Characteristics of Transplanted Mouse Myeloproliferative Disease Developed after Repeated Injections of Granulocytic Colony-Stimulating Factor

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Transplanted myeloproliferative disease developed in mice against the background of repeated injections of granulocytic CSF was characterized using morphological and molecular biological methods. It was demonstrated that transplanted myeloproliferative disease had a non-viral nature and is probably induced by repeated injections of granulocytic CSF. Tumor cells actively populate the liver of sick animals, which leads to their rapid death. Expression of Myc, Abl, G-CSF, and MPO genes is enhanced, which is typical of myeloid neoplastic transformation.

Key Words: *granulocytic colony-stimulating factor; myeloproliferative disease; tumor transformation; gene expression*

Evaluation of the state of donors receiving repeated injections of granulocytic CSF (G-CSF) for mobilization of hemopoietic stem cells into peripheral blood showed that appreciable, but transient changes in the expression of various genes regulating cell cycle, proliferation, adhesion, and signaling in the cell appear after course injections of this factor [5]. Course administration of G-CSF to healthy donors induced various side effects such as bone pains, headache, and fatigue. Acute respiratory distress was observed in some cases; a case of acute lung damage on day 4 after administration of G-CSF was also reported. Moreover, splenomegaly and (in rare cases) splenic rupture as well as clusters of hemopoietic cells in the liver were observed in healthy donors [14]. A case of severe thrombocytopenia was described [7]. Tetraploid cells of the myeloid lineage were found in the peripheral blood of some donors after G-CSF courses [2]. A case of acute myeloleukemia (M1 subtype) was diagnosed in a healthy donor 14 months after mobilization of hemopoietic stem cells with G-CSF [9]. Thus, the study of delayed consequences of G-CSF administration is required for the evaluation of the safety of its use.

The mechanisms of tumor development and the efficiency of antitumor therapy are often studied on immunodeficient and transgenic animals. However, immunodeficient animals are not always adequate models of the pathogenesis of the studied diseases.

Delayed consequences of administration of G-CSF to healthy mice were studied. Twenty-two of 40 experimental animals died within 20 months, 8 mice developed hematological and tumor diseases. One mouse developed a myeloproliferative disease. Bone marrow cells of this mouse induced tumors in syngeneic recipients.

Here we studied morphological and biomolecular characteristics of transplanted myeloproliferative disease developed in a mouse after repeated courses of G-CSF in non-mobilizing doses.

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MATERIALS AND METHODS

G-CSF (Neupogen 48 Mio U; F. Hoffmann-La Roche Ltd.) was dissolved in physiological saline with 0.1% BSA (Sigma) and injected subcutaneously to $(C57Bl/6\times CBA)F_1$ female mice weighing 23-25 g in a dose of 25 μg/kg (in 0.2 ml). Control animals received physiological saline with 0.1% BSA. The preparations were administered once a day for 4 days every month.

Peripheral blood smears were fixed with methanol for 7 min and stained after Romanowsky. Bone marrow imprints were processed similarly. Fragments of the liver tissue were fixed in Bouin fluid and embedded in paraffin. The sections were stained with hematoxylin and eosin. The preparations of the liver for electron microscopy were fixed in 2.5% glutaraldehyde (Sigma) and embedded in epon. Nonserial microsections were analyzed under an electron microscope.

RNA from bone marrow cells was isolated routinely using guanidine isothiocyanate. The first cDNA strand was synthesized by reverse transcription using 2 μg total RNA (per reaction), poly-T primers, and revertase (Promega).

Some studied genes were found in the library enriched with sequences differentially expressed in tumor cells and obtained by the method of subtractive hybridization. Other genes were chosen on the basis of their functions in normal and tumor cells.

The presence of cDNA of the required gene was detected by PCR with specific primers (Table 1) at working concentration of Mg^{2+} within 1-4 mM (depending on the analyzed gene), 0.25 mM deoxynucleoside triphosphates, and 0.5 pmol/μl corresponding primers. Denaturation was carried out at 94°C for 30 sec, hybridization of the matrix with primers for 30 sec, elongation at 72°C for 60 sec, preliminary denaturation at 94°C for 5 min, and after the end of PCR elongation at 72°C for 5 min was performed.

