The effect of c-KIT and NPM 1 mutations on myeloblast proliferation and differentiation

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of malignancies arising from myeloid stem cell (1,2). The outcome of AML is ominous most of the time. The prognosis and treatment of AML (and other leukemias as well) is dependent on many genetic mutations and chromosomal aberrations present with the disease. The classification of AML is also based on morphology, cytochemistry and genetic markers. Except for the recurrently defined chromosomal aberrations such as t(9;21), t(15;17), inv(1)6, t(9;11), the majority (more than 50%) of AML carries normal karyotype with no underlying recurrent genetic abnormality (1,2). Two novel mutations identified in some haematological and non haematological neoplasia include mutations in c-KIT gene and NPM 1 gene.

c-KIT is a proto-oncogene which encodes a transmembrane tyrosine kinase III receptor (3,4). With its ligand stem cell factor, c-KIT receptor has been shown to be important for cell growth, function and survival of cells. It belongs to the same family of receptors, in Monocyte Colony Stimulating Factor (M-CSF), PDGF and FLT 3. It is expressed in many different human cells including normal haemopoietic precursor cells, mast cells, germ cells and melanocytes etc. Its activation leads to a cascade of phosphorylations in the cytoplasm. It has been implicated in many none haemopoietic malignancies including cancers in breast, lung, ovaries etc (3,4,5,6,). Almost all primary systemic mastocytosis patients show c-KIT mutations specially one named as c-KIT 816 (D816V). Over-expression of c-KIT receptor in myeloblasts was observed in 60-80% of AML. Point mutations of c-KIT gene were exclusively associated with Core Binding Factor AML (CBF-AML) which constitutes about 33-45% of AML and showed higher relapse rate. This could be related to direct effect of mutated c-KIT gene. The relapse and worse prognosis in AML sub types with inv (16) and t (8;21) were associated with c-KIT mutations even though these subtypes fall in to good prognosis category (5-12).

Some experimental studies support the hypothesis that these fusion proteins impair myeloid differentiation and expand haemopoietic stem cell pool. Some of these mutations specially 816 when present in Chronic Myeloid Leukemia (CML) showed increased resistance and treatment failure to Imatinib Mesylate, the current best therapy available for CML (13,14).

Therefore, study of effects of these mutations on myeloblasts could be important to understand disease outcome and to assess possibilities for therapeutic interventions by blocking mutated protein as it is one major research in leukaemia therapeutics (15-19).

On the other hand, nucleophosmin (nucleoplasmin) (NPM) is a nucleocytoplasmic protein found in both normal and tumour cells (20,21). It was shown in studies that a mutation in NPM (NPM 1) is more abundant in nuclei of tumour cells than normal cells (20,21). Mitogenic activation or growth of cells is associated with increased nuclephosmin protein in nucleus in many folds. NPM regulates p 53 and tumour suppressor pathways both positively and negatively. It is involved in ribosome

biogenesis and centrosome duplication as well (20,21,22).

The NPM 1 mutation has been associated with different haemopoietic malignancies by virtue of its involvement in translocations. Recently in 35.2% of primary AML, it was shown aberrant cytoplasmic localization of nucleophosmin protein. It was associated with many different subtypes of AML and interestingly most were of normal karyotype (20-23). AML with recurrent genetic abnormalities and secondary AML were devoid of NPM1 mutations thus cytoplasmic localization of nucleophosmin (22,23,24).

The immortalized murine myeloblast (32D) cells have been widely used in research and assessed for stability in in-vitro cultures. These cells are easy to manipulate in nucloefection procedures. The characteristics of the 32D cells are well defined (25,26).

Considering these facts, it can be argued that C-KIT mutations and NPM 1 mutation can play a significant role in leukemogenesis. Therfore, this study was planned to assess effects of these mutations on two most important features in tumourgenesis; excessive uncontrolled proliferation and inhibition of differentiation. To study effects of genetic mutations, 32D cells are used in many researches showing its applicability in laboratory setting thus selected in this study.

Objectives

Objectives of this study was to assess differences in proliferation, differentiation and autonomous proliferative capacity of normal and c-KIT & NPM 1 mutated murine myeloblasts.

Method

Immortalized murine myeloblasts (32D cells) were selected for the study. The 32 D cells are derived and established from long term bone marrow cultures of C3H/HeJ mice infected with Friend of Murine Leukaemia virus. These cells are immortalized and are constitutively dependent on IL 3 to proliferate continuously and on G-CSF to differentiate in to neutrophils. In the absence of growth factors these cells decline proliferative capacity and undergo apoptotic death within 24 hours of culturing in the absence of growth factors (IL 3 & G-CSF).Unmutated (wild type 32 D cells) were selected as negative control.

Using plasmid vectors and mutated gene sequence available, murine myeloblasts were nucleofected with c-KIT and NPM 1 mutations using standard protocol. Wild type 32 D cells were mixed with nucleofection media containing highly purified plasmids and were incubated providing standard conditions.

