IL-3 and GM-CSF in combination alters protein tyrosine kinase activity of splenic macrophages in leukemic mice

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Abstract
Objectives: Murine macrophages were playing a key role against microbes and foreign particles as well as help in solid tumorigenesis in the host body. In this experiment we were trying to evaluate the protein tyrosine kinase (PTK) activity in macrophages from experimentally induced leukemic animal model.

Methods: Balb/C mice were divided into four groups, two groups were challenged with N-N' ethyl nitrosourea (ENU) and two groups remained without ENU challenged condition. After confirmation of leukemia induction, one normal and one ENU challenged groups were received rmIL-3 and rmGM-CSF in combination for 4 consecutive days. Disease was confirmed by histological studies of peripheral blood and bone marrow smear. PTK activity assay were done using universal tyrosine kinase assay kit.

Results: Protein tyrosine kinase (PTK) activity of macrophages was increased in leukemic animal significantly which were reduced after combination of IL-3 and GM-CSF treatment.

Conclusion: Results suggest that, in leukemia reduced macrophage number can be increased by IL-3 and GM-CSF administration in combination but PTK activity is not involved to increase the number of macrophages. PTK activity may be involved in macrophage activation process in leukemic animal.

Keywords: Macrophages, protein tyrosine kinase, interferon-γ, leukemia, N-N’ ethyl nitrosourea, cytokines, interleukin-3, granulocyte-macrophage colony stimulating factor

1. Introduction

Protein tyrosine kinase is the key protein for cell signaling pathway including receptor tyrosine kinase (RTK) and non-receptor tyrosine kinase (NRTK). In human around 20 RTKs families and 9 groups of NRTK have been identified which includes 90 tyrosine kinases. RTKs bound to ligand as different growth factors including VEGF, EGF, PDGF, FGF etc. to transfer signal from membrane to cytoplasmic molecule and finally to nucleus for gene transcription. NRTKs are either attached with cytoplasmic portion of receptors or in the cytoplasm which mediate signals upon stimulation from membrane receptor. Activation of this enzyme leads to proliferation, differentiation, metabolism, vasculogenesis, T-cell and B-cell activation, apoptosis etc. In general this enzyme prevents the deregulating proliferation signal, but in cancer cells altered signals are dominated to establish proliferation resulting defective signaling network[1].

NRTK has specific role in regulation of immune function. Src oncogene encodes one NRTK, having an important role in cancer. Jak families of NRTK are activated upon stimulation of cytokine receptor like IFN-γ and activate Stat family for transcription of specific genes. Fes oncogene encodes another NRTK which is also responsible for macrophage like myeloid cell development and their activity.

Different populations of macrophages are the key cell in cell mediated immunity. Some populations of macrophages are antimicrobial, some are wound healer and some populations are anti-inflammatory or regulatory macrophage [2]. Macrophage-derived cytokines promote the phagocytic response through recruitment and production of fresh phagocytes and opsonizing molecules [3]. Different types of cytokines specifically interferons, which are induced by viral infection, can activate natural killer (NK) cells, to
contribute to innate host defense against viruses and other intracellular pathogens [4]. Activated T helper-1 cells, cytotoxic T cells and natural killer cells produces interferon-gamma, a key factor for macrophage activation [5]. This cytokine converts resting macrophages into potent cells with increased antigen presenting capacity, increased synthesis of pro-inflammatory cytokines and toxic mediators, and augmented complement-mediated phagocytosis. Thus, macrophages acquire the capacity for killing of bacteria, especially intracellular pathogens, and perhaps tumors[6]. Recent Reports suggest that monocyte derived macrophage have the capacity to lyse leukemic cells[7]. It was also reported that, treatment of chronic granulomatous disease (CGD) mice with IFN-γ also enhanced uptake of apoptotic cells by macrophage in vivo via the signalling pathway. Importantly, during acute sterile peritonitis, IFN-γ treatment reduced excess accumulation of apoptotic neutrophils and enhanced phagocytosis by CGD macrophages. Impaired phagocytosis ability of macrophages was impaired against apoptotic cells by CGD are reversed by IFN-γ in a Nitric Oxide-dependent manner[8]. During the disease condition, impair the ability of the bone marrow to produce red blood cells and platelets[9]. For CML, the target is the unique protein called the BCR-ABL tyrosine kinase enzyme. BCR-ABL is a constitutively activated tyrosine kinase that is associated with chronic myeloid leukemia [10]. Tyrosine kinase activity is crucial for the transformation of BCR-ABL. Therefore inhibiting the PTK with imatinib, dasatinib, nilotinib etc. improves CML symptoms. GM-CSF alone can induce the macrophage differentiation and proliferation [11,12]. IL-3 alone can induce the macrophage activation and proliferation [13]. These cytokines regulate the growth, differentiation, migration and effector function activities of many hematopoietic cells in bone marrow, blood and sites of inflammation. [14]. To investigate the changes of PTK in macrophage in ENU induced leukemia condition, IL-3 and GM-CSF in combination were used. GM-CSF and IL-3 stimulates myeloid lineage stem cells to differentiate granulocytes and monocytes. In peripheral blood granulocytes are activated through different cytokines released from Th cells. PTK is one of the signalling mediators to differentiate and proliferate of macrophages. But in leukaemia PTK activity in macrophage is not explored in details. In this study, PTK activity of macrophages in leukemic animal and after treatment with IL-3 and GM-CSF were evaluated.

