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RESEARCH ARTICLE

ROLE OF G-CSF PLUS IL-15 ON NEUTROPHIL POPULATION IN PERIPHERAL BLOOD MODULATING PROTEIN TYROSINE KINASE ACTIVITY IN LEUKEMIC MOUSE

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ABSTRACT

Protein tyrosine kinase (PTK) is an enzyme which mediates cell function, growth, metabolism and apoptosis. The present work has an objective to evaluate the involvement of PTK activity on neutrophil population in the peripheral blood upon stimulation with G-CSF plus IL-15 in leukemic animal. N-N'-Ethyl-nitrosourea (ENU), a carcinogen was used to induce Leukemia in BALB/C mice where in combination with Granulocyte colony stimulating factor (G-CSF) and Interleukin-15 (IL-15) were treated. To determine the PTK activity in normal and leukemic animal universal tyrosine kinase assay kit were used. Appearance of blast cells in peripheral blood and bone marrow smear confirmed the Leukemia. Neutrophils were isolated from spleen using percoll gradient method. Cell population was measured in both normal and leukemic animal before and after treatment of combination of cytokines. ENU induced mice model showed mixed type Leukemia dominating lymphoblastic in nature. The data indicated that treatment with IL-15 and G-CSF in combination to leukemic mice, increased the number of neutrophil population in periphery and under the regulated control of protein tyrosine kinase activity. Results may hint for cell based therapeutic approach to improve neutrophil population in Leukemia.

INTRODUCTION

Tyrosine kinases are important components of signal transduction process, leading to cell differentiation, proliferation, migration, metabolism and programmed cell death. Protein tyrosine kinases consists of both receptor and non-receptor cytosolic type of protein which catalyzes phosphorylation of select tyrosine residues in target proteins, using ATP. This covalent post-translational modification is an important component of normal cellular communication and maintenance of cellular homeostasis (Hunter, 2000; Schlessinger, 2000). Tyrosine kinases also play an important role in several steps of neoplastic development and progression. Tyrosine kinase signaling pathways normally prevent deregulated proliferation or contribute to sensitivity towards apoptotic stimuli. These signaling pathways are often genetically or epigenetically altered in cancer cells to impart a selection advantage to the cancer cells. Thus, it is no wonder that aberrant modulation in signaling emanating from tyrosine kinase endows these enzymes a dominating oncoprotein status, resulting in the malfunctioning of signaling network (Blume jenson and Hunter, 2001).

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Leukemia is a cancer of blood-forming cells in the bone marrow (Ebata *et al.,* 2006) characterized by an abnormal increase of immature white blood cells called "blasts". These immature cells accumulate in blood and organs but all of them are not able to carry out the normal functions of blood (Pierson and Miller 1997). ENU, an alkalyting agent, (chemical formula $C_3H_7N_3O_2$), is a highly potent mutagen and ENU can induce 1 new mutation in every 700 loci. It acts by transferring the ethyl group of ENU to nucleobases (usually thymine) in nucleic acids (Ogui and Odashima, 1982). ENU can induce several types of cancer like brain cancer (Yabuna T *et al.* 1998). Specific doses of ENU can induce mixed type of leukemia (both the myelogenous and lymphogenous) (Druckrey *et al*.,1966; Koestener *et al.,* 1971). Neutrophils, the most abundant immune cell in blood quickly arrive at sites of infection and form the first line of defense following infection. The key role of neutrophils is the antimicrobial effector functions and the ability to produce cytokines to initiate inflammatory responses and chemokines to induce trafficking of immune cells (Ymashiro *et al*., 2001). During the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, environmental exposure (Jacobs *et al.,* 2010), and some cancers (Waugh and Wilson, 2008), neutrophils are one of the first-responders of inflammatory cells to migrate towards the site of inflammation. In leukemia, the immune system is in depressed condition and the infection