After completion of nucleofection, cells from each nucleofection group were transferred to culture dishes. Culture dishes were incubated under standard conditions and isolation of clones were done under strict sterile conditions avoiding mixing or contamination.

The eukaryotic green fluorescence protein (EGFP) gene was used in the procedure of nucleofection to verify success of the procedure.

All the mutated and unmutated murine myeloblasts (32D cells) were propagated and cultured using standard technique. At the end of the initial incubation phase, cultures were assessed for positivity of green fluorescence under fluorescent microscope. Sets of cell cones positive were considered successful and further propagated in RPMI 1640 (Rosewell Park Memorial Institute) media (by Cambrex Bio Science - Walkersville, Inc.) with antibiotics (penicillin and streptomycin) to avoid contaminant bacterial and fungal growth. The media was mixed with G-CSF and or IL 3 (depending on assay) and foetal calf serum to provide required basic factors for cell survival and propagation. The most stable and cell count optimal, clones with adequate surviving cells were selected and transferred to $25cm^3$ corning culture flasks with angled neck (CORNING Flasks, polystyrene, sterile) with RPMI media to perform proliferation and incubation assay.

All the clones incubated over night at 37^0C with 5% CO_2 and other standard conditions. Differentiation assay and cell count were performed using standard techniques at the beginning of each day, in each cultured clones.

A cytospin preparation was prepared every day and stained with Giemsa method using standard protocol and kept labelled with mutation and the date.

After taking the cell count every morning, a standard quantity of cells (1.5 x 10^6/ml RPMI solution) re transferred for culture propagation to be continued overnight. Excess cells and media were removed, destroyed and discarded using standard protocol. Thus every day the proliferation started with the same cell number as per the unit volume of media.

The different clones propagated in the study are given in the table 1 with abbreviations.

The clones in proliferation assay were propagated only with IL 3 as the growth factor. The clones were propagated for 7 days. Cell counting was performed every morning using Casey cell counter. The cells were observed for fungal or bacterial growths and for dead cells using the inverted microscope.

The differentiation assay was performed in the absence of IL 3 but with G-CSF as the growth factor. Cell counting, cytospin preparation and replacement of media continued.

To assess autonomous proliferation ability clones were propagated in the absence of both IL 3 and G-CSF.

When more dead cells are present in clones, they were removed using density gradient cell sorting system using standard protocol (with lymphoprep solution) to minimize negative effect of dead cell constituents on cell proliferation.

Presence of mutations in research cell clones was confirmed using following three techniques.

1. Examination under UV fluorescent microscope for positive green fluorescence (Figure 1).
2. Flowcytometry using C-KIT antibodies to check expression level with fluorescence activated cell sorting (FACS) system. This confirms expression of C-KIT gene in cell cones.
3. Western blot analysis was used to assess degree of expression of nucleophosmin. A control phoenix cells (murine fibroblasts) with NPM 1 mutation were taken as the control. Cells from each selected clone were lysed to extract proteins and purified (to retain nucleophosmin). The protein blots prepared were incubated with radiolabeled antinucleophosmin antibodies and X-ray exposure films were taken to confirm expression of NPM 1 in selected clones.

Table 1: Clones of 32 D cells and abbreviations used

Clone number	*Clone*	*Abbreviation used*
1,9,17,25	32 D cells – negative control – wild type	32D WT
2,10,18,26	32 D cells with EGFP nucleofection	32D EGFP
3,11,19,27	32 D cells with wild type C-KIT gene	32D KT WT
4,12,20,28	32 D cells with wild type NPM1 gene	32D NPM1 WT
5,13,21,29	32 D cells with mutated NPM 1 gene	32D NPM1MUT
6,14,22,30	32 D cells with C-KIT mutations – insertion/deletion 1	KIT ID 1
7,15,23,31	32 D cells with C-KIT mutations – insertion/deletion 2	KIT ID 2
8,16,24,32	32 D cells with C-KIT 816 mutation	KIT 816

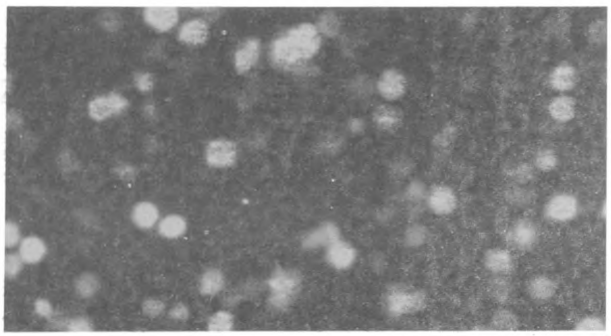

Figure 1: Demonstration of green fluorescence of nucleofected cells.

Results

Proliferation assays

The proliferation assay showed an almost similar proliferation pattern and rate in all the cell lines. There was no difference in rate of proliferation observed in wild type over mutated ones. Table 2 shows the proliferation of different cell clones over 10 days in culture media.

