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Comparative Studies on the Antioxidant Enzymes in Bone Marrow Precursor Cells and Mature Rat Polymorphonuclear Leukocytes

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Abstract- Continuous blood cell production throughout the lifetime of an individual is ensured by the hematopoietic stem cells (HSCs), which are bone marrow cells that possess extensive self renewal capacity and ability to differentiate to all the blood cell lineages. The redox status of the bone marrow stroma is an important factor determining whether the HSCs would differentiate or remain quiescent. Lower levels of ROS (reactive oxygen species) are required to maintain HSC quiescence, whereas higher ROS levels drive the HSCs out of quiescence and reduces their self renewal capacity. The ROS levels in the bone marrow should therefore be regulated to maintain the self renewal capacity of HSCs. Endogenous antioxidants play an important role in regulation of the ROS levels. The present study was therefore undertaken to examine the changes in the cellular detoxifying defences against superoxide and H2O2 i.e. superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), in the precursor cells from the rat bone marrow and in the rat blood polymorphonuclear leukocytes. An increase in the Catalase and GPx activities in the precursor cells over the differentiated cells was seen. However, the SOD levels in the differentiated cells were greater than in the precursor cells. The levels of Nitric Oxide (NO)- an antioxidant and a regulator of superoxide generation- in the precursor cells were also found to be greater than those in the differentiated cells. The results of the present study thus indicate an up regulation of the endogenous antioxidants in hematopoietic precursor cells, which might be related to the maintenance of their self- renewal capacity.

Keywords: *bone marrow precursor cells, polymorphonuclear leukocytes, antioxidants, nitric oxide.*

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Abstract- Continuous blood cell production throughout the lifetime of an individual is ensured by the hematopoietic stem cells (HSCs), which are bone marrow cells that possess extensive self renewal capacity and ability to differentiate to all the blood cell lineages. The redox status of the bone marrow stroma is an important factor determining whether the HSCs would differentiate or remain quiescent. Lower levels of ROS (reactive oxygen species) are required to maintain HSC quiescence, whereas higher ROS levels drive the HSCs out of quiescence and reduces their self renewal capacity. The ROS levels in the bone marrow should therefore be regulated to maintain the self renewal capacity of HSCs. Endogenous antioxidants play an important role in regulation of the ROS levels. The present study was therefore undertaken to examine the changes in the cellular detoxifying defences against superoxide and H_2O_2 i.e. superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), in the precursor cells from the rat bone marrow and in the rat blood polymorphonuclear leukocytes. An increase in the Catalase and GPx activities in the precursor cells over the differentiated cells was seen. However, the SOD levels in the differentiated cells were greater than in the precursor cells. The levels of Nitric Oxide (NO)- an antioxidant and a regulator of superoxide generation- in the precursor cells were also found to be greater than those in the differentiated cells. The results of the present study thus indicate an up regulation of the endogenous antioxidants in hematopoietic precursor cells, which might be related to the maintenance of their self-renewal capacity.

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Abbrebiations:
Ros Reactive Oxygen Species
Nos Nitrogen Oxygen Species
GR Glutathione reductase
SOD Superoxide Dismutase

I. Introduction

The metabolic activities have variant degree of differences on the basis of tissue or organ where they are expressed, which leds to differences in their energy requirement. ROS are implicated in expression of several diseases affecting tissues and systems. ROS are mainly Free radicals, considered to have atoms having lone pair of electrons, which is formed via breaking of covalent bond [1]. Ros can be categorized in two broad groups Oxygen derived free radicles and Nitrogen derived free radicals. These reactive entities are produced outside as well as inside of cell and cell organelles, such as mitochondria [2]. Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) produced via the enzymatic activity of nitric oxide synthase (NOS) and NADPH oxidase respectively. Nitrosative stress is one of the mechanism where (ROS) and (RNS) works together to damage microbial cell. Therefore, these two species are often collectively referred to as ROS/RNS. Functions of various aminoacids can be modified by ROS that may leads to inappropriate actions of proteins like dimerization and interaction with Fe-S [3]. It may also causes impairment of cell function and development of morbit condition [4]. Inspite of all the anomiles it causes studies have also showed that ROS are required in a certain amount to maintain proper functioning of signalling pathways and metabolism [5]. Change in redox state by these reactive species might be responsible for proper defferentation of cell. Changes in the redox balance during differentiation appear to be due to an increase in the rate of O_2^- generation. The effective concentration of oxidants depends on cellular levels of antioxidants-molecules which counteract the oxidants the cells possess several antioxidant enzymes such as superoxide dismutase (which reduces $O_2^{-\cdot}$ to H_2O_2), catalase, and glutathione peroxidase (which reduces H_2O_2 to H_2O). In current work we have indirectly investigated the role of ROS and RNS via doing comparative analysis of antioxidant enzymes in the precursor cells of bone marrow and differentiated cells of blodod. Since ROS and RNS play a role in cellular proliferation and differentiation during hematopoiesis, our aim was to compare the level of antioxidant enzymes in the precursor cells of bone marrow and the differentiated cell of blood.