2. Materials and Method
2.1. Chemicals
ENU, trypsin obtained from Sigma Aldrich, USA. RPMI-1640, Hank’s balanced salt solution (HBSS), Antibiotic solution from Himedia, India. Percoll from GE Healthcare, USA. IL-3, rmGM-CSF from ImmunoTools GmbBH, Germany. PTK assay kit from Clontech,Takara Biotech In., Japan. IFN gamma ELISA kit from Life technologies, USA. Leishman’s stain from LOBA Chemicals. PBS, Starch and the other chemicals were obtained from SRL, India.

2.2. Animals
Male Balb/C mice, 3weeks old, were obtained from National Institute of Nutrition, Hyderabad, India. Mice were housed in a virus-free animal facility for the duration of the experiments as per guidelines of Institutional Animal Ethics Committee, Tripura University (Ethical Clearance Ref. No.: TU/IAEC/2014/VIII/3-2; Dated: 12-09-2014). These mice were kept and maintained specific pathogen free condition with proper humid (60-65%), and temperature 25-28°C in Tripura University Animal House. Food, dietary supplements and water were provided ad libitum.

2.3. Numbers of animal used
Four groups (two groups as control and two groups as treated) were used for entire experiment having six animals in each group.

Group I: Normal control group introduced with PBS only.
Group II: Challenged with ENU for leukemia induction.
Group III: Normal mice received only PBS and treated with rmGM-CSF and rmIL-3 in combination starting at 5days before sacrifice at 24 hr. of interval.
Group IV: Mice challenged with ENU, confirmed leukemia after 5 months and treated with rmGM-CSF and rmIL-3 in combination starting at 5 days before sacrifice at 24 hr. of interval.

2.4. Treatment and maintenance of animals
PTK activity of splenic macrophages was assayed from both treated and non-treated mice. Leukemia control mice are produced by challenging in intraperitoneally injected with ENU at 80 mg/Kg body weight at -3 weeks old condition twice in one week interval[15,16].

2.5. Cytokine supplementation
After five months from the first injection of ENU, leukemia was confirmed by peripheral blood and bone marrow smear study. After confirmation of leukemia induction, all the mice from group III and IV were received rmIL-3 at the dose of 5 µg/kg/d and rmGM-CSF at the dose of 5 µg/kg/d for 4 days (ImmunoTools GmbBH, Germany)[17-19].
2.6. Sample collection

After completion of treatment all the mice were sacrificed as per guidelines of Institutional Animal Ethics Committee. Mice were dissected and peripheral blood was collected using a sterile syringe into a 2 ml micro centrifuge tube containing heparin solution. The spleen was collected in 1X PBS for macrophage isolation. The bones from upper and lower legs were dissected and kept into 1X PBS for bone marrow smear.

2.7. Blood and bone marrow smear preparation

Clear, fresh and grease free slides were used for blood film smear from previously collected heparinized blood and bone marrow smear from previously collected bone. The blood and bone marrow smear were stained with Leishman’s stain for differential analysis of blood cells count.

2.8. Total count

Small amount of blood were used for total count of blood cells using Neubeur’s Hemocytometer Chamber.