by different microorganism in that condition is prevented generally by the neutrophils. In acute leukemic condition the number of neutrophil decreased significantly (Silver *et al*., 1957). Neutrophils migrate through the blood vessels, then through interstitial tissue, following chemical signals such as Interleukin-8 (IL-8) and C5a by the process called chemotaxis (De Larco *et al*., 2004). Protein kinase C (PKC) is reported to play a role in maturation of the myeloid cell and functions of the mature neutrophil. The neutrophils in chronic myeloid leukemia (CML) exhibit defects in several functions (Balasubramanian *et al.,* 2002). Cytosol-membrane distribution of PKC was significantly altered in leukemic neutrophils as compared to normal cells. Cytosolic levels of all PKC isoforms were reduced in CML neutrophils (Balasubramanian *et al*., 2002). Granulocyte colony stimulating factor (G-CSF) is a lineage specific hematopoietic growth factor that initiates the differentiation and proliferation of committed progenitor cells into mature neutrophils. G-CSF exerts its proliferative effect mainly at the stage of myeloblast-promyelocyte, and also stimulates the release of mature bone marrow neutrophils from storage pools into the peripheral circulation (Ulich *et al.,* 1998; Lorde *et al.,* 1989; Hollingshead *et al*., 1991; Hariss *et al.,* 1994). In vivo studies of G-CSF administration reveals enhanced adhesion on nylon wool, phagocytosis, luminal enhanced chemiluminiscence, degranulation and expression of cell surface antigens (Rapoport *et al*., 1992; Krest *et al.,* 1993; de Hass *et al*.,1994; Xu *et al.,* 1996). One study also showed promotion of neutrophil survival in vitro (Adachi *et al.,*1994).

IL-15 can induce phagocytosis, cytoskeleton rearrangement, gene expression, *de novo* protein synthesis and can delay apoptosis in human neutrophils (Girard *et al.,* 1996). Production of chemokines, cytokines and natural inhibitors is increased in IL-15 induced neutrophils, including CXCL8 (IL-8) (Musso *et al.,* 1998; McDonald *et al.,*1998), IL-1beta, IL-1RII and IL-1Ra (Jablonska *et al.,*2001; Bouchard *et al.,* 2004). IL-15 has also been shown to induce the redistribution of ICAM-3 and P-selectin glycoprotein ligand-1 (PSGL-1) to the uropods in neutrophil (Alonso- Lebrero *et al*., 2000). The mechanisms involved in IL-15-induced activation of human neutrophils are not fully understood. However, IL-15 was shown to activate NFkB (McDonald *et al.,*1998), and to induce the phosphorylation of Syk and its physical association with IL-15R alpha (Rathhe *et al.,* 2004). As reported by Ratthé C et. al., in 2004, IL-15 can induce the functional activity of neutrophil and alone G-CSF can increase the population of neutrophil (Deshpande *et al.,* 1997). We have studied the combination treatment in leukemic condition. Our focus of this study was to find out the neutrophil population in ENU induced leukemic animal model modulating the PTK activity during pre and post treatment in combinations of G-CSF and IL-15.

MATERIALS AND METHODS

Materials

RPMI-1640, Fetal Bovine Serum was purchased from Hi-media, India. Leishman's stain is purchased from Loba chemicals, India. N-ethyl-N'-nitrosourea from Sigma, Recombinant mouse interleukin-15 (rm IL-15), Recombinant mouse granulocyte colony stimulating factor (rmG-CSF) from ImmunoTools, Germany, Percoll from GE Health Care Life Sciences, Sweeden and Universal tyrosine kinase assay kit was purchased from Clontech Laboratories Inc/Takara Bio Inc. Japan.

Animal

Healthy BALB/C male mice were obtained from National Centre for Laboratory Animal Sciences, (NCLAS), NIN, Hyderabad, India. Animals were divided into four groups having six animals in each group. Groups were divided as Mock control, ENU challenged, and control received only combination cytokine and ENU challenged mice received combination cytokine. These mice were kept and maintained specific pathogen free condition in Tripura University Animal House as per guidelines of Institutional Animal Ethical Committee. Food, dietary supplements and water were provided *ad libitum*.

Induction of leukemia

Leukemia were induced by injecting N-ethyl-N'-nitrosourea (ENU) (Sigma Aldrich, USA) (i.p.) to 7-10 days old mice, at the dose of 80mg/kg body weight concentration at two times in one week interval (Law *et al.,* 2003a). After five months of ENU introduction the peripheral blood and bone marrow slides were prepared for both control and treated group and stained with Leishman's stain. Total count and differential count of leukocytes were also done in all the four groups.

Cytokine treatment

After 5 months of ENU injection Leukemia was confirmed by peripheral blood smear observation. The two cytokines, rmIL-15 at the dose of 5µg/ kg body weight (Choi *et al.,* 2004; Verri *et al.,* 2007) and rm G-CSF at the dose rate of 10µg/ kg body weight (Girard *et al.,* 1998; Jilma *et al.,* 2000) (ImmunoTools, Germany) were injected intra-peritoneally in animals of one control and one ENU treated group after conformation of leukemic induction for consecutive five days. Every 24 hours from first cytokine treatment, peripheral blood smear were prepared to have total count and differential count of leukocytes until 5 days program completed.

