European Journal of Cell Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Research paper

Epigenetic and microenvironmental alterations in bone marrow associated with ROS in experimental aplastic anemia

Ritam Chatterjee, Sujata Law*

Stem Cell Research and Application Unit, Department of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine, 108, C.R Avenue, Kolkata, 700073, West Bengal, India, India

ARTICLE INFO

Keywords:
Aplastic anemia
Hematopoiesis
Hematopoietic stem/progenitor cells
Niche
ROS
Epigenetics

ABSTRACT

Aplastic anemia or bone marrow failure often develops as an effect of chemotherapeutic drug application for the treatment of various pathophysiological conditions including cancer. The long-term bone marrow injury affects the basic hematopoietic population including hematopoietic stem/progenitor cells (HSPCs). The present study aimed in unearthing the underlying mechanisms of chemotherapeutics mediated bone marrow aplasia with special focus on altered redox status and associated effects on hematopoietic microenvironment and epigenetic status of hematopoietic cells. The study involves the development of busulfan and cyclophosphamide mediated mouse model for aplastic anemia, characterization of the disease with blood and marrow analysis, cytochemical examinations of bone marrow, flowcytometric analysis of hematopoietic population and microenvironmental components, determination of ROS generation, apoptosis profiling, expressional studies of Notch-1 signaling cascade molecules, investigation of epigenetic modifications including global CpG methylation of DNA, phosphorylation of histone-3 with their effects on bone marrow kinetics and expressional analysis of the anti-oxidative molecules viz; SOD-2 and Sdf-1. Severe hematopoietic catastrophic condition was observed during aplastic anemia which involved peripheral blood pancytopenia, marrow hypocellularity and decreased hematopoietic stem/progenitor population. Generation of ROS was found to play a central role in the cellular devastation in aplastic marrow which on one hand can be correlated with the destruction of hematopoiesis supportive niche components and alteration of vital Notch-1 signaling and on other hand was found to be associated with the epigenetic chromatin modifications viz; global DNA CpG hypo-methylation, histone-3 phosphorylation promoting cellular apoptosis. Decline of anti-oxidant components viz; Sdf-1 and SOD-2 hinted towards the irreversible nature of the oxidative damage during marrow aplasia. Collectively, the findings hinted towards the mechanistic correlation among ROS generation, microenvironmental impairment and epigenetic alterations that led to hematopoietic catastrophe under aplastic stress. The findings may potentiate successful therapeutic strategy development for the dreadful condition concerned.

1. Introduction

Long term and short term hematopoietic stem cells (HSCs) are at the top of the hematopoietic hierarchy which has the ability of self-renewal, proliferation and differentiation into different lineages of blood cells passing through various transient progenitor stages (Mikkola and Orkin, 2006; Weissman et al., 2001). Hematopoietic disruption due to bone marrow injury is a common late effect of chemotherapeutic tumor management which involves the development of myelosuppressive condition leading to aplastic anemia (Mauch et al., 1995; Testa et al., 1984; Wang et al., 2006; Shao et al., 2013; Young, 1988; Scatena et al., 2010; Bowcock et al., 1989; Shepherd et al., 1994).

Under homeostatic condition the osteoblastic niche, adjacent to endosteal bone surface provides a special environment to the HSCs that facilitates self-renewal (Shao et al., 2013; Guerrouahen et al., 2011; Zhang et al., 2003; Calvi et al., 2003a). The intricate-interactions promote self renewal of hematopoietic cells in a part by keeping them quiescent as due to low metabolic activities HSCs produces less reactive oxygen species (ROS) which would otherwise cause oxidative damage to hematopoietic mass (Takubo et al., 2010). Moreover, endosteal osteoblastic niche is mostly hypoxic as it is relatively remote from blood flow (Winkler et al., 2010). Increase of ROS can cause oxidative damage to HSC population impairing self-renewal ability and inducing HSC senescence which promotes premature exhaustion of HSC leading to long term bone marrow suppression (Shao et al., 2013; Wang et al., 2010; Ito et al., 2006, 2004). Apart from affecting basic hematopoietic

* Corresponding author at: Department of Biochemistry and Medical Biotechnology, Calcutta School of Tropical Medicine, Kolkata-700073, India.
E-mail address: msuj2002@yahoo.co.in (S. Law).

https://doi.org/10.1016/j.ejcb.2017.11.003
Received 5 September 2017; Received in revised form 4 November 2017; Accepted 20 November 2017

population, redox imbalance in bone marrow is also reported to have negative impact on hematopoiesis supportive microenvironment as well (Mangialardi et al., 2014; Khatri et al., 2016). In addition to the stem-stromal disruption in bone marrow, ROS has the generalized property of altering epigenetic regulation over cell. On one hand, ROS is associated with global CpG hypomethylation of DNA and on the other hand can facilitate aberrant histone-3 (H3) phosphorylation that leads to apoptotic elimination of cell (Wu and Ni, 2015; Tikoo et al., 2001). It is a well established fact that cellular exposure to certain stresses viz; ionizing radiation or chemotherapeutic agents results in the increase in ROS production and this initial oxidative stress not only produces immediate damage to cells, but more importantly perturbs cellular metabolism that disturbs the normal oxidation/reduction (redox) reactions, leading to a persistent and prolonged elevation in ROS production (Shao et al., 2013; Balaban et al., 2005; Richardson et al., 2015). Our present study aims in establishing the correlation between the alteration of bone marrow redox status and the shift of stem-stromal equilibrium as well as epigenetic modification in hematopoietic stem/progenitor cell (HSPC) compartment during chemotherapeutic drug induced bone marrow aplasia.