The number of PCR cycles for each gene was chosen in such a way that band intensity for the PCR product in the agarose gel linearly depended on the number of cycles. The intensity of bands was evaluated semiquantitatively using GelPro 4.0 software.

For comparison of gene expression in bone marrow cells from sick and healthy mice (RNA samples from 5 mice in each group were pooled) using GelPro 4.0 software, we calculated the ratio of band brightness of sick/healthy animals (coefficient k_x). For evaluation of the data scatter obtained in independent PCR repetitions, k_x in 5 and more PCR was determined and the mean and standard error of the mean were calculated.

For normalization of cDNA content in initial samples we used housekeeping genes encoding HPRT1 (hypoxanthine phosphoribosyl transferase I), RPL13A (ribosomal L13A protein), and UBC (ubiquitin C). For each of these genes, a PCR series with the corresponding primers was carried out; in this reaction, cDNA obtained after reverse transcription was used from sick and healthy mice. Standardization factors for each gene were calculated:

$$K_{RPL13a}=\frac{b}{a},\ K_{HPRT}=\frac{d}{c},\ K_{UBC}=\frac{g}{f},$$

where b, d, g are fluorescence intensity of the bands corresponding to amplification products for the studied cDNA from cells of sick mice, while a, c, f are the corresponding parameters for healthy mice. Then, the mean standardization coefficient by 3 housekeeping genes were calculated:

$$NF=\frac{K_{RPL13a}+K_{HPRT}+K_{UBC}}{3}.$$

For semiquantitative evaluation of the relative expression, the coefficients k_x were divided by standardization factors:

$$\overline{k_{xn}}=\frac{k_x}{NF},$$

where $\overline{k_{xn}}$ is a standardized relative expression of genes.

RESULTS

During 20-month observation, 22 of 40 mice died (18 during treatment courses and 4 after their completion), 10 animals were sacrificed because of suspected hematological or other diseases. In one mouse, a myeloproliferative disease with elements of histiocytic sarcoma was detected (according to classification [6]). The number of leukocytes in the peripheral blood of this mouse sharply increased after 4 courses of G-CSF. The percent of granulocytes in the peripheral blood was 72.5% (*vs.* 36.1± 2.7% in control animals). The liver was sharply enlarged (>4 g *vs.* 1.4±0.3 g in controls). Hemorrhages and albescent plaques were seen on the surface of the liver, separate clusters of hepatocytes with highly eosinophilic cytoplasm (apparently ne-

