

INTRODUCTION

Strict regulation of proliferation, differentiation and apoptosis of bone marrow progenitor cells is indispensable for the homeostasis of the hematopoietic system. Leukemia originates from a single pathologic stem cell that continuously proliferates without responding to signals for differentiation or apoptosis. *In vitro* differentiation of leukemic cell lines has been in the focus of intensive research for several decades. Today, differentiation dependent apoptosis of malignant cells is already a widespread clinical therapeutic target. Understanding the differentiation of leukemic cell models at the molecular level is fundamental for the development of novel efficient therapies.

In chronic myeloid leukemia (CML) the Philadelphia chromosome, developed in a primitive hematopoietic stem cell the pathological Bcr-abl fusion protein is present in numerous cell types of the bone marrow well before the blastic transformation. Myelodysplasia (MDS) is also characterized by clonal instability, reduction of differentiation potential and leads to acute myeloid leukemia (AML). The regulation of intracellular events leading to blastic transformation is hitherto largely unknown.

The TF-1 cells, investigated in the thesis are human, GM-CSF or IL-3 cytokine dependent cells, established from a patient with an MDS/AML transformation. Due to chromosomal translocation TF-1 cells have a mutant erythropoietin (Epo) receptor lacking C-terminal 96 amino acids. This intracellular part plays an important role in the signal transduction of the full length Epo receptor that governs the proliferation, survival and differentiation of a distinct set of erythroid progenitor cells. The signaling pathways directing the distinct physiological responses are still controversial. The TF-1 cell line is thus a unique human cell model that enables the investigation of GM-CSF receptor mediated signals leading to survival and cell growth and Epo induced erythroid differentiation as completely separate cellular events.

The CML derived human leukemia cell line *bcr-abl*⁺ K562 is an excellent model to study *in vitro* the role of the Bcr-Abl fusion tyrosine kinase activated pathways in influencing erythroid differentiation.

OBJECTIVES

The aim of the studies presented in the thesis was to elucidate the activity of signal transduction pathways already known to be involved in cell survival and proliferation. The role of such pathways in erythroid differentiation in the leukemic model cell lines was not clear.

I have used myelodysplasia (TF-1) and chronic myeloid leukemia (K562) derived human myeloid leukemia cell lines to investigate erythroid differentiation induced by distinct approaches. Furthermore, I have explored the correlation of activity alterations in cytokine stimulation induced signaling pathways linked to cell proliferation and development of myelodysplasia; as well as the correlation among the activity of the analyzed pathways and the transformation of MDS into acute leukemia.

Major lines of the studies presented in this thesis were designed to:

- distinguish the signal transduction pathways involved in differentiation from those involved in cell growth and survival of TF-1 cells.
- investigate the activity alterations of cytokine receptor mediated pathways in myelodysplasia with an emphasis on Epo-dependent signaling events and to assess cytokin dependent signaling mechanisms following blastic transformation of myelodysplasia into acute myeloid leukemia.
- investigate the role of activity changes in the survival and proliferation pathways mediated by the Bcr-Abl fusion protein during the erythroid differentiation of K562 cells to ascertain the relationship between apoptosis and differentiation.

METHODS

Cell lines: K562 cells were grown in nucleoside free RPMI 1640 medium, complemented with 10% (v/v) FCS, 2mM glutamine, 50 U/ml penicillin and 50 μ g/ml streptomycin. Cell cultures were maintained at 37°C, 5% CO₂ and 100% humidity. TF-1 cells were cultured under similar conditions in the presence of 2.5 ng/ml hrGM-CSF.

Analyses of clinical samples: Patients were diagnosed by the collaborating physicians. All cases were classified according to the FAB (French American British) criteria. With the approval of the Ethical Committee 3 ml bone marrow was obtained for the experiments. Mononuclear fractions were separated by the use of Ficoll-Hypaque solution.

Cell number and cell viability was determined by trypan blue exclusion assay and cell counting. In certain cases the presence of cell death was confirmed by propidium iodide incorporation using flow cytometry.

Investigation of erythroid differentiation: the benzidine staining technique was used to determine the haemoglobin content of the cells and Light Cycler system (Roche) was used for the determination of the β -globin gene group changes at the mRNA level.

Upon the investigation of apoptotic processes caspase-3 activity was determined by a fluorimetric method. The oligonucleosomic DNA degradation (DNA ladder) was detected on agarose gels. The proteolysis of PARP (Poly-ADP-Ribose Polymerase) and the changes of anti-apoptotic proteins (Bcl-2 and Bcl-xL) were followed by Western-blot analyses.

Detection of protein activation was achieved by Western Blot analyses by means of phospho-specific antibodies recognizing P-ERK1/2, P-Elk-1, P-STAT-5 and a phospho-tyrosine specific antibody (4G10) was used to detect Bcr-Abl activation. PVDF membranes were also developed by an antibody that recognized all forms of the respective protein under investigation. The data obtained by semi-quantitative optical density analyzes were normalized to the above mentioned immunostaining. The DNA-binding activities of the AP-1, Egr-1, c-Myb, Stat-5 and NF- κ B transcription factors were examined by EMSA (Electrophoretic Mobility Shift Assay).