II. Materials and Methods

a) Experimental animal

Closed-colony-bred male rats, 4-6 weeks of age and weighing 80-100 g were used. They were kept in plastic cages with paddy husk bedding in a temperature between 25-28° C.

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b) Animal sacrifice and sample collection

i. *Isolation of PMNs from Blood*

Isolation of PMNs was carried out by the method of Boyum, 1976[6], Rat blood was collected under ether anaesthesia by cardiac puncture in sodium citrate (0.129 M, pH 6.5, 9:1 v/v). Blood was centrifuged at 1500 rpm for 20 min at 20°C and the buffy coat was carefully layered onto HiSep gradient and centrifiuged at 2,000 rpm for 20 min at 20°C .The leukocyte rich layer was transferred to a fresh tube and centrifuged at 2,000g for 20 min at 20°C . Pellet was washed and suspended in 2 ml PBS {Composition (mM): NaCl 138; KCl 2.7; Na_2HPO_4 8.1; KH_2PO_4 1.5; pH 7.4]. PMNs were counted in Neubaur's chamber and the viability of the cells was determined by the trypan blue dye exclusion test.

ii. *Isolation of Precursor Cells from Rat Bone Marrow (BMCs)*

Both the femurs were surgically removed. Ends of the femur bones were snipped open with fine scissors. A 23-gauge needle fitted on a 5 ml sterile plastic disposable syringe (Dispovan, Hindustan Syringes & Medical Devices, Faridabad, India) containing 2 ml of Dulbecco's Modified Eagles Medium (DMEM, GIBCO, USA) was inserted inside the femur from one end and its content was forcefully ejected into the femoral cavity. As a result, the marrow plug was flushed from the femoral shaft. The sample was collected in graduated plastic centrifuge tubes. The procedure was repeated two times to ensure collection of all the cells from the femur. Precursors from bone marrow were isolated according to method described by [7].Following sedimentation of debris (5–10 min), the clear supernatant containing leukocytes was removed and the cells were recovered by centrifugation (2000rpm for 10 min at room temperature). The pellet was washed and then resuspended in PBS. Cell counting was done using the Neubaur's chamber and the viability of the cells was determined using the trypan blue dye exclusion test.

iii. *Isolation of Platelets*

The platelets were obtained by centrifuging whole blood at 1,500 rpm for 20 min at 20°C. The platelet rich plasma so obtained was used as the source of platelets. Cell counting was done using the Neubaur's chamber and the viability of the cells was determined using the trypan blue dye exclusion test.

iv. *Nitrite Estimation*

Nitrite content in the rat platelets, neutrophils and precursors (BMCs) was measured by using the Griess reagent. Hypotonic lysis of the cells was carried out for 5 mins on ice followed by centrifugation at 2,000rpm for 20 min at 4°C. The supernatant thus obtained was used for nitrite estimations using the Griess reagent (1% sulphanilamide, 0.1% N-1 naphthylethylene diamine and 2.5% orthophosphoric acid. Briefly, the samples were treated with Griess reagent and incubated for 30 min at 37°C. Concentration of nitrite was estimated by measuring the absorbance at 548 nm using sodium nitrite as standard [8].

v. *Myeloperoxidase Activity*

MPO activity was evaluated following the method of Pember *et al*, 1983[9],Neutrophils/ BMCs were freeze–thawed consecutively for three times and then they were hypotonically lysed for 5 mins. Cetyltrimethyl ammonium bromide (0.3%) was incubated with the cell lysate at 37°C for 10 min followed by centrifugation at 3,000rpm for 20 min at 20°C. Supernatant was taken for evaluation of enzymatic activity. Enzyme kinetics was run for 3 min at 15 s intervals using TMB (tetra methyl benzidine 0.5%) and H_2O_2 (0.2μM) at 37°C in the presence of 1.0 M citrate buffer pH 6.0). One unit of enzyme activity is defined as the amount of enzyme which causes an increase in absorbance of TMB by 0.4 in 2 mins.