TABLE 1. Primers Used in the Study

Primers	Sequences	Melting temperature, °C	Number of cycles
Actb, sense	CCAAGGCCAACCGCGAGAAGATGAC	60	19-21
Actb, antisense	AGGGTACATGGTGGTGCCGCCAGAC		19-21
mHPRT1 s	GACCTCTCGAAGTGTTGGATACAG	65	24-28
mHPRT1 a	GAAATCGAGAGCTTCAGACTCGTC		24-28
mRPL13a s	CTCCGGAAGCGGATGAATACCAAC	65	19-24
mRPL13a a	TTGGTCTTGAGGACCTCTGTGAAC		19-24
mUBC s	CGAGCCCAGTGACACCATTGAG	65	24-28
mUBC a	CTGTCTTCCTGTTTGACCTTCTTG		24-28
mcIAP2 s	CAAGGGCCATCACTGAACAG	57	27-30
mcIAP2 a	TCCAGGCTGGCCTCAAGTTC		27-30
mBcl2 s	GTCGCTACCGTCGTGACTTC		33-35
mBcl2 a1	TCAACCAGACATGCACCTAC	55	33-35
mBcl2 a2	AGCCAGGAGAAATCAAACAG	53	33-35
Atp5b s	GAGCACGGTCAGAACTATT	56	22-23
Atp5b a	CTGGGTAAAGCGGAAGAT	54	22-23
Huwe1 s	TCACCTACACCATCAATCCA	58	25-27
Huwe1 a	CAATGTCGATGGTAGGCAG	58	25-27
IL1-r2 s	TGTCAGTTTCGTGGCAGAGA	60	27-28
IL1-r2 a	CCTAGTGATGTTGTATTCCTGG	60	27-28
CathepsinK s	TTGTGGACTGTGTGACTGAG	60	28-29
CathepsinK a	CAGGCGTTGTTCTTATTCC	60	28-29
mLy6e s	TCTGCCACTTCCAACATGAGAGTC	65	19-25
mLy6e a	GTAGCCAGTACCACTTCAACAGAG	65	19-25
mMpo s	CCAATGACCCTCGAATCAAG	51	20-24
mMpo a	GTCGTTGTAGGATCGGTACTG	55	20-24
mSH3BGRL3 s	GTCTACAGCACGTCGGTCAC	55	25-27
mSH3BGRL3 a	AAGCAACAGAAATGCTCATC	47	25-27
mCD45 s	ATGACTCATGTGCTCCAGC	49	25-29
mCD45 a	AGGTTTAGATACAGGCTCAG	49	25-29
N-Cadherin s	CAAGAGCTTGTCAGAATCAGG	53	32-36
N-Cadherin a	GATCCTGTACCGCAGCATTC	53	32-36
p53 s	CTGCATCCCGTCCCCATCAC	57	25-30
p53 a	GGGGTAGGGTGAGATTTCATTG	57	25-30
Abl s	GGCACTCTATGATTTTGTGG	58	25-29
Abl a	TGGGTAGTGGAGTGTGGTGA	62	25-29
Myc s	TTCTCAGCCGCTGCCAAGCTGGTC	65	23-27
Myc a	GGTTTGCTGTGGCCTCGGGATGGA	65	23-27
Junb s	CCCGTCTACACCAACCTCAGCAGT	50	20-23
Junb a	GGGGGCCATGTAAACCT	50	20-23
Csf3 s	CAAGTGAGGAAGATCCAGGC	58	34-38
Csf3 a	CGGAAGTGGAGAGAATGATC	56	34-38
Csf3r s	CTCAAACCTATCCTGCCTCATG	57	22-25
Csf3r a	TCCAGGCAGAGATGAGCGAATG	57	22-25
Granulin-s	CTGCAATGTGAAGGCGAGG	60	23-25
Granulin-a	ACAGTGACGTCCATCTCTAC	60	23-25
Csf1r-s	TCGAAACGTGCTGTTGACC	58	26-27
Csf1r-a	AGGACGAGTCAGCTTGGAGA	60	26-27
Fibronectin-s	CACAGAGATACAATCAGAGA	56	24-26
Fibronectin-a	AAGGTCTTGGGTACTAGCAT	58	24-26
3110001A13-s	GAG CCA GAC TAC ATA GAA GA	58	24-26
3110001A13-a	ACA ATG GTA AAT CAA ACG CA	54	24-26
PU.1 s	GGATGACTTGGTTACTTACGAT	62	21-24
PU.1 a	ATGGGAGTATCGAGGACGTG	62	21-24

