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Alteration of classical and hematopoiesis specific p53 pathway in the bone marrow hematopoietic stem/progenitor compartment facilitates leukemia progression in experimental mice

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ABSTRACT

Downregulation of p53 is associated with most of the neoplasms, however it claims additional significance for hematopoietic malignancy due to its supplementary role during hematopoiesis. Apart from the classical role as tumor suppressor, p53 during steady state hematopoiesis is associated with the maintenance of quiescent cell population in bone marrow by upregulating necdin (Ndn) and Gfi-1. We felt, it is necessary to delineate its attribution towards malignant conversion of hematopoietic system during leukemogenesis from all the possible angles. The present study deals with the characterization of *N-N′* Ethylnitrosourea (ENU) induced mouse model of leukemia by peripheral blood hemogram, bone marrow cytology, histology, cytochemical staining (MPO) and scanning electron microscopic study. We further investigated the alteration of conventional and hematopoiesis specific p53 pathways by flowcytometric expressional analysis of ATM, Chk-2, p53, p21, Ndn, Gfi-1 and Tie-2. The disruption of classical p53 pathway was observed in leukemic hematopoietic stem/progenitor population which involved downregulation of ATM, Chk-2, p53 and p21. Moreover, the expressional decline of Ndn and Gfi-1 hinted towards the mechanism of hindrance of hematopoietic quiescency in leukemic bone marrow. Increased expression of Tie-2 due to reverse correlation with p53 was found to be responsible for pathological angiogenesis in bone marrow together with increased blast burden in bone marrow during leukemia. The study presents the mechanistic scenario of the alteration of both classical as well as hematopoiesis specific p53 pathways in HSPC compartment triggering leukemic pathophysiology.

1. Introduction

Bone marrow (BM) acts as the major post natal repertoire of hematopoietic stem and progenitor cell (HSPC) population for the production of different blood cell lineages, a process referred to as hematopoiesis [26]. The dynamic process is orchestrated by the complex cross talk of stem-progenitor cells with highly specialized niche or microenvironment comprised of mesenchymal derivatives viz; stromal fibroblasts, osteoblasts, multilaminar cells etc. [38,10,40]. Abnormalities in the normal programme of blood cell formation may lead to various malignant and non-malignant pathophysiological conditions such as leukemia, myelodysplasia, aplastic anemia etc. [18,7,6]. Leukemia is the most common hematopoietic disorder that involves malignant transformation of hematopoietic stem/progenitor cells (HSPCs) under the support of altered microenvironment [3]. Pathophysiological changes of hematopoietic machineries during leukemia results into the overshoot of abnormal immature population in bone marrow as well as in peripheral circulation at the cost of proper functional cells [20,33,34].

Like other malignancies, leukemia also involves aberrant cell cycle checkpoint regulation resulting in the hyperproliferation of hematopoietic cells [30,4,15]. Our previous work demonstrated the reduction of hematopoietic quiescency during leukemia [7]. Neoplastic transformation of cells in most of the tumor is found to be associated with the expressional modulation of tumor suppressors viz; p53, check point kinases (Chk), ataxia telangiectasia mutated (ATM) etc. [31]. In normal cells, the coordinated action of the mentioned molecules in stressed condition is often associated with limiting the cellular proliferation and triggering apoptosis. Following the accumulation of DNA damaging stress in cells, sequential activation of ATM and Chk-2 leads to the up-regulation of p53

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which restricts cell division as well as eliminates them by apoptosis [5,13,32]. For the purpose, p53 often rely on classical cell cycle inhibitor of wif/kip family, the p21 [42,43,11,9]. Apart from this general role, p53 pathway has some extra significance for hematopoietic system. Recent studies established the role of p53 behind the maintenance of hematopoietic quiescency in bone marrow during steady state hematopoiesis by promoting transcriptional up-regulation of two downstream players: Necdin (Ndn) and Gfi-1 [24,23]. Due to constitutive down-regulation of p53 pathway in neoplastic condition, proliferation of carcinogenic stress assaulted cells fails to be restricted and instead they go on producing more and more abnormal clones resulting and disrupt the normal physiology [14,22,44]. Alteration of general p53 pathway is well established in most of the tumors but due to additional significance of p53 for hematopoietic system, its modulation particularly in case of hematopoietic malignancy deserves more importance and still remained as a less discussed issue.

The present study holds mechanistic intervention about the alteration of general as well as hematopoisis specific role of p53 pathways in HSPC rich marrow compartment of ENU-induced leukemic mice. Moreover, the work aimed to unearth the correlation between p53 alteration and increased blast burden in leukemic marrow. Understanding the conventional as well as non-conventional mechanisms of leukemogenesis by p53 modulation may pave the way for developing new bone marrow response modifying strategies for the disease concerned.