Isolation of splenic neutrophils

The spleens were removed from mice after sacrifice by cervical dislocation of the four groups. The splenic suspension was made by smashing the spleen gently by two frosted glass slides in a sterile petri dish containing 37^0C RPMI-1640 media containing 10% FBS (Himedia, India). Tissue debris and fat droplets were removed by an initial low speed centrifugation. Two layered Percoll^{TM} gradient was used to isolate neutrophils (Boxio *et al.,* 2004; Sroka *et al.,* 2009). In a 15ml centrifuge tube 1.11 gm/ml density percoll taken followed by 1.089 gm/ml density layered. On the top of the tube splenic suspension cells are layered. Cells are centrifuged at 900g for 30 minutes at room temperature. The interphase between 1.11gm/ml and 1.089 gm/ml consists of neutrophils and were collected. After washing in PBS three times finally suspended

in complete RPMI-1640 media. Total number of cells for all the four groups was counted by haemocytometer.

Protein tyrosine kinase assay

The protein tyrosine kinase assay of neutrophil was done using universal tyrosine kinase assay kit, according to the manufacturer's instructions. In brief, 3x 10^6 cells from each group were used for this assay in triplicates. Cells were pelleted down by centrifugation at 1500 rpm for 5 minutes and 1ml of extraction buffer was added and mixed gently. The solution was centrifuges at 10,000x g for 10min at 4^0C and the supernatant was used as sample. 40µlof the sample was used for each group in triplicate. In each 40µl of the sample, 10µl of 40mM ATP-2Na solution in each well, mixed well and incubate for 30min at 37^0C. The samples were removed and the wells were washed 4 times with washing buffer before addition of antibody. Added 50µl of Anti-phosphotyrosine (PY20)-HRP solution into each well and incubated at 37^0C for 30 minutes. Before adding the substrate, the antibody solution was discarded and washed the wells 4 times with wash buffer. The substrate reaction started by adding 100µl of HRP substrate solution in each well and incubated for 15 minutes at 37^0C. Color reaction was stopped by adding 100ul of stop solution to each well. The optical density was measured at 450nm using Synergy H-1 hybrid plate reader, Biotech, India. This experiment was done in triplicate. A standard curve was prepared according to the manufacturer's instruction and activity of PTK was measured in the unit of 10^{-5} unit/µl of sample.

Statistical analysis

Statistical calculations was done using MS excel 2010 software and only student's t-test was done using online graphpad software.

RESULTS

ENU induced Leukemia reduced the number of neutrophils in animal model

After 5 months of ENU challenge, occurrence of Leukemia in animals were confirmed by appearance of numerous blast cells in peripheral blood smear (Figure 1B) and bone marrow smear (Figure 1D) in comparison to control (Figure 1A and 1C). Acute death occurred in 2 animals out of 12 animals in the experiment. Total counts of leukocytes were increased in ENU challenged group compared to control animals (Figure 2A). After treatment with IL-15 and G-CSF in combination to the leukemic animals the number of leucocytes was increased since 3rd day of treatment (Figure 2A). Total isolated neutrophil count was also reduced in leukemic group which were significantly increased again after combined cytokine treatment (Figure 2B). The blood smear showed the appearance of mixed type of blast cells, lymphoblast cells were dominating (18%) in the peripheral blood. The liver and spleen size of the Leukemia group increased compared to control counterparts. Some secondary infections were detected including foot and mouth infection, hair fall, redness in mouth etc. in Leukemia group. Differential count of lymphocytes showed an increased number from day 3 to day 5 following cytokine treatments (peripheral blood smear count data not shown). Number of immature blast cells, both lymphoblast and myeloblast were increased in peripheral blood in ENU challenged mice of approximately 20% of the total leukocyte.