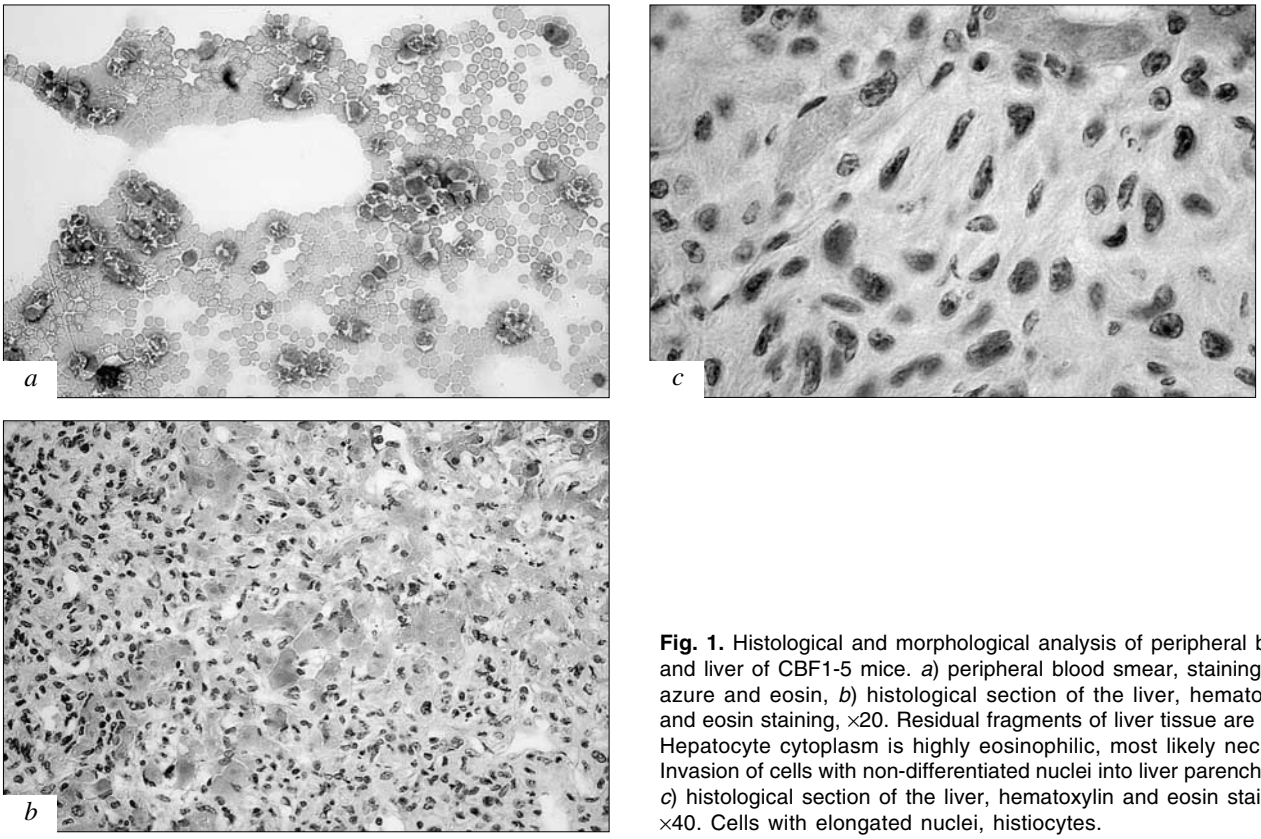

Fig. 1. Histological and morphological analysis of peripheral blood and liver of CBF1-5 mice. *a*) peripheral blood smear, staining with azure and eosin, *b*) histological section of the liver, hematoxylin and eosin staining, ×20. Residual fragments of liver tissue are seen Hepatocyte cytoplasm is highly eosinophilic, most likely necrotic. Invasion of cells with non-differentiated nuclei into liver parenchyma. *c*) histological section of the liver, hematoxylin and eosin staining, ×40. Cells with elongated nuclei, histiocytes.

crotic) were seen, tissue structure in these clusters was not preserved. The major part of the organ was presented by cells with undifferentiated nuclei; granulocytes and cells with elongated nuclei resembling histiocytes were also seen (Fig. 1). Bone marrow imprints showed cells typical of this disease (cells with ring-shaped nucleus with fine uncondensed chromatin and basophilic cytoplasm). The difference in the percentage of these cells on bone marrow imprints from healthy and sick animals was 8.63%. The percent of stab and segmented granulocytes was 23.2% (*vs.* 25.8% in the con-

TABLE 2. Studied genes

Symbol	Name	Presumable function
N-cadherin	N-cadherin	Cell adhesion to extracellular matrix, cell-cell contacts
p53	Oncogen p53	Tumor suppressor, transcription factor Induces apoptosis upon damage to DNA, stops cell cycle in G1 and G2 phases
Myc	Protooncogene c-Myc	Regulation of cell apoptosis, division, growth, and differentiation [11]
JunB	Oncogene JunB	Tumor suppressor [10]
Abl	Oncogene 1 of Abelson murine myeloproliferative disease	Protein phosphorylation
G-CSF (Csf3)	Colony-stimulating factor 3 (granulocytic)	Proliferation of granulocyte precursors [13]
G-CSFr (Csf3r)	Csf3 receptor	Transmission of G-CSF signal into the nucleus
CD45	Common leukocyte antigen	Marker of hemopoietic cells (expressed on all hemopoietic cells except mature erythrocytes)
Bcl2	Factor of B-cell lymphoma 2	Inhibition of apoptosis by preventing the release of cytochrome C from mitochondria into cytoplasm [3]
cIAP2	Factor 3 containing baculoviral IAP repeat	Inhibition of apoptosis via inactivation of caspases [4]

trol), *i.e.* the percent of mature granulocytes in the bone marrow did not change (in contrast to peripheral blood).

