Splenectomy Modulates the Erythrocyte Turnover and Basigin (CD147) Expression in Mice

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Abstract The present study was designed to study the splenectomy induced modulation of erythrocyte turnover in mice. We have also studied the modulation of reactive oxygen species (ROS) and basigin (CD147) expression level on erythrocytes in splenectomized condition. The erythrocyte turnover was studied by a newly developed double in vivo biotinylation (DIB) technique. This technique enables to discriminate three different age (young, intermediate and old) groups of erythrocytes. The expression level of ROS and CD147 was studied by staining with CM-H₂DCFDA stain and anti-mouse CD147 monoclonal antibody followed by flow cytometry. We observed that intermediate and old age groups of erythrocytes were randomly eliminated in splenectomized condition. A marked surge in the blood reticulocyte count was observed in splenectomized mice. Splenectomy induced the level of ROS and CD147 expression on erythrocytes. The expression level of ROS was induced up to 35 days, but it reversed to basal level by 42 days indicating the emergence of refractoriness to splenectomy. The CD147 expression was significantly higher on day 7, 21 and 28 but it also normalizes on later time points. We conclude that erythrocyte turnover is significantly modulated in splenectomized mice. The enhanced level of ROS and CD147

expression may be a possible cause to increase erythrocyte removal in splenectomized mice.

Keywords Erythrocyte · Splenectomy · Flow cytometry · Double in vivo biotinylation · Reactive oxygen species · Basigin

Introduction

Erythrocytes have a definite life span of 60 days in murine peripheral blood circulation. Approximately 2% erythrocytes are removed every day and are replaced with an equal number of fresh erythrocytes in mice [1]. Erythropoietic activity ensures that an equal number of new erythrocytes are added to blood circulation so that overall erythrocyte count in the blood remain constant [2]. During its survival erythrocytes travel hundreds of miles and are subjected to oxidant and osmotic stress with a limited repair mechanism to maintain itself. Under normal condition 80–90% of normal erythrocyte destruction activity is considered to be extravascular that takes place within tissue-resident macrophages of the spleen and to a lesser extent in the liver and bone marrow [3, 4]. Erythrocytes are phagocytosed at the rate of 2×10^6 per second by the macrophages. Each macrophage of spleen, liver and bone marrow phagocytose on average one erythrocyte per day [5]. The immunoglobulin M (IgM) mediated erythrocyte clearance occurs inside spleen and to a smaller extent in the liver. The IgG induced erythrophagocytosis occurs through FCYR and complement receptor by the kupffer cells in the liver [6]. Neutrophils and activated endothelial cells also phagocytose old or damaged erythrocytes [7, 8].

The 10–20% removal of erythrocytes occur intravascular and it has certain special features [9].

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CD147 or extracellular matrix metalloproteinase inducer (EMMPRIN) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. It is an adhesion molecule expressed on several cells like erythrocytes, leucocytes, endothelial cells and tumor cell lines [10]. On erythrocyte surface, CD147 acts as a determinant of the Ok blood grouping system and play a crucial role in the recirculation of mature erythrocytes from the spleen into circulation [11, 12]. CD147 is also an essential molecule for the invasion of *P. falciparum* into erythrocytes [13]. The over expression of CD147 has been reported in sickle cell disease and Myelodysplastic syndrome [14, 15]. The oxidative stress also leads to enhance expression of CD147 on erythroid cells in mice [16]. The expression level of CD147 decreases with the maturation of erythrocytes [17]. The CD147 interacts with monocarboxylate transporter-1 (MCT-1) and helps in lactate transport [18].

Spleen is also a primary erythropoietic organ in neonates and under acute anemic conditions [19]. It has also been shown that erythropoiesis switches to liver in splenectomized mice [20, 21]. Splenectomy is an effective therapy for the treatment of a number of diseases such as immune thrombocytopenic purpura (ITP) [22, 23], hereditary anemia, malignant tumors of spleen, chronic lymphoid leukemia (CLL), hereditary spherocytosis, hodgkin lymphomas, splenic myelofibrosis, sickle cell disease, myelodysplastic syndromes and in a number of abdominal traumas and splenic injuries [24, 25]. There are concerns regarding post-splenectomy infections and thrombotic complications. The scenario of erythrocyte turnover in asplenic condition has also not been clearly understood. In this study, we have analyzed the erythrocyte turnover in splenectomized condition by utilizing the newly developed double in vivo biotinylation (DIB) technique [26–28]. The DIB technique involves two steps of in vivo biotinylation of blood erythrocytes that results in categorization of blood erythrocytes into three discrete populations, biotin^{high} (old), biotin^{low} (intermediate) and biotin^{neg} (young) populations of erythrocytes. Using the DIB technique, we have studied the survival kinetics of blood erythrocytes as well as the kinetics of changes in ROS and CD147 expression on erythrocytes in splenectomized conditions.

Materials and Methods

Animals

All experiments were conducted on Inbred C57Bl/6 male mice (8–12 weeks old, 20–25 g body weight). Animals were maintained in a microbes free environment in the

animal house facility at Jawaharlal Nehru University (JNU), New Delhi or obtained from the National Institute of Nutrition, Hyderabad. The animals were housed in positive-pressure air conditioned units (25 °C, 50% relative humidity) and kept on a 12 h light/dark cycle. Water and mouse chow were provided *ad libitum*. All the experimental protocols were performed strictly in compliance with the Guidelines as notified by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate change, Government of India (CPCSEA guidelines, www.envfor.nic.in/divisions/awd/cpcsea_laboratory.pdf). The study was duly approved by the JNU Institutional Animal Ethics Committee (IAEC Approved Project Code: 5/2010).