2.9. Isolation of macrophages

In a sterile petridish, spleen suspensions were made using frosted slide in RPMI-1640 culture media. The cells were suspended in RPMI-1640 and were allowed to adhere on plastic surface of the bottom for 1 hour in 37°C incubator, non-adherent cells were removed [20]. The adherent cells were marked as macrophage and cells were detached from plastic surface of the bottom using 1X trypsin solution.

2.10. Protein tyrosine kinase assay

Protein tyrosine kinase activities were done by using universal tyrosine kinase assay kit (Cat # MK 410, Clontech, Takara Bio. Inc. Japan) as per manufacturer’s instruction. Absorbance readings were taken at 450nm in microplate reader (Biotek, USA).

2.11. IFN-gamma release assay

IFN-gamma release in serum by different immune cells was assayed by IFN-γ ELISA Kit (Cat # KMC 4021, Invitrogen, USA) as per manufacturer’s instruction. Absorbance readings were taken at 450nm using by microplate reader (Biotek, USA).

2.12. Statistical analysis

All the readings were taken 3-5 times repeats of same experiments. Data were expressed as means ± SEM. Data were analyzed Student’s t test for test of significant using GraphPad Prism Software. All statistical test were considered as significant in p values as follows (**p < 0.001, *p < 0.01, *p < 0.05).

2.13. Ethical permission

All the animal experiments were done with proper permission and according to the guidelines of Institutional Animal Ethics Committee of Tripura University.

3. Results

3.1. Leukemia induction in mouse

After 5 months of the ENU challenge mice were showed some secondary infection including hair fall, foot and mouth infection, red color of lower portion of nose. Two mice out of twelve treated mice were dead within five days of ENU introduction. Peripheral blood smear as well as bone marrow smear showed appearance of blast cells in ENU challenged mice (Fig: 1) which were absent in control group, confirmed the leukemia. This is one of the conventional diagnosis processes for the leukemia patient. Both lymphoblast and myeloblast cells are present in the smear. The total leukocyte count was much higher (Fig: 2) compared to control group after 5 months of ENU injection which was the other evidence of leukemia induction by conventional method of diagnosis.

Fig 1: Peripheral blood (a-b) and bone marrow smears (c-d) with Leishman’s stain. (a) Normal control group, (b) ENU challenged leukemic group, (c) Normal control group, (d) ENU challenged leukemic group. The arrow indicates the blast cell.
3.2. Total leukocyte count

The other conventional diagnostic process is to count the total cell number. In ENU challenged mice the total count increased around six fold compared to control mice (Fig 2). This happens may be due to presence of both myeloblast and lymphoblast in peripheral blood.

Fig. 2: Total count in peripheral blood. In ENU challenged animal total count is 6.5 times higher than control group. The counting of cells were done in three repeats and results were calculated in average of all repeats ± SD (**p < 0.001).

3.3. Macrophage count restored after cytokine treatment in leukemic animal

Splenic macrophages were isolated from all the groups of animals after the experiment was over. The total splenic macrophage count was almost four fold decreased in ENU induced leukemic animal which was restored again after treatment with IL-3 and GM-CSF in combination for consecutive four days (Fig 3).

3.4. Protein tyrosine kinase activity of splenic macrophages was upregulated in ENU induced leukemia and restored after treatment with IL-3 and GM-CSF

After leukaemia induction and followed by treatment with combination of IL-3 and GM-CSF, we determined the PTK activity of splenic macrophage. In this study, as a whole the PTK activity (Both non-receptor protein tyrosine kinase and receptor protein tyrosine kinase) was determined. PTK activity in macrophage was increased around two fold in ENU challenged mice in compared to control group. After treatment with IL-3 and GM-CSF in combination, the PTK activity reduced 1.6 fold and came into almost normal level (Fig. 4).

3.5. Serum IFN-γ level increased in Leukemic animal and restored after IL-3 and GM-CSF treated leukemic mice

After sacrificing the animals serum was collected from each group. There was a significant change of IFN-γ level in serum among the groups. The level was significantly increased in ENU treated mice in comparison to control. After IL-3 and GM-CSF treatment the level was modestly decreased (Fig: 5).

Fig 4: PTK activity of macrophages from ENU challenged mice was significantly increased which came into normal after treatment of those mice with combination of IL-3 and GM-CSF cytokines. All the experiments were done three repeats and results were calculated in average of all repeats ± SEM (**p < 0.01).