The developed myeloproliferative disease was transplantable. Syngeneic recipients receiving intravenous injection of 10^6 bone marrow or liver cells from the sick mice developed similar symptoms. A total of 9 retransplantations of bone marrow cells were performed. The properties of the tumor cells gradually changed, which manifested in shortening of the disease development from 28-32 to 16-18 days after transplantation.

In order to rule out viral nature of the observed myeloproliferative disease, we performed a series of experiments. Bone marrow and liver cells were homogenized in phosphate buffered saline (cell membranes were destroyed). The homogenate was filtered through Nylon filters (0.45 μ pore diameter) permeable for the majority of virus particles, and 0.5 ml filtrate was injected intravenously to intact syngeneic mice irradiated in a dose of 6 Gy. None recipients of the filtrate developed the disease. Electron microscopy revealed no viral particles on ultrathin sections of the damaged liver and bone marrow. Bone marrow and liver cells (10^6) from sick (CBA×C57Bl/6) CBF_1 mice were transplanted to parental CBA and C57Bl/6 animals. The transplanted cells were rejected, none recipients developed the

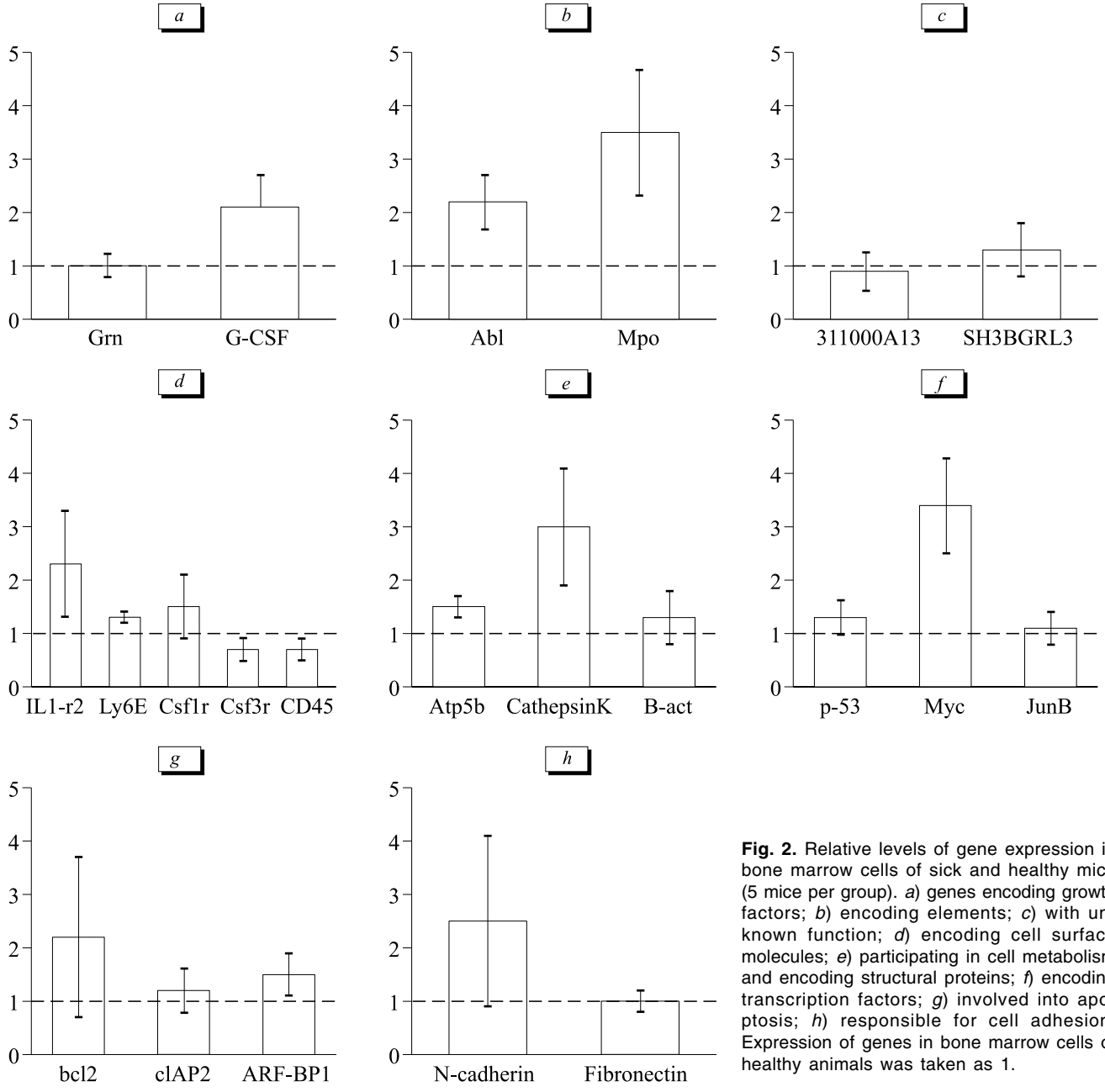

Fig. 2. Relative levels of gene expression in bone marrow cells of sick and healthy mice (5 mice per group). *a*) genes encoding growth factors; *b*) encoding elements; *c*) with unknown function; *d*) encoding cell surface molecules; *e*) participating in cell metabolism and encoding structural proteins; *f*) encoding transcription factors; *g*) involved into apoptosis; *h*) responsible for cell adhesion. Expression of genes in bone marrow cells of healthy animals was taken as 1.

disease. Thus, using three independent approaches we proved that the disease has a nonviral nature and is transmitted by bone marrow and liver cells.

For evaluation of the cause and mechanism of this disease, bone marrow cells inducing the myeloproliferative disease were analyzed at the molecular level. To this end, expression of some genes presumably involved in the development of this disease was compared using semiquantitative methods.

On the basis of the results of subtractive hybridization, the following genes were chosen for the analysis: ATP-synthase β-subunit (Atp5b), type II receptor for IL-1 (IL1r2), catepsin K (CtsK), Mcl-1 ubiquitin ligase (ARF-BP1), myeloperoxidase (Mpo), SH3BGRL3 gene, Ly6E gene, granulin, fibronectin 1, receptor for M-CSF (Csf1r), and a gene with unknown function 311000A13. Additionally, genes presumably involved into the development of myeloproliferative disease were chosen for PCR analysis (Table 2).

