [2].

To analyze the potency of the cell strains to maintain hemopoiesis, the bone marrow cells were explanted onto preliminary irradiated cell monolayer (2 millions cells per 25 cm^2 plate). To this end, we used the nonadherent fraction of bone marrow cells obtained after 2-h adhesion.

RESULTS

Adherent cell strains were obtained from SF of LTBMC TNF-/- cells after 75-week culturing. A significant decrease in the number of hemopoietic CD45+ cells was observed in the culture, although the total number of cells in SF increased in some cases (Fig. 1). At late terms of culturing, most SF cells were nonhemopoietic cells, some of which being capable to produce adherent cell strains. The cells were passaged, but as a rule, they endure no more than 40 passages. Each strain was cloned, and more than 100 clones were obtained. The efficiency of cloning depended on the environment: it was 1.6-14.3% in the medium for

TABLE 1. Primer Sequences Used to Detect Gene Products in Probes

Gene	Primer sequence
Integrin β1	5'-ttcccagtgatgtgtgtg-3', 5'-ctagttcgagacagagca-3'
β-Actin	5'-accgtgaaaagatgacccag-3', 5'-cgttgccaatagtgatgacc-3'
SCF	5'-gatgattcagttgttggagc-3', 5'-catatgccgtggtattatgc-3'
c-kit	5'-taccagactgtcaccagtt-3', 5'-acccacatgtaacgtgac-3'
Lif	5'-cttagaagcacagtcagg-3', 5'-gtctcagttaccatcagg-3'
Lif-R	5'-ccaaaggacctcaagactaac-3', 5'-agtaggcacagaaatcagcac-3'
VEGF	5'-gatccttcgaggagcactttg-3', 5'-cgagctctacaggaatccca-3'
FGF1	5'-ccaggcagtgtccatagtta-3', 5'-ggcattagcactctgaac-3'
BMP4	5'-cattgtgcagaccctagtca-3', 5'-ccagctatagggaagcagtt-3'
SDF-1α	5'-ttgtccctgagtcctata-3', 5'-atacaccgtggctgacac-3'
Osteomodulin	5'-gcaagcattctacattccaag-3', 5'-gccactcttgcatctcatag-3'
BMP4	5'-cattgtgcagaccctagtca-3', 5'-ccagctatagggaagcagtt-3'
COMP	5'-cagagtgacagtgatggtga-3', 5'-ctgaagtcggtgagggtgac-3'
Desmin	5'-ggtcgacgtggagcgtgaca-3', 5'-tgtaggactggatctggtgt-3'
PECAM-1	5'-cgagaactttgtgctcatgga-3', 5'-actggctttggagatacggt-3'
β3-tubulin	5'-cattctggtggacttggaac-3', 5'-agctggtggatggacagggt-3'

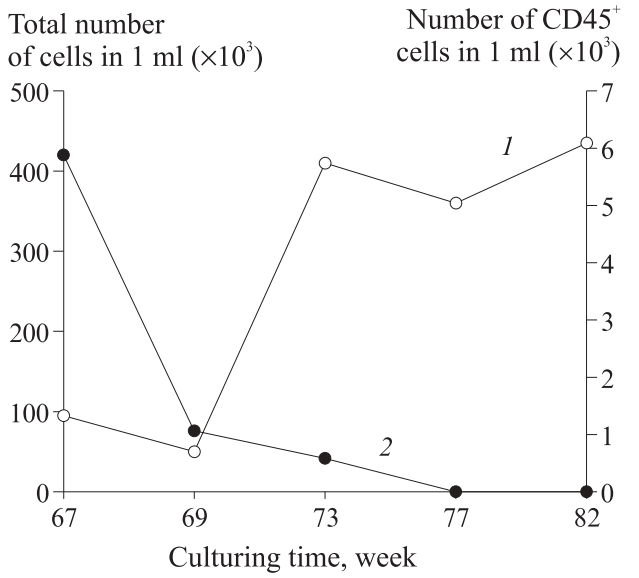

Fig. 1. Timeline of the total number of cells (*1*) and the number of CD45⁺ cells (*2*) in suspension fraction (SF) of long-term bone marrow culture (LTBMC) deficient for TNF during culturing weeks 67-82.

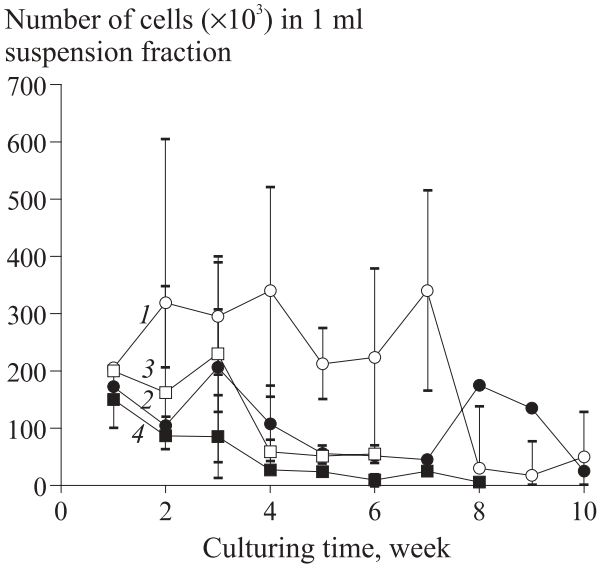

Fig. 2. Maintenance of hemopoiesis in various sublayers of the adherent cells (functional test). *1* – sublayer of cells from SF LTBMC of wild-type mice and of the mice deficient for TNF; *2* – experiment 26, *3* – experiment 36, *4* – experiment 41.

growing LTBMC on irradiated macrophages and 28.7-98% in the dexamethasone medium for growing LTBMC on irradiated cells of the initial strains.