c) Antioxidant enzyme Assays

i. *Superoxide Dismutase (SOD)*

The activity of superoxide dismutase (SOD) was assayed by the method of Kakkar, *et al*, 1984 [10].Briefly the assay mixture consisted of sodium pyrophosphate buffer (0.052 M, pH 8.3), phenazine methosulphate (6.5μM), NBT (30μM), NADH (52μM) and lysed neutrophil , BMC or platelet supernatant. SOD activity was estimated as the inhibition of chromogen formation due to NADH mediated PMS dependent reduction of NBT and is expressed in terms of U/ml/10^6 cells. One unit of enzyme is defined as the amount of enzyme, which causes 50% inhibition of chromogen formation under the experimental conditions.

ii. *Catalase*

Catalase enzyme activity was estimated by the method of Aebi *et al*, 1974 [11]Briefly 100μl of the lysed neutrophil, BMC or platelet supernatant was added to 2.9 ml of buffered substrate (50mM phosphate buffer, pH 7.0 containing 10 mM H_2O_2). The decrease in absorbance was monitored at 37°C for 3mins at intervals of 15 secs. The activity was calculated using the coefficient for H_2O_2 at 240 nm (0.041 $M^{-1}cm^{-1}$) and is reported as μM of H_2O_2 utilized per minute per 10^6 cells.

iii. *Glutathione Reductase (GR)*

The activity of glutathione reductase in the neutrophil, BMC, or platelet lysate was carried out by the method of Sheokand *et al*., 2008 [12]Incubation mixture for GR enzyme assay consisted of 0.1 M phosphate buffer (pH 7.5), 5 mM oxidized glutathione (GSSG), 0.2 mM NADPH and 100 μL enzyme extract in a final volume of 1.5 mL. Addition of GSSG, initiated the enzyme reaction. The decrease in absorbance at 340 nm due to oxidation of NADPH was monitored. The enzyme activity was calculated by using the extinction coefficient value

of 6.2 mM^{-1} cm^{-1} for NADPH. One unit of enzyme activity was equivalent to one nmol of NADPH oxidised during the reaction.

III. Results

a) Catalase

In bone marrow cells amount of enzyme activity is 0.933 Unit/10^6 cells and in neutrophils it is 0.365 Unit/10^6 cells and 0.232 Unit of enzyme/10^6cells in platelets. The finding shows that the activity of catalase is much more in the precursor cells and it decreases in differentiated cells.

b) Nitrite (stable end product of nitric oxide synthase)

Enzyme activity in bone marrow cells is 0.509, which is decreasing in neutrophil and platelets as 0.112 and 0.009 μM/ 10^6 cells.

c) Myeloperoxidase

In case of bone marrow cells myeloperoxidase activity is 1.6894 units/10^6 cells and in neutrophil it is 1.0717 units/10^6 cells. Amount of enzyme activity is decreasing in mature cells.

d) Superoxide dismutase

Superoxide dismutase activity in bone marrow cells was 0.071units/10^6 cells and in neutrophil it was 0.104 units/10^6 cell. But in platelet the level again decreased to 0.004 units/10^6 cells.

e) Glutathione reductase

Activity of enzyme in bone marrow cell is 0.066 and it is 0.0001 and 0.0003 in neutrophil and platelets respectively. Enzyme activity of glutathione reductase is decreasing in mature cells.