Analysis revealed a 3.5-fold increase in the expression of Myc transcription factor (Fig. 2). Myc is an oncogene and activated expression or increased stability of its product (*e.g.* in Burkitt lymphoma) leads to enhanced cell proliferation, because Myc stimulates cell entry into S phase of the cell cycle. Myc activation induces the release of transcription factors E2F from the complex with tumor suppressor Rb and synthesis of proteins involved into DNA replication. Enhanced expression or amplification of Myc gene is often associated with the development of myeloproliferative diseases [8]. Considerable increase in Myc expression attests to its involvement into the development of the studied pathology.

Expression of Abl and Mpo genes was also increased in the bone marrow of sick mice. Abl gene encoding non-receptor tyrosine kinase is involved into the development of chronic myeloleukemia (CML). In CML, kinase activity of Abl in the hybrid protein Bcr-Abl increases several fold [12]. In this case, no chromosome translocation (9;22) was noted, but the expression of Abl increased 2-fold. Expression of Mpo in the bone marrow increased 3.5-fold. On the one hand, increased expression of Mpo is observed in inflammatory focuses and leads to potentiation of antibacterial activity of granulocytes via induction of oxidative stress. On the other hand, increased level of Mpo in the bone marrow of sick animals probably reflects enlarged pool of granulocyte precursors, which can be a result of G-CSF treatment.

Expression of the gene encoding G-CSF also was 2-fold increased in the bone marrow of sick mice, which indirectly confirmed the involvement of G-CSF into pathogenesis of the tumor.

In bone marrow cells of sick animals, the expression of CtsK was 3-fold increased, which can attest to active protein metabolism in tumor cells. Moreover, increased level of CtsK and other cysteine proteases is often associated with increased capacity of tumor cells to dissociation from the microenvironment and invasion.

Of genes encoding surface molecules, only expression of type II receptor of IL-1 was 2.3-fold increased. This receptor competes with type I receptor for binding with IL-1, but does not transmit the signal into the cell. Thus, type II receptor of IL-1 blocks the signal of IL-1, thus suppressing the development of specific immune response, which is also typical of tumor cells.

Possible changes in the expression of various genes were detected, but it remains unclear, whether they induce the described disease or are its consequences. This question requires further studies.

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