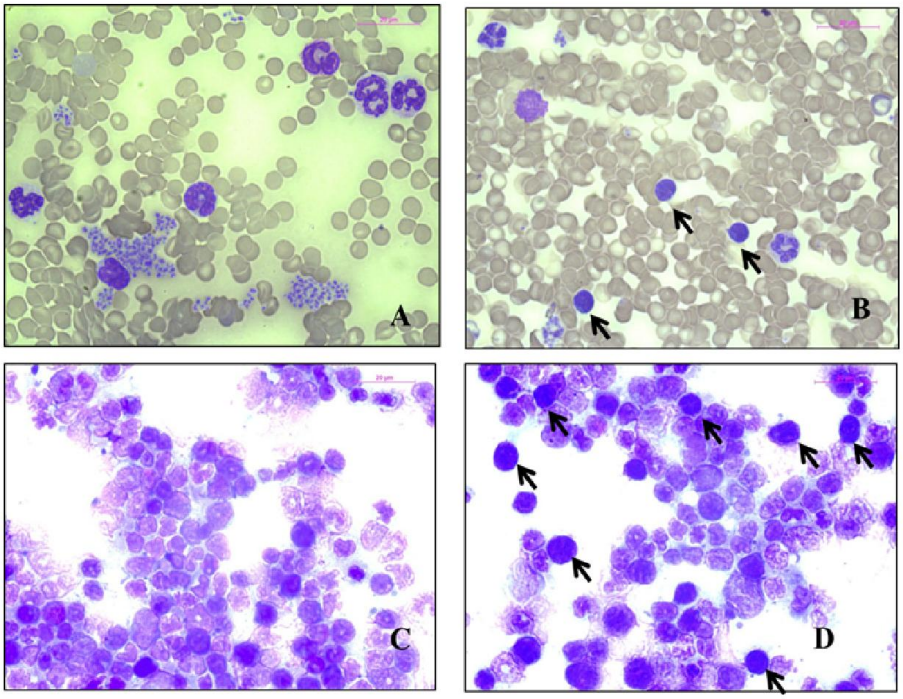

Figure 1. Leishman's staining of peripheral blood and bone marrow smear of mice

A and C are peripheral blood and bone marrow smear with Leishman's stain of control mice. B and D are blood and bone marrow smear after 5 months of ENU challenged respectively. Arrows are showing the presence of blast cells. Experiment was done in triplicate and results are expresses as mean ±SD. "N"=6.

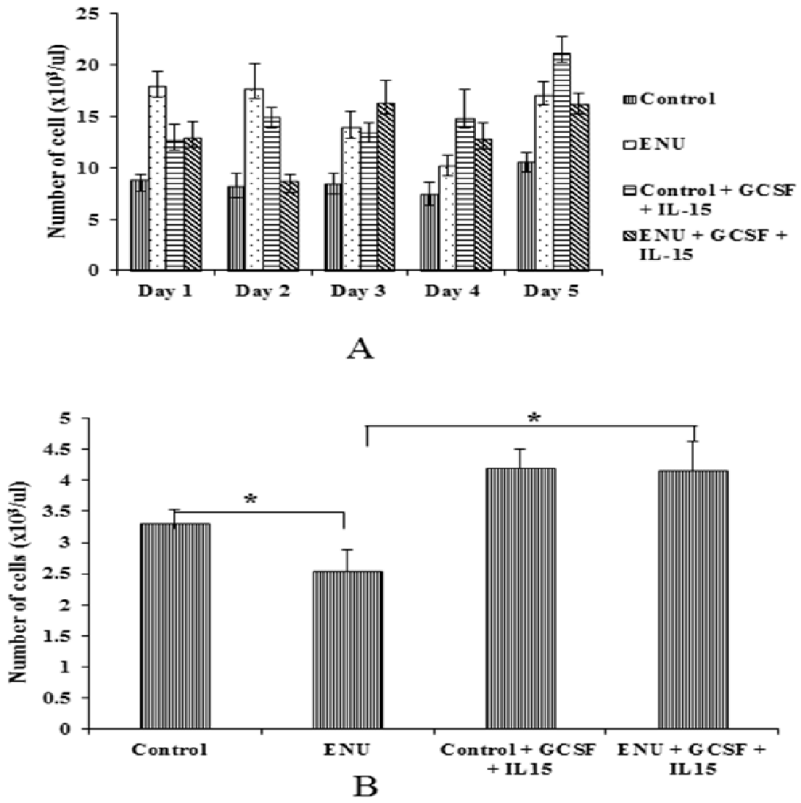

Figure 2. Cell counts

A. Total count of leukocytes after treatment in combinations of cytokines IL-15 and G-CSF for consecutive five days. Blood smear prepared after every 24 hours of the first dose of G-CSF and IL-15 treatment from each group. **B.** Total count of neutrophil from spleen after percoll isolation at the end of 5 days treatment. Experiment was done in triplicate and results are expresses as mean ±SD.”N”=6.

