Transforming Activity of Flt3 in 32D Cells


Introduction

The type III receptor tyrosine kinase Flt3 (Matthews et al., 1991) and its ligand FL (Hannum et al., 1994) play an important role in survival and self renewal of early multipotent hematopoietic progenitors, of monocytic precursors and in early lymphoid development (Lyman and Jacobsen, 1998).

Incubation of leukemic blasts with FL results in enhanced DNA synthesis in some, but not all cases of AML and in a reduced rate of spontaneous apoptosis of AML blasts. Interestingly, the proliferative response to FL is not necessarily predicted by surface expression of the receptor (Stacchini et al., 1996).

Since the description of Flt3 expression on leukemic blasts (Drexler, 1996) speculation arose that it might play a role in malignant transformation of hematopoietic progenitors. Reports about a proliferative and antiapoptotic response to FL supported that hypothesis (Drexler, 1996; Lisovsky et al., 1996). We have reported previously that Flt3 receptor of two third AML patients shows ligand dependent phosphorylation and 10% AML patients show ligand-independent autophosphorylation of Flt3 receptor. The mechanism of the observed activation remains unknown. Internal tandem repeat (ITR) mutations involving exon 11 of Flt3, which cause an insertion of several amino acids in the juxtamembrane region of the protein and are detectable in about 20% of AML samples, have been suggested as a mechanism of Flt3 activation (Kiyoi et al., 1998). However, we have shown that ITR mutations do not correlate with Flt3 autophosphorylation (Fenski et al., submitted for publication). An activating point mutation in the kinase domain of two other receptor tyrosine kinases has been linked to neoplastic diseases. Substitution of Asp 814 by Valin (Furitsu et al., 1993) in the structurally and functionally closely related protein c-kit (Qiu et al., 1988) has been associated with systemic mastocytosis (Longley et al., 1996). A similar mutation (Met 918 Thr) in the protooncogene ret is found in germ line of families with multiple endocrine neoplasia type 2b and as a somatic mutation in about 30–40% of sporadic cases with medullary thyroid carcinoma (reviewed in Kolibaba and Druker, 1997). In order to analyse the consequences of constitutive activation of Flt3 in myeloid cells, we introduced the wildtype receptor into 32D cells, a murine IL-3-dependent hematopoietic cell line without endogenous Flt3 expression. Furthermore, we constructed a mutation of Flt3, substituting Asp 838 with Valin, (Flt3D838V), which we also transfected into these cells. This mutation is homologous to the activating mutation found in c-kit in neoplastic mast cell disorders. Here, we show that Flt3 signalling can mediate proliferation and survival of 32D cells, and that the D838V mutation confers ligand independent proliferation of these cells. We also demonstrate possible signalling pathways and in-vivo effects of Flt3 activation in 32Dcl3 cells.

Materials and Methods

cDNA Constructs and Expression Systems

The cDNA of murine Flt3 was kindly provided by Dr. Ihor Lemischka (Princeton, NJ, USA). The complete coding sequence was
subcloned into the retroviral vector pGD at the Bcl I site under the control of the long terminal repeat of the myeloproliferative sarcoma virus (MPSV). After site-directed mutagenesis of Flt3, integrity of the complete Flt3 coding region was confirmed by sequence analysis. Hybrid receptor containing the extracellular domain of mouse c-kit and the transmembrane and intracellular domains of human Flt3 was constructed by PCR-based methods and the construct was cloned into pcDNA3.1 (Clontech). For transfection, 10 μg plasmid DNA of either plasmid was linearized by HindIII digestion and added to the cell suspensions. Samples were electroporated with a Gene Pulser (Biorad) in 0.4 cm cuvettes at 300 V and 960 μF and were selected with 0.6 mg/ml G418. 14 days later the Geniticin resistant cells were purified twice with a magnetic cell sorting (MACS)-column (Miltenyi Biotec) using rat anti-mouse-Flt3 monoclonal antibody and goat anti-rat-ferrobenoids according to the manufacturer’s instructions. COS-1 cells were transiently transfected by the DHEA-method.

Proliferation and Apoptosis Assays

Proliferation was measured by 3H thymidine incorporation into cells, which had been arrested in G0/G1 prior to the assay. Apoptosis was induced by growth factor and serum deprivation of the cells for the indicated times combined with ionising irradiation when indicated. For this purpose, cells were γ-irradiated with 10 Gy and FL or IL-3 was added as indicated. Inhibition of cytokine activities was done by 15 min-preincubation with 100 nM wortmannin (Sigma) and/or 40 μM PD98059. Apoptosis assay was done with the Annexin-V assay kit (Genzyme). Annexin-V− Propidium Iodide− cells were counted as viable cells.

Immunoprecipitation, Western blot and MAPK Assays

After starving from cytokines cells were lysed 50 mM HEPES pH 7.4, 10% Glycerol, 150 mM NaCl, 1% Triton x-100, 1 mM EDTA, 1 mM EGTA, 50 μM ZnCl2, 25 mM NaF, proteinase inhibitors, 1 μM peptain and 1 mM sodium orthovanadate) and immunoprecipitation was performed with the indicated antibodies. MAPK activity was measured by adding MBP as a substrate and GGG[32P]-ATP. The kinase reaction was allowed to proceed for 20 min at 30°C. Reactions were spotted onto phosphocellulose paper, washed extensively with 0.85% phosphoric acid and incorporated radioactivity was determined. For western blot analysis, immunoprecipitates were separated by SDS-PAGE (7%), blotted onto PVDF membrane and immunoblots were performed using the indicated antibodies and ECL (Amersham) as a detection system.

In vivo Tumorigenicity

C3H/HeJ-mice were injected with 1x10⁶ cells of 32Dcl3/Flt3 or 32Dcl3/Flt3D838V. Control mice received 32Dcl3/vector. The animals were observed on a daily basis. Moribund animals were sacrificed and bone marrow samples were smeared onto glass slides and stained with May-Grünwald Giemsa stain.

Results

In order to analyse the effects of activating mutations of Flt3, we stably transfected Flt3 and Flt3D838V into 32D cells (Fig. 1). Similar levels of surface expression could be reached for both protein isoforms. 32Dcl3/Flt3 cells responded to addition of FL by increased 3H thymidine incorporation, whereas 32Dcl3/vector were not influenced by addition of FL. In contrast to these two cell lines, 32Dcl3/Flt3D838V cells proliferate without exogenously added growth factors, and exogenously added FL does not influence DNA synthesis in these cells (Fig. 2). 32Dcl3/Flt3D838V continued to grow for several weeks without exogenously added growth factors (data not shown). Thus, Flt3D838V expression causes factor-independent growth of 32Dcl3 cells.

32Dcl3 cells are IL-3 dependent cell lines, and IL-3 deprivation induces apoptosis on these cells. When depleted from IL-3 and serum, 90% of 32Dcl3 cells die by 40h (Fig. 3A). Ionising irradiation induces more rapid death in 32Dcl3 cells, and after 16 h over 90%