Establishment of a FDC-P1 Murine Cell Line with Human KIT N822K Gene Overexpression

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Received July 26, 2019; in final form, December 09, 2019
DOI: 10.32607/actanaturae.10938

ABSTRACT The mechanism of resistance of leukemia cells to chemotherapeutic drugs remains poorly understood. New model systems for studying the processes of malignant transformation of hematopoietic cells are needed. Based on cytokine-dependent murine acute myeloid leukemia (AML) FDC-P1 cells, we generated a new cell line with ectopic expression of the KIT gene encoding mutant human receptor tyrosine kinase (N822K). We investigated the role played by overexression of the mutant KIT in the survival of leukemia cells and their sensitivity to therapeutic drugs. We also generated a co-culture system consisting of FDC-P1 murine leukemia cells and a HS-5 human stromal cell line. Our data can be used for a further comprehensive analysis of the role of KIT N822K mutation in the cellular response to anti-leukemic drugs, growth factors, and cytokines. These data are of interest in the development of new effective therapeutic approaches to the treatment of acute leukemia.

KEYWORDS receptor tyrosine kinase KIT, FDC-P1, acute myeloid leukemia (AML), KIT N822K, stromal cells.

ABBREVIATIONS SCF – stem cell factor; AML – acute myeloid leukemia; FAB M2 – acute myeloblastic leukemia with maturation (AML subtype according to the French–American–British classification); CD34 – cluster of differentiation 34; HPI – Heinrich Pette Institute, Leibniz Institute for Experimental Virology; GFP – green fluorescent protein.

INTRODUCTION Expression of the KIT gene encoding receptor tyrosine kinase is often observed in various malignant diseases: leukemia, neuroblastoma, gastrointestinal stromal tumors (GISTs), mast cell tumors, and melanoma [1–3]. In normal hematopoiesis, KIT is expressed in a subpopulation of immature hematopoietic progenitor cells positive for CD34 (a marker of stem cells and early progenitor cells) [4].

Mutations in the KIT kinase gene are found in cells from patients with acute myeloid leukemia (AML) and mastocytosis; most frequently those are D816V and N822K mutations localized in the tyrosine kinase domain and leading to constitutive activation of tyrosine kinase. The activating D816V and D814V mutations significantly reduce cell sensitivity to the KIT inhibitor imatinib (Gleevec; Novartis Pharma AG, Basel, Switzerland) both in vitro and in vivo [5, 6]. Mutations in the KIT kinase domain are associated with an increased relapse rate of FAB M2 AML after chemotherapy [7]. Imatinib is a highly selective inhibitor of the Abl, BCR-ABL, PDGFRα/β, and KIT kinases; it is used in BCR-ABL-positive leukemia and GISTs carrying mutations in KIT and PDGFR. Imatinib is used in combination with other anti-leukemic drugs in AML. Imatinib was shown to have a strong effect on AML cells in high-risk populations. However, high-dose imatinib monotherapy often leads to severe cytopenia, and, therefore, is not recommended [8].

A new, continuous cell line overexpressing mutant human kinase KIT (N822K) has been generated using FDC-P1 cytokine-dependent murine acute myeloid leukemia cells with a low expression level of the wild-type KIT gene. We investigated the role of the mutation, which is located in the tyrosine kinase domain, in maintaining the malignant status of leukemic cells: their survival, sensitivity to drugs, and growth rates when in contact with stromal cells.

EXPERIMENTAL Cell cultures Continuous FDC-P1 cells were cultured in IMDM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and a 7% WEHI-3B conditioned medium containing mouse interleukin-3 (IL-3) at 37°C and 5% CO₂. HS-5 human stromal cells...
were cultured in a RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 2 mM L-glutamine. All reagents were purchased from Gibco, Thermo Fisher Scientific (USA). Cell lines were obtained from the Heinrich Pette Institute, Leibniz Institute for Experimental Virology (HPI, Hamburg, Germany), and tested for the absence of mycoplasma contamination.

**Immunocytochemical analysis**

For qualitative analysis, FDC-P1 cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 15 min then washed with PBS and treated with PBS containing 0.2% Triton X-100 and human KIT-specific antibodies conjugated to PerCP/Cy5.5 (PerCP/Cy5.5, Abcam, ab157320, 1 : 50). After washing with PBS, the cells were embedded in a SlowFade Gold mounting medium (Invitrogen, USA, s36936) containing 1 μg/ml DAPI (Sigma-Aldrich, USA); the slides were sealed with nail polish. The samples were analyzed using a Leica TCS SP5 confocal microscope (Leica, Germany) and an HCX PLAPO CS lens. For quantitative assessment of the staining intensity, the FDC-P1 cells were treated with human KIT-specific antibodies conjugated to PerCP/Cy5.5 (PerCP/Cy5.5, Abcam, ab157320, 1 : 50) and analyzed on an LSRFortessa flow cytometer (BD Biosciences). Data analysis was performed using the FlowJo software.

**Quantitative real-time PCR and primer design**

RNA was isolated using the Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. RNA concentration and purity were determined on a spectrophotometer (NanoDrop). Complementary DNA was synthesized using a reverse transcription kit (Thermo Fisher Scientific, USA) (random primers). Real-time PCR was performed using Maxima SYBR Green Supermix (Thermo Fisher Scientific) on a CFX96 Real-Time System (Bio-Rad, USA). Expression of the target genes was normalized to that of β-actin in each sample. The Ct and relative expression level were calculated using the Bio-Rad CFX manager 3.1 software. The Ct and relative expression level were calculated using the Bio-Rad CFX manager 3.1 software. The Ct and relative expression level were calculated using the Bio-Rad CFX manager 3.1 software.