Mouse model can be a powerful tool to screen the underlying complexities of drug mediated aplastic anemia. Alkylating drugs viz; busulfan (BU), cyclophosphamide (CP) are used to develop the hematopoietic catastrophic condition in experimental animals for decades (Morley and Blake, 1974; Fried et al., 1977; Ottolander et al., 1982; Boyd et al., 1986; Gibson et al., 2003a; Gibson et al., 2003b; Chen, 2005; Chatterjee et al., 2008; Chen et al., 2014; Chatterjee et al., 2016a). BU and CP mediated aplastic anemic condition in mice recapitulates many features of chemotherapy mediated long term marrow failure in human (Chatterjee et al., 2016a; Chatterjee et al., 2009; Chatterjee et al., 2010). In the present study, the novel mouse model was used for the extrapolation of the mentioned mechanistic interventions.

2. Materials and methods

2.1. Animals

Healthy, inbred Swiss albino mice weighing 20–25 gm, aged 8 weeks of both the sexes were maintained in the animal house of the Calcutta School of Tropical Medicine in proper conditions (12 h light–dark cycle, 22 ± 2 °C temperatures, and humidity) and provided with standard food and water *ad libitum*. Animal maintenance and all the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) of Calcutta School of Tropical Medicine.

2.2. Induction of aplastic anemia

Use of chemotherapeutic drugs like busulfan and cyclophosphamide is in practice to develop mouse model of aplastic anemia for decades (Morley and Blake, 1974; Boyd et al., 1986; Gibson et al., 2003a; Gibson et al., 2003b; Chen, 2005; Chatterjee et al., 2008; Chen et al., 2014; Chatterjee et al., 2016a; Chatterjee et al., 2009; Chatterjee et al., 2010; Chatterjee et al., 2016b). In our experimental study, mice were divided into control and aplastic anemia groups each containing 30 animals. Aplastic anemia was induced in mice by i.p. injections of two doses each of busulfan (20 mg kg^{-1} b.w.) and cyclophosphamide (80 mg kg^{-1} b.w.) at an interval of 28 days. The animals were kept for three months post second dose for the development of long term marrow injury condition as per the protocol of Chatterjee et al., 2016a. Control group of mice received comparable volume of aqueous saline solution. (Fig.1A–C)

2.3. Peripheral blood hemogram

Total count and differential count of blood cells were done following the methodologies of Pereira and Law, 2017. In brief, 200 µl of blood was collected in heparinized vial by tail vein puncture from both the control and experimental groups of mice. Total RBC and WBC counts were determined by standard laboratory techniques using hemocytometer chamber (Rohem, India). Reticulocyte count was determined by brilliant Cresyl blue staining. Estimation of hemoglobin content was done by colorimetric method using Drabkin's reagent (Stanbio Reagent, India). Differential leukocyte counts were determined by the analysis of blood films after Leishman staining.

2.4. Bone marrow histology

Histological preparation of bone marrow was done as described previously (Chatterjee et al., 2016c). In brief, long bones were collected immediately after the sacrifice of mice, fixed with 10% buffered formalin, decalcified with 10% formic acid, dehydrated with ascending grades of alcohol, embedded in paraffin, cut into 5 µm thick sections and finally subjected to routine H & E staining. Stained tissue sections from both the groups of mice were analysed by light microscopic observations. Tissues where < 30% of intertrabecular space remained occupied by hematopoietic cells were considered to be hypo-cellular (Kong et al., 2013). Tissue sections were also subjected to Mallory's trichrome staining following the protocol of Chatterjee et al., 2016b; Humason, 1979.

2.5. Marrow isolation and single cell preparation

Marrow cells flushed out from the long bones of 6 mice in RPMI-1640 (Sigma, USA) using 26 gauge needled syringe were pooled and made into single cells by repeat pipetting. Cells were thoroughly washed in ice-cold media several times to remove the debris. Finally, the cell suspension was passed through 100 µm cell strainer.

2.6. Oil red O staining

Assessment of bone marrow adipocyte content in control and aplastic mice was done by Oil Red O staining according to the protocol used by Chattopadhyay et al., 2016a. In brief, air dried bone marrow smears from both the groups were subjected to 10% formaldehyde fixation for 10 min and thereafter stained with Oil Red O solution (Oil Red O: water; 3:2) for 15 min followed by the removal of excess stain by washing and counter staining with Harris Hematoxiline for microscopic analysis. Adipocytes were identified by red coloration.

2.7. Measurement of bone marrow ROS

ROS level in the bone marrow was measured by Dihydroethidium (DHE; Sigma Aldrich, USA) staining (Rothe and Valet, 1990). In brief, bone marrow cells at a density of 10^6cells/ml were treated with 20 µl of 30 mM DHE and incubated for 30 min in dark. The intensity of the resultant red fluorescence was determined by FACS analysis (BD-FACS Calibur, Cell Quest Pro software; BD Bioscience, USA).

2.8. Nuclear chromatin condensation study

To access the chromatin condensation status, 1×10^6 marrow cells from both the group of mice were transferred on glass slides, mounted with DAPI shield (Sigma Aldrich, USA) and examined under laser scanning confocal microscope (FV1200, Olympus). Observations of 20 random fields were taken into consideration in both the cases.

2.9. Global DNA CpG methylation analysis in HSPC population

Global DNA CpG methylation was evaluated by the classical method of staining cells with monoclonal antibody against 5-methylcytosine (Cell signaling Technology, USA) (Milutinovic et al., 2003). Briefly,