Development of the retroviral short hairpin RNA (shRNA) construct silencing the Bcr-Abl fusion protein and the nonsense (not coding) control

The retroviral constructs involved EGFP (enhanced green fluorescent protein) as a reporter gene and puromycin resistance gene as selection marker. The DNA template needed for shRNA production was operated by the H1 promoter. The double DNA strands were sequenced and amplified by means of a double packaging cell system to obtain viruses competent to infect K562 cells. From the third day posttransduction K562 cells were selected with puromycin. The presence of EGFP⁺ cells was detected by flow cytometry and viability of cells was determined by propidium-iodide incorporation. The relative level and activity of the Bcr-Abl protein and ERK1/2 kinases were determined by Western blot till the 10th day following transduction.

RESULTS (THESIS) AND DISCUSSION

In my thesis, I have presented my work on hematopoietic differentiation and related signal transduction mechanisms using model cell lines suitable to analyze the different segments of the underlying mechanisms. I have made the following observations:

- In a human erythroleukemia cell line (TF-1) Epo is unable to induce differentiation the presence of the growth factor, GM-CSF, i.e. the synthesis of hemoglobin is impeded whereas differentiation can be carried out with the inhibition of ERK1/2 kinases.
- In an experimental condition suitable for differentiation Epo can not protect the cells from GM-CSF deprivation induced apoptosis.
- GM-CSF activates the Raf/MEK/ERK/Elk-1 MAP kinase pathway, the transcription of the immediate early response genes *c-Fos* and *Egr-1*, as well as the DNA-binding potential of Egr-1, AP-1, NF- κ B, c-Myb and STAT-5 transcription factors during proliferation. In contrast, Epo does not activate the signaling pathways leading to cell proliferation and survival; moreover GM-CSF inhibits the Epo induced differentiation through the activation of the above mentioned pathways.
- The elevation of intracellular calcium concentration in TF-1 cells inhibits Epo induced cell differentiation through the activation of the ERK1/2 MAP kinases.
- The increase of the intracellular calcium concentration induces hormone independent cell survival or proliferation in TF-1 cells.
- The calcium-induced growth factor independent cell survival and proliferation depend exclusively on the activity of the ERK1/2 kinases.

As a summary, the activity of the signaling pathways essential for proliferation and survival is not only dispensable for Epo induced erythroid differentiation, but they even inhibit it in TF-1 cells. The functional mechanism of the Epo receptor in TF-1 cells is identical to the short Epo receptor variant typical of erythroid progenitors. The latter one does not mediate survival and proliferation but exclusively assists differentiation. ERK1/2 mitogen activated kinases are the major preventive factors

among survival-related and mitogen pathways that inhibit erythroid differentiation in TF-1 cells.

- Investigating clinical samples I have found that Epo does not activate the STAT-5 and ERK1/2 pathways in the refractory anemic group of myelodysplastic syndrome (MDS); this may explain the Epo resistant anemic phenotype of the patients with MDS.
- Following development of acute leukemia from MDS in proliferating leukemia cells STAT-5 activation together with c-Fos and Egr-1 expression increases and becomes independent from cytokine regulation. This may contribute to the unregulated cell growth typical of leukemia and may partake in the impairment of certain differentiation processes.
- In case of myelodysplasia STAT-5 activation does not pursue erythropoietin stimulation in each sample; whereas all samples display cytokine independent STAT-5 DNA binding following leukemic transformation.

The above observations may assist the development of novel diagnostic assay systems appropriate to distinguish MDS from the early period of AML.

- By studying K562, a Philadelphia chromosome positive myeloid leukemia cell line I have found that inhibition of ERK1/2 activity leads to increased hemoglobin synthesis without inducing cell cycle arrest or apoptosis.
- The stable retroviral production of shRNA targeting the Bcr-Abl fusion protein does not trigger the cell death of K562 cells even at above 90% transduction efficiency, yet it is enough to induce transient reduction of Bcr-Abl levels and differentiation.

Accordingly, the reduction of steady-state ERK1/2 activity resulted in increased hemoglobin synthesis of K562 cells independent from the experimental approach. Erythroid differentiation as marked by enhanced globin expression and apoptotic processes are completely independent processes in K562 cells.

To sum up the results, ERK1/2 kinases might be universal inhibitors of the erythroid differentiation process at a very early phase of the erythroid differentiation cascade. Down-regulation of steady-state ERK1/2 activity may be a common mechanism for erythroid differentiation accompanied by the appearance of hemoglobin. Thus, the ERK1/2 MAPK pathway may control such a gene-regulator system that negatively influences the differentiation towards the erythroid lineage in hematopoietic cells.

**THE REGULATORY ROLE OF MITOGEN SIGNAL
TRANSDUCTION PATHWAYS DURING ERYTHROID
DIFFERENTIATION**

Ph. D. thesis

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