**RESULTS AND DISCUSSION**

Generation of continuous murine leukemia cells overexpressing a mutant human KIT N822K gene

In order to analyze the oncogenic potential of KIT receptor tyrosine kinase with a N822K mutation in the kinase domain (and its ability to influence the proliferation of leukemia cells of myeloid origin in particular), the FDC-P1 N822K cell line was obtained. For this purpose, IL-3-dependent mouse FDC-P1 cells were transduced with a retroviral vector expressing human KIT receptor tyrosine kinase with the N822K mutation. The vector also contained the GFP reporter gene (Fig. 1a). The vector was kindly provided by Mrs. Carol Stocking (HPI, Hamburg, Germany). The control cell line was transduced with the original retroviral vector lacking KIT N822K.

The population of cells with the highest fluorescence intensity in the FITC channel, which corresponds to the high GFP expression level, was isolated by cell sorting (S3e Cell Sorter, Bio-Rad) (Fig. 1b). The expression level of KIT was determined by real-time PCR in the selected cells (Fig. 1d). FDC-P1 N822K cells express the KIT protein on their surface (Fig. 1c). The presence of the human KIT protein in FDC-P1 KIT N822K cells was confirmed by confocal microscopy, while no human KIT expression was detected in the FDC-P1 cells transduced with the control vector: neither at the mRNA nor the protein level (Fig. 1e).

KIT N822K mutation results in IL-3-independent growth of FDC-P1 cells

The control cells and FDC-P1 N822K cells were seeded at the same density. Cell counting was performed for 6 days. Introduction of the mutant KIT N822K did not affect the growth rate of FDC-P1 cells in the presence of IL-3 (Fig. 2a).

Overexpression of the mutant KIT in FDC-P1 cells leads to their factor-independent growth (Fig. 2a). The growth rate of FDC-P1 control cells is significantly lower in a medium with a reduced content of a IL-3-conditioned medium (0.25%).

We observed no significant changes in the cell growth rate after treatment with the KIT–SCF ligand (Abcam) (Fig. 2b). The control and FDC-P1 N822K cells were treated with the antitumor drugs imatinib (5 and...
10 μM) and cytarabine (75 and 100 nM). The amount of cells was counted on day 3 after addition of the drugs. The imatinib concentration inhibiting the growth of the FDC-P1 control cells by 50% (IC50) was 10 μM. FDC-P1 cells overexpressing KIT N822K turned out to be more sensitive to this drug concentration (Fig. 2c). Cell sensitivity to cytarabine remained the same as that in the control cells (Fig. 2d).

Our data are in line with the finding that mutation in the KIT tyrosine kinase domain, in particular D816V, enhances cell sensitivity to imatinib [9].

**Generation of a co-culture of leukemic and stromal cells**

The factors that facilitate the production of stromal cells are involved in the stimulation of hematopoietic cell proliferation, the regulation of the cell cycle, and apoptosis. Meanwhile, the processes occurring when stromal cells come into contact with leukemic cells remain poorly understood.

Continuous HS-5 human stromal cells were seeded at 5,000 cells per well. On the next day, the culture medium of HS-5 cells was changed to IMDM containing...
FDC-P1 cells (500 cells per well). The number of cells in the suspension fraction was counted 3 and 5 days after seeding. Direct interaction between leukemic and stromal cells leads to a reduction in the growth rate of the control FDC-P1 cells (Fig. 2a,b) but not the FDC-P1 cells overexpressing KIT N822K.

Cytokines and growth factors produced by stromal cells (including HS-5 cells) can modulate KIT expression in co-cultured leukemic cells [10]. Apparently, the growth rate of FDC-P1 cells with ectopic expression of KIT N822K does not change upon overexpression of the kinase. Moreover, the growth rate can vary due to...
differences in the adhesion of control FDC-P1 cells and FDC-P1 N822K cells.

CONCLUSION
The IL3-dependent murine cell line FDC-P1 is widely used to study the oncogenic effect of kinases, transcription factors, as well as the effectiveness of anti-leukemic drugs [11, 12]. We have obtained and characterized the FDC-P1 cell line overexpressing the mutant human *KIT* N822K gene. It has been shown that N822K mutation in *KIT* increases the sensitivity of FDC-P1 cells to imatinib. The D419A mutation in the extracellular domain of the KIT receptor also increases cell sensitivity to imatinib [9]. It was shown that the growth rate of control cells that come into contact with the stroma decreases, which is not typical of FDC-P1 cells expressing the mutant *KIT* N822K gene. Closer attention should be paid to the study of the mechanisms of interaction between leukemic and stromal cells in order to establish any possible contribution of stromal cells to the response of leukemic cells to chemotherapeutic agents. Our model can be used to test various anti-leukemic drugs, including co-cultivation of leukemic and stromal cells.

*This study was supported by the Russian Foundation for Basic Research: the experiments involving obtaining genetic constructs were performed under project No. 18-29-09151; cell cultures work was carried out under project No 17-04-01555.*

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