Fig 5: IFN-γ secretion in the blood serum separately collected from all group of mice showed induced level in ENU treated group in comparison to control group and came into normal level after cytokine treatment to ENU challenged group. All the experiments were done three repeats and results were calculated in average of all repeats ± SEM. ( **p < 0.01, *p < 0.05 )
4. Discussion

In animal leukemia model, induced by the carcinogen ENU [15], the classical role of macrophage is tumoricidal or antimicrobial. In disease condition, secondary infection is common and accordingly normal macrophages become non-specifically activated to fight against the leukemic cell or to protect the bacterial infection having proper proliferation and differentiation during disease state. PTK is one of the mediators of these processes. This signaling process is tightly regulated through tyrosine kinase and tyrosine phosphatase activity. Action of the PTKs is fundamental due to its conserved role in signal transduction pathway [1]. Protein tyrosine Kinase (PTK) has an important role in pathogenesis. Abnormal PTK activity and other factors induce acute myeloid leukemia [21]. In disease condition, especially in cancer and other proliferative disease, PTK activity alters and deregulated. Increased tyrosine phosphorylation is necessary for tumoricidal activity of macrophages [22]. Tyrosine kinases are normally under close-fitting control and have low basal activity; but they are activated briefly in response to specific stimuli [23]. Expressions of some Src PTK subtypes including Hck, Lyn, and Fgr are increased by numerous inflammatory stimuli, with lipopolysaccharide (LPS) and interferon-γ, in mature monocytes and macrophages [24]. Moreover, the skill of activated forms of Src family members to make DNA synthesis and cell proliferation indicates that activation of the wild-type kinases can stimulate pathways leading to cell proliferation. But in this study the low macrophage score in leukemic animal does not correspond to higher PTK activity. This may due to inhibitory effect of macrophage function or inhibitory effect of transformed oncogene products activity under the influence of carcinogen ENU. It may happen that altered signal selected which do not correspond to proliferating gene expression rather apoptotic gene expression. On the other hand, leukemic mice showed the higher concentration of IFN-γ level compared to control group. This may suggest that macrophage activation followed through induction of IFN-γ which up-regulate the PTK activity [22]. It was also reported that, tyrosine kinases and phosphatases regulate actin dynamics leading to macrophage chemotaxis and phagocytosis[25].

Recent study showed that, enhanced susceptibility to apoptotic death on exposure to the microbial and immune inflammatory signals bacterial LPS and interferon-gamma in vitro in bruton’s tyrosine kinase (Btk)-knockout macrophages [26]. Furthermore, a combination of LPS and IFN-γ induced expression of Hck and Lyn in murine bone marrow-derived macrophages [24]. These earlier studies established that the expression of Src family PTKs could be encouraged by inflammatory stimulations in monocytes and macrophages. More recent studies demonstrate that the Src PTK activities are also regulated during inflammatory responses. From this hypothesis we may suggest that increase IFN-γ level in serum upregulates the PTK activity of macrophages in leukemic mice.

In shortly, increase IFN-γ levels in blood serum of leukemic mice enhances the binding of IFN-γ with its receptor, then IFN-γ receptor activation induces protein-tyrosine kinases of the JAK family. Through JAK-STAT pathway activated STAT moves towards the nucleus to activate the transcription of IFN-inducible gene expression associated with apoptosis, cell cycle arrest or cell proliferation [27]. Recent reports suggest that over activation of PTK can leads the cells towards its apoptosis [23,26]. In contrary, our result showed less number of macrophage cells in leukemic mice after isolation compared to normal mice may be due to apoptosis of macrophage in connection with tumor-effector interaction or may be due to altered oncogenic products under the influence of carcinogen ENU. IL-3 and GM-CSF are similarly responsible for differentiation and proliferation of hematopoietic stem cells of myeloid lineage. In our result, the treatment of both cytokines leukemic mice showed decrease PTK activity but significantly higher number of macrophage count.

5. Conclusion

In animal leukemia model induced by carcinogen ENU, macrophage number was increased significantly with the treatment of IL-3 and GM-CSF which was significantly reduced in leukemia. PTK activity of macrophages from leukemic animal increased significantly may be due to increase the macrophage activation process for either the tumor killing or inflammation or killing bacterial infection or due to higher IFN-γ level.

Conflict of interest

The authors declare no conflict of interest for this study.

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