Reagents and Other Supplies

Biotin-X-NHS Ester was from Calbiochem (La Jolla, CA). Streptavidin APC, Anti-mouse CD71-PE, anti-mouse IgG1κ-PE, anti-mouse CD147-FITC, anti-mouse IgG2bκ-APC, and anti-mouse IgG2bκ-FITC were procured from e-biosciences (San Diego, CA, USA). 5-(and 6) chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was purchased from molecular probes. Fetal bovine serum (FBS) was obtained from Hyclone (South Logan, UT). RPMI, HEPES, dimethylformamide (DMF), ketamine, xylazine and other reagents were from Sigma-Aldrich (India).

Splenectomy Procedure

Mice were anesthetized by an intra-peritoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). An incision was made in the skin and body muscles. The spleen vessels were tied and then spleen was excised off. The abdominal wall was sutured with nylon fiber and skin closed with wound clips. All mice were kept under a heating lamp until they came out of anesthesia. Seven days after the surgery, the wound clips were removed. Splenectomized mice were allowed to recover for 15 days before their use in experiments.

Double In Vivo Biotinylation (DIB) Technique

The DIB technique can be used to study the erythrocyte turnover and age related changes [26–28]. This technique is used for study the changes in cohort of erythrocytes that enters the blood circulation within a defined 5 days window. In DIB technique, the first step of high intensity biotinylation of all erythrocytes in circulation is carried out by administration of biotin-X-NHS ester (BXN) (three daily intravenous injection of 1 mg of biotin-X-NHS Ester

(BXN) dissolved in 20 µl of dimethylformamide (DMF) and 250 µl of phosphate buffered saline (PBS)). After 5-days rest, the new erythrocytes that entered the blood circulation after first biotinylation step were biotinylated by a low dose second biotinylation step. Mice were given 0.6 mg of BXN dissolved in 12 µl of DMF and 250 µl of PBS. At any time points after the second biotinylation step, the biotin intensity on circulating erythrocytes could be analyzed by staining the cells with streptavidin colored with fluorochrome followed by flow cytometry. Three different aged group of erythrocytes can be identified; Biotin^{negative} cell in circulation would represent the naive erythrocytes entered in blood after second biotinylation step. Biotin^{low} erythrocytes would represent the cohort of erythrocytes released in blood between the first and second biotinylation step. Biotin^{high} erythrocytes would represent the older population of erythrocytes that were present in the blood at the time of first biotinylation [26–28].

Measurement of Intracellular ROS

ROS levels were analyzed by staining with CM-H₂DCFDA stain. Briefly, erythrocytes were washed and suspended in pre-warmed PBS supplemented with 2% FBS and incubated with CM-H₂DCFDA stain (5 µM) in the dark for 30 min at 37 °C in an atmosphere of 5% CO₂ in air. The oxidative conversion of CM-H₂DCFDA to its fluorescent product by ROS was quantified immediately by Flow Cytometry [16, 29].

Measurement of Basigin (CD147) Expression

CD147 expression was analyzed by staining with anti CD147 monoclonal antibody. Erythrocytes were washed and suspended in pre-warmed PBS supplemented with 2% FBS and incubated with CD147FITC antibody for 20 min at 4 °C [26]. CD147 expression on erythrocytes was determined based on the intensity of the CD147 fluorescence intensity profiles. After incubation, cells were washed and analyzed on BD FACS Calibur flow cytometer using Cell Quest software for acquisition and analysis [16, 17].

Flow Cytometric Analysis

Mouse blood was collected in PBS in the presence of EDTA (5 mM/ml) and washed 3 times with ice cold normal saline containing HEPES buffer (pH 7.4) and 1% FBS. Biotin labeled erythrocytes were stained with streptavidin-APC antibody and analyzed on a flow cytometer as described before [26–28]. Reticulocyte proportions were estimated by staining with anti-transferrin (CD71) monoclonal antibody followed by flow cytometric analysis [29].

Statistical Analysis

Statistical analysis was carried out by using the Sigma plot software. All data were expressed as mean ± SEM. Standard t-tests were used to calculate the significant levels between groups. A *p* values less than 0.05 were considered significant.

Results

Modulation of Erythrocytes Turnover in Splenectomized Mice

Turnover of erythrocytes of different age groups in blood circulation in control and splenectomized mice was studied by using the DIB technique. In this technique a cohort of erythrocytes entering blood circulation within a defined window of 5 days can be tracked as it ages in blood circulation. This cohort is represented as erythrocytes subpopulation in box Y (biotin^{low} subpopulation in Fig. 1a (control mice) and b (splenectomized mice). Erythrocytes in box Z (biotin^{high} subpopulation of erythrocytes) represents the older aged erythrocytes. The erythrocytes in box X represents the (biotin^{negative} subpopulation of erythrocytes) younger erythrocytes of age lower than the tracked cohort of erythrocytes (Fig. 1a, b). Decline in biotin^{high} subpopulation from 61.08% in control to 53.80% (12% decline) in splenectomized mice after 14 days suggest that the older aged erythrocytes are removed at a much faster rate than control.

Time kinetics of changes in different age group of erythrocytes has been shown in Panel C and D. Our data suggest that a significant enhancement in relative proportions of young erythrocytes occurred during early time period of splenectomy that came down to normal at later time points. Proportion of young erythrocytes in splenectomized mice was significantly higher than control on day 2 (165% of control), 7(119%), 14 (109%) and 21 (109%) of observation (Fig. 1c). Moderate decline in relative proportions of old erythrocytes was seen on earlier time points that normalized on later time points. Percentage of old aged erythrocytes in blood circulation of splenectomized mice were significantly higher on day 35 (117% of control) and 42 (118%) of observation (Fig. 1d). These results suggest that the pattern of erythrocyte turnover is significantly altered in splenectomized mice.

Changes in