Table 2: Proliferation assay - results are given number of cells X 10^9/ml media

	Day of the Culture									
Cell clone	*D1*	*D2*	*D3*	*D4*	*D5*	*D6*	*D7*	*D8*	*D9*	*D10*
1	3.2	4.5	6.4	6.7	9	8	8.5	8	8.8	8.3
2	3.4	6	6.5	6.9	8.2	7.8	8.4	7	6.3	8
3	6	9.6	3.5	5.6	5.4	9	8	8.3	8.3	8
4	3.3	6.3	5.4	6.5	7.4	8.6	9	8.7	7.8	8
5	9.4	5.2	9.6	10	13	10	12	9.6	9.5	10
6	5.5	4	8.3	13	6.6	9.4	8	9.3	7.8	7.6
7	5	6	5.2	10	7.6	12	7.4	8	9.6	8.2
8	3.2	5	7.8	6.7	10	9.7	9	9.8	8.9	8
9	3	7.4	6.2	9.1	9.4	11	7.6	9.5	8.6	9
10	3.8	6.9	5.2	7.5	7.8	10	7	7	7	8.8
11	2.9	8.8	8.3	8	9.9	9.5	10	10	8.5	8.9
12	2.7	5.8	6.4	6	9.2	9	6.7	7	7.4	7
13	2.8	5	6.3	5.8	10	9.4	8.5	9	7.9	8.4
14	5.8	5.7	7.8	7.5	10	10	7.5	8.2	7.9	7
15	4	6.8	4.3	7.7	9	9.7	9.2	8.4	9	10
16	2	6.8	5.5	9	8.6	9.8	7.9	6.4	8	7.5

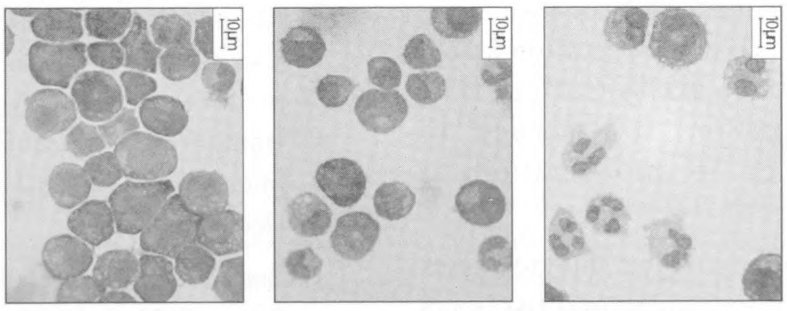

Figure 2: 32D cells in different stages of differentiation assay.
A. 32D cells with c-KIT 816 mutation on day 2, **B.** 32D cells with c-KIT mutation on day 10, **C.** Wild type 32D cells in day 10

Differentiation assay

Interestingly, the differentiation assay showed impaired differentiation compared to 32D WT cells. This was reflected by the presence of large percentage of blasts compared to 32D WT even towards the end of the assay. Specially the cell cone with c-KIT 816 showed highest difference compared to wild type. Even on day 12, mutated clones showed over 50% blasts in the presence of 100% differentiation in to neutrophils in wild type clones by day 10. Figure 2 shows different 32D cells at different stages of differentiation assay.

Interestingly, both mutated and wild type clones failed to sustain autonomous proliferation in the absence of growth factors and undergo complete apoptosis by day 2. FACS analysis of cell cones showed c-KIT positivity. However, the signals were weak, could be related to the degree of expression.

In addition, more apoptotic cells were noted in wild type clones while less apoptosis was noted in mutated clones in differential assay. However, apoptosis was not assayed in the study to ascertain exact details.

Discussion

The proliferation of wild type cells and mutated cells showed almost equal degree and rate of proliferation in this study. The 32D cells used in the study are immortalized blasts having enormous capacity to proliferate thus, the proliferation assay may not be sensitive enough to identify the differences. The insensitive nature could have been the reason for absence of an observable difference in proliferation assay. Therefore, more sensitive assays (such as H3 - Thymidine incorporation) are needed to ascertain differences in proliferation at molecular level.

Surprisingly both wild type and mutated ones failed to sustain proliferation beyond two days without growth factors. This needs further evaluation to ascertain the exact nature of activity of the mutations studied and need for additional supportive mutations.

In this study, the most conclusive results are obtained in differentiation assay. Those support the effect of c-KIT mutation in propagation of leukemia by inhibiting differentiation. Even at the last day of the assay, mutated clones had a significant quantity of undifferentiated blasts in the culture. In c-KIT 816 clone, it was more than 50%. The clones with pure wild type cells and wild type gene had shown rapid differentiation and marked apoptosis within a few days after commencement of assay. This could be utilized to explain ominous nature of the c-KIT mutation in leukaemias.

However, the expression of c-Kit appears weak in clones studied. If the expression of c-KIT was optimal the results could have been more decisive.

The NPM 1 mutation however, failed to show any influence either on proliferation or differentiation of myeloblasts.

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