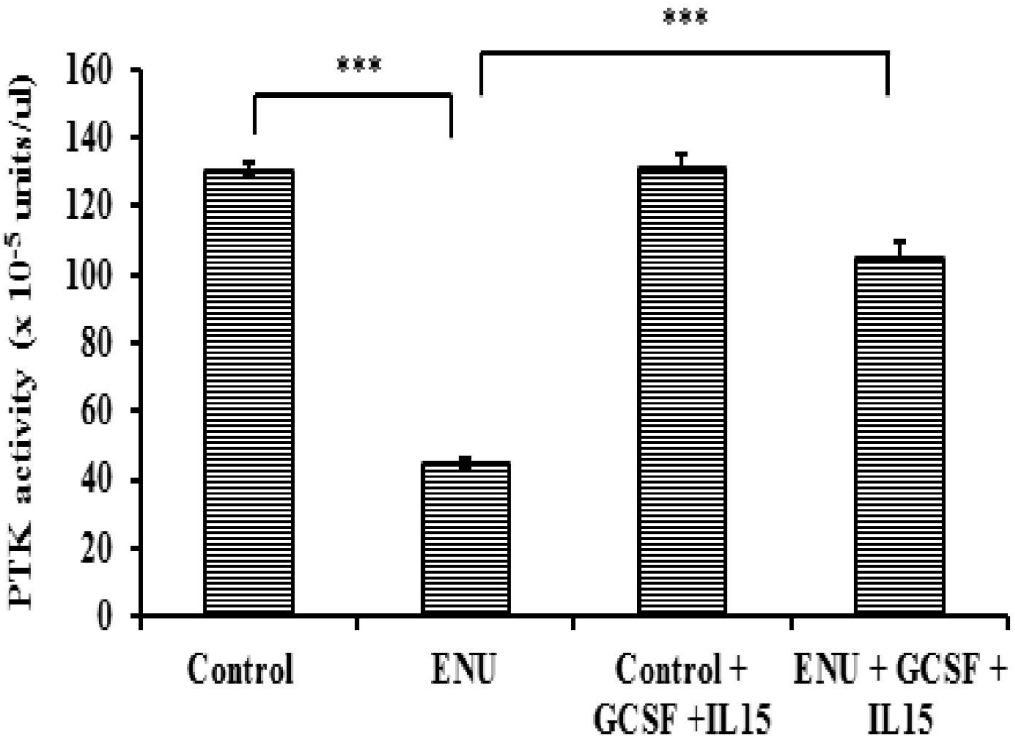

Figure 3. Protein tyrosine kinase assay

Bar diagram showing changes in protein tyrosine kinase activity of neutrophils between control and ENU induced leukemic group before and after G-CSF and IL-15 treatment. Experiment was done in triplicate and results are expresses as mean ±SD. (* indicates p< 0.05, * * indicates p< 0.01, ***indicates p< 0.001). ”N”=6.

The blast cell count reduced subsequently with the advancement of cytokine treatment from day 1 to day 5. The total reduction was approximate 5% of the total lymphocytes count in the end of 5 days (data not shown).

Protein tyrosine kinase (PTK) activity was reduced in animal Leukemia model

Protein tyrosine kinase is an enzyme responsible for cell proliferation, differentiation, metabolism and apoptosis. In tumorigenesis, PTK has important role. Both receptor and cytoplasmic PTK phosphorylate tyrosine residue in different substrates and show increased activity in tumour progression. Our results demonstrated that the phosphorylation activity of this enzyme in neutrophils in leukemic mice was significantly reduced more than 50% ($p< 0.001$) compared to the control group and was restored back again after treatment with combination therapy by IL-15 and G-CSF ($p< 0.001$) (Fig 3). This results support the data of neutrophil population in peripheral blood.