Karyotyping of the resulting cell strains revealed genomic instability (aneuploidy) and chromosomal rearrangements, which differed in the clones and initial cultures. The clones obtained from three independent LTBMC had chromosomal rearrangements, in chromosome IV (in experiments 26, 36, 41: der(4), t(4;7), t(X;4), respectively).

The cells of all strains obtained, most of which were fibroblast-like cells, were well-spread on the substrate, and expressed the markers of various stromal differentiations (Table 2).

The high level of Sca-1 expression, absence of CD45 marker, and the presence of intracellular collagens indicate stromal nature of cells [4]. Intracellular localization of collagen and simultaneous appearance of different types of collagen are also characteristic of stromal cell strains of different origin [4]. At the same time, the cells were not terminally differentiated by any pathway of stromal differentiation, which were corroborated by RNA analysis (Table 3).

Evidently, the cells of the obtained strains were not differentiated: they simultaneously expressed the markers, which were characteristic of cartilaginous, endothelial, or neural cells (experiment 26) or osseous and muscle cells (experiment 30).

TABLE 2. Immunophenotypic Characteristics of Strains Obtained from Various LTBMC TNF-/-*

Characteristic	Experiment 26	Experiment 30	Experiment 36	Experiment 41
Collagen I	+	+	+	+
Collagen II	+	+	+	+
Collagen IV	+	+	+	+
CD45	—	—	—	—
CD44	±	+	—	—
PECAM-1	+	—	—	—
CD105	+	n	±	±
Sca-1	++	++	++	++
Mac-3	—	—	—	—

Note. *The data are averaged across all subclones of the given culture. Here and in Table 3: (++) high level of expression in all subclones; (+) pronounced expression in all subclones; (±) expression was observed not in all clones; (—) expression was not detected, (*n*) expression was not tested.

TABLE 3. Expression of Stromal Differentiation Markers by Cells

Source, marker gene		Sublayer	Experiment			
			26	30	36	41
Bone	BMP 4	+	—	+	—	—
	osteomodulin	+	—	+	+	±
Catrilage						
	COMP	++	+	±	—	+
Endothelium	PECAM-1	++	++	—	++	+
Muscle	desmin	—	—	+	—	±
Nerves	tubulin-β3	±	++	±	++	+

The cells maintained hemopoiesis (Fig. 2), since they produced certain cytokines needed for the growth of hemopoietic cells. This inference is also corroborated by other physiological tests: the conditioned media of the test strains maintained growth of granulocyte-macrophagal colonies in agar (Fig. 3). Analysis of expression of cytokines and growth factors showed that virtually all subclones intensively expressed the stromal growth factor SDF-1 and stem cell growth factor SCF. In addition, they expressed (with various degree of intensity) LIF, VEGF, FGF1, LIF-R, and c-kit.

Thus, the long-term culturing of SF LTBMC TNF-/- cells yields non-differentiated stromal precursor cells, which under certain conditions can produce adherent cell strains. The cells in these strains are also non-differentiated; they express mixed sets of differentiation markers. Such cells are never detected in SF LTBMC WT culture, which attests to the role of TNF in the regulation of maturation of the stromal cells.

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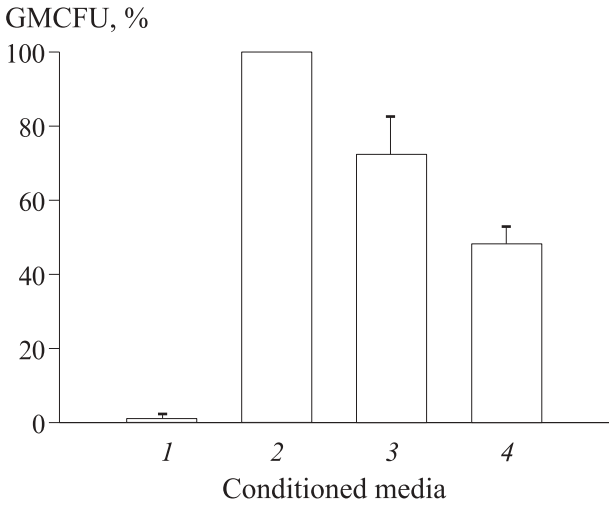

Fig. 3. Incidence of granulocyte-macrophagal colony-forming units (GMCFU) of bone marrow in the presence of various conditioned media. *1* – without the growth factors; *2* – L929+WEHI3B; *3* – experiment 26, *4* – experiment 36.

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