IV. Discussion

This study has demonstrated a comparison of the antioxidant enzymes in precursor cells of bone marrow and the differentiated neutrophils and platelets. The level of the antioxidants like catalase, nitric oxide, and glutathione was greater in the precursor cells than in the differentiated cells. In contrast, superoxide dismutase level was much elevated in the differentiated neutrophils than in the precursor bone marrow cell population. The difference in the antioxidant profiling can be attributed to the varying ROS and RNS concentration in the bone marrow microenvironment where precursor cells reside. Hematopoietic stem cells in bone marrow are maintained in the quiescent stage in hypoxic condition in the osteoblast niche [13] whereas in the relatively more oxygenic vascular niche, due to the proximity to blood circulation, stem cells actively proliferate and differentiate [14,15,16]. Which might increase the intracellular ROS level [17] However, increased ROS may result in accumulation of DNA damage and unscheduled activation of senescence mechanisms in the stem cell compartment in the long term. Unregulated accumulation of ROS in hematopoietic stem cells leads to abnormal hematopoiesis (Yalcin *et al*, 2008) [18]. Thus, tight regulation of oxidative stress in hematopoietic stem cells is essential for normal control of homeostasis in hematopoietic tissues and this may be obtained by regulating the level of antioxidant enzymes. Catalase enzyme is compartmentalized into small granules in both bone marrow precursor cells and peripheral blood neutrophils [19]. Myeloid, erythroid, and megakaryocytic cells all contain substantial amounts of catalase [20][21]. The number and size of the diaminobenzidine (DAB)-reactive catalase-containing compartments decreased with differentiation of immature myeloid, erythroid, and megakaryocytic cells progressed, [19][22]. This supports our finding of reduced catalase activity in mature peripheral blood neutrophils and platelets than in precursor cells of bone marrow. Similar trend of reduction was found for the level of nitric oxide which was measured in the form of nitrite level in bone marrow cells and peripheral blood neutrophils and platelets. The level was several times elevated in the bone marrow precursor cells probably because in the bone marrow it may have a role in normal and malignant hematopoietic cell differentiation. Earlier studies have also reported that NO can modulate cellular differentiation [23].The enzyme myeloperoxidase (MPO) is synthesized only in myeloid and monocytic cells, making it an important marker of the myeloid lineage. The elevated MPO level in bone marrow precursor cell from our present study is supported by the earlier finding that transcription of the MPO gene is turned on early during the myeloblast stage of myeloid differentiation and is turned off when myeloid precursors are induced to differentiate along any one of a number of pathways [24] Superoxide dismutase (SOD) is responsible for dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide. Catalase, the subsequent enzyme in this pathway, converts hydrogen peroxide into water and molecular oxygen. High levels of SOD1 activity in neutrophils as reported from our study, without a concomitant increase in catalase, could cause escalation of the Fenton reaction producing hydroxyl radical from hydrogen peroxide[25][26] H_2O_2 produces hydroxyl radicals ($OH^{\cdot}$) were generated by a Fenton reaction, involving an ADP-Fe^{2+} (or ATP-Fe^{2+}) complex. It has been reported that a portion of the $OH^{\cdot}$ free radicals derived from H_2O_2, produced by the action of SOD, play a role in differentiation [27] This may account for the increased SOD level in differentiated cells revealed from our study. Our study also has shown a decline in the glutathione reductase activity in the peripheral blood neutrophils and platelets. Previous studies have reported that glutathione reductase activity decrease as erythroid cell differentiate [28] The higher level of the enzyme in bone marrow precursor cell indicates the greater importance of this enzyme in the precursor cell.

V. Conclusion

Cellular oxidants, called reactive oxygen species (ROS), are constantly produced in animal and human cells. Excessive ROS can induce oxidative damage in cell constituents and promote a number of degenerative diseases and aging. Cellular antioxidants protect against the damaging effects of ROS. However, in moderate concentrations, ROS are necessary for a number of protective reactions. ROS are essential mediators of antimicrobial phagocytosis, detoxification reactions carried out by the cytochrome P-450 complex, cellular differentiation and apoptosis which eliminates cancerous and other life-threatening cells this effect of ROS is dose dependent. Endogenous antioxidants do not completely remove ROS in animal and human cells. This raises the question of why, despite the existence of a powerful cellular system of antioxidants, the short-living ROS are not removed entirely and are permanently present in cells. The reasonable explanation for this phenomenon is that continuously produced ROS are needed to perform some important biological functions .Seemingly, the cells are tuned to remove excessive ROS and to leave the required level of oxidants. Thus, an interply of oxidants and antioxidants decides the role of free radicals beneficial or deleterious. This depends on the basal concentration of free radicals and antioxidants in the cells. With this aim in mind, the present study was undertaken to find out the oxidant and the antioxidant status in precursor and differentiated cells. It was found that with the exception of SOD, basal level of all other antioxidants–catalase, NO and GR levels were higher in the precursor cells than in the differentiated cells, pointing towards the role of cellular antioxidants in maintaining the self-renewal characteristics of the bone marrow cells. Current study is undertaking the elaborative role of ROS in keeping the naive undifferentiated blood cell confined to that realm itself.On the contrary antioxidants are helping in differentiation of the blood cells.

Declaration of competing interest

The authors declare that they have no competing interests.

Contribution of authors

Swechha Mishra carried out all the experiment and also contributed in designing this whole experiment. Sonia Chadha, Sayali Mukherji, Sangeeta singh carried out formulation of the methods used in this experiment and also helped in drafting the manuscript via reviewing it.

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