DISCUSSION

Simple animal Leukemia model is very helpful to study the details of Leukemia progression as well as the treatment by inducing activity of various immune cells in this debilitating disease. ENU has been reported to induce brain and other types of cancers. Leukemia is one the major events in this process. Even though ENU has a pleotropic effects on the generation of other kinds of cancer cell formation. We have been using ENU to induce Leukemia in the mice model successfully (Law *et al.,* 2001, 2003a). The blood cell formation from single stem/progenitor cells is always under continuous stimulation of various cytokines and growth factors secreted from its surrounding accessory cells (Can 2007). Any alteration in the signalling cascade of differentiation and proliferation leads to hematopoietic disorders which may accelerative or decelerative in nature. Under this circumstance, the normal hematopoietic cells can be transformed into leukemic cells. It results in accumulation and alteration in the leukemic cells that shifts the balance between cell proliferation and cell death, and leading to a cumulative increase of leukemic cells within bone marrow and peripheral blood (Kobari *et al.,*1991). N-N' Ethyl nitrosourea (ENU) is being used as a potent carcinogenic agent (Dexter *et al.,* 1974; Justice *et al.,* 1999) which can induce several cancer including brain tumour (Briançon-Marjollet *et al.,* 2010), reproductive tumour (Stoica G, 2002), Leukemia etc. in animal model. In this present study, BALB/C mice were used to induce Leukemia by injecting ENU (Chatterjee *et al.,* 2009). The cellular mechanism through which ENU induces Leukemia in animals may be due to carcinogenic activity (Chen D *et al.,* 2011) or due to causing immuno-suppression (Vquero *et al.,* 1998; Basak *et al.,* 2010). Simultaneously, it is an inhibitor of cellular kinetics with certain limitations; mostly dose dependent activity. Residual effect of ENU mediated inhibition leads to selective suppression of the bone marrow derived cells and finally tumour burden appeared (Basu *et al.,* 2012). Therefore, tumour cells predominates in number, whereas, the pluripotent bone marrow cells remains subsided. In our study, the peripheral blood smear as well as in bone marrow environment were dominated by the tumour cells, called 'blast'. Both myelogenous and lymphoid types of tumour cells were appeared. After treatment with combination of cytokines, the total number of tumour cells increased than only. ENU challenged animals which was expected results due to the effect of G-CSF. G-CSF mobilized immune cells into the peripheral blood which composed of both tumour and normal.
PTK, a key component in cellular signaling mechanism, and has a big role in pathogenesis. PTK both receptor or cytoplasmic has the function to phosphorylate tyrosine residue at different substrate. This function is smoothly balanced by tyrosine kinase and tyrosine phosphatase. Non-receptor PTK may be transformed by different mutagenic agent and can deregulate the normal function of other enzymes which may hamper the cell division, growth and cell death (Paul *et al.,* 2004). In response to G-CSF, PTK activity has increased in neutrophil resulted in proliferation of precursor cells to mature neutrophil (Deshpande *et al., 1997*).

In some cases of ovarian cancer Src tyrosine kinase expression and activity reduced. It has been reported that malignant human ovarian cancer tumour growth in a nude mouse model is inhibited by decreased Src tyrosine kinase activity (Weiner *et al.,* 1999). Though the total tumor growth is not completely abrogated but is reduced. The activity of Src is controlled by Csk is also another protein tyrosine kinase which inhibits the Src, a non-receptor tyrosine kinase (Irby RB and Yeatman TJ, 2000). Another protein tyrosine kinase, syk also found to regulate the cancer homeostasis, especially in breast cancer. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells by the mechanism of inhibiting microtubule formation and ultimately effects mitosis. (Coopman *et al.,* 2000). Though the mechanism of inhibition of tumour growth by reduced activity of PTK is not understood well, in our studies also the PTK activity in neutrophil of ENU induced leukemic mice is reduced. ENU may inhibit the expression of PTK or the transformed PTK does not show the normal activity in neutrophil from leukemic mice. Our data supports findings of Despande *et al.* where G-CSF increase the PTK activity resulted proliferation of the progenitor cells of neutrophils and may be the functional activity. After the administration of both G-CSF and IL-15, the deleterious effect of ENU on PTK is reduced by both the cytokines together to increase PTK level and also normal cellular functioning like proliferation, differentiation and signaling function of neutrophils. Treatment with combination of cytokines increased the production of neutrophil through normal hematopoiesis. Which PTK is involved exactly in this increased population of neutrophil in leukemia is not yet explored but increased number of neutrophil may relate to improve immune system to fight against tumour cells.

Conclusion

Though most of the cases of solid tumour, PTK activity increases to support the growth of tumour, our carcinogen induced leukemia study, PTK activity of neutrophil was significantly reduced. After combination of G-CSF plus IL-15 treatment, it is restored again. Further investigation using specific inhibitor of specific PTK including Syk, Src or c-Yes may open some mechanism by which PTK help in treatment of Leukemia.

Competing interest

The authors declared no competing interest.

Authors' contributions

BB performed the experiment, analyze the data and wrote the manuscript. AKS performed the experiment. DM conceived the plan of work, designed the experiment, standardize some the experiment and wrote the manuscript.

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