2. Materials and methods

2.1. Animal maintenance

Inbred Swiss albino mice (*Mus musculus*) were maintained at the animal house of Calcutta School of Tropical Medicine abiding by the regulations of Institutional Animal Ethical Committee (IAEC). Animals were kept in sterile condition, with proper temperature ($22 \pm 2\,^{\circ}C$), humidity and 12 h light and dark cycle. Maximally six animals were kept in each cage and provided with proper diet and water *ad libitum* throughout the experimental period.

2.2. Disease induction and animal grouping

For the experimental purpose two groups of animals (60 animals in each group) were maintained:

Group-I: Litter pups of 10–14 days weighing 4–5 gm were challenged with intra-peritoneal injection of ENU, a potent carcinogen, at a dose of 80 mg/kg body weight for developing leukemic condition in 6–8 months as confirmed by peripheral blood hemogram [19,2,3,6].

Group-II: Control group of mice received equal volume of saline in similar condition.

2.3. Histopathological preparation of bone marrow

For histological preparation of BM, long bones were dissected out from leukemic as well as control groups and fixed in 10% buffered formalin for 24 h. Bones were decalcified by 24 h treatment with 10% formic acid and then gradually passed through ascending alcohol gradations for dehydration and finally embedded in paraffin. From the paraffin blocks 5 μm thick histological sections were cut and processed routinely for routine hematoxylin and eosin (HE) staining and observed under light microscope (Olympus, Japan).

2.4. Bone marrow isolation and single cell preparation

Bone marrow was flushed out from dissected long bones into RPMI-1640 (Sigma, USA) media using 26 gauge sterile needles. A part of the marrow was kept intact and rest were made into single cells by constant repeat pipetting and washed several times in media for removing debris. The suspension was finally passed through 100 μm cell strainer.

2.5. Bone marrow smears study

BM smears were prepared from the intact marrow by conventional method and stained with Giemsa for observation under light microscope (Olympus, Japan).

2.6. Myeloperoxidase (MPO) staining of bone marrow smears

Bone marrow smears were fixed in Buffered Formal Acetone and thereafter treated with Sorenson buffer (pH 7.4) containing 3-3\ diaminobenzidine (DAB) for 20–25 min. Smears were counterstained with Geimsa and photographed under light microscope (Olympus, Japan).

2.7. Scanning electron microscopy of bone marrow

Pieces of bone marrow tissue from both control and leukemic groups were subjected to overnight fixation in 2.5% gluteraldehyde. This was followed by the dehydration of the samples by passing through ascending grades of alcohol (30%, 50%, 70%, 90% and 100%). Finally the tissues were dried, coated with gold in vacuum and subjected to scanning electron microscopy (Zeiss, Germany).

2.8. Flowcytometric analysis

For flowcytometric study, the prepared bone marrow single cell suspension from both control and leukemic groups were fixed in 1.5% paraformaldehyde for 15 min in dark at 37 °C. Cells were then washed twice with phosphate buffered saline (PBS) and divided into two parts. One part of the non-permeablized cells was directly suspended in FACS fluid to detect the surface proteins (Becton-Dickinson, USA). Another part was treated with 90% chilled methanol for membrane permeablization at 4 °C for 20 min, then washed thoroughly with PBS and suspended in FACS fluid for the detection of intracellular signaling molecules (Becton-Dickinson, USA). From the suspension of non-permeabilized cells, 2×10^6 cells/ml were collected in respective sorting tubes, and incubated for 30 min with 2 μl of PE-tagged anti-Tie-2 antibody (Abcam, UK). Permeabilised cell populations from both the groups were divided into six FACS tubes each containing 2×10^6 cells/ml. 2 μl of antibodies against ATM (Cell signaling Technology, USA), Chk-2 (Biolegend, USA), p53 (BD bioscience, USA), p21 (Cell signaling Technology, USA), Ndn (Santa Cruze Biotechnology, USA), Gfi-1 (Santa Cruze Biotechnology, USA) were then added to respective tubes and incubated at 37 °C for 30 min. This was followed by the addition of respective secondary antibodies tagged with AlexaFluor-488 (Invitrogen, USA) and incubation for 30 min at 37 °C in dark. Finally the expressional analyses of respective proteins were done in bone marrow cells (10,000 events) by BD-FACS Calibur (Becton-Dickinson, USA) using Cell Quest Pro software (Becton-Dickinson, USA). Next, histogram overlay analysis was performed to represent the comparative expression of the aforesaid proteins on the basis of a low SSC/low FSC virtual gate drawn on the flowcytometry scatterogram which is reported to be enriched with Sca-1 positive primitive hematopoietic stem/progenitor cells [27,7,8].

2.9. Statistical analysis

All the quantitative mean values were subjected to unpaired Student's *T*-test and probabilities of $P < 0.05$ and $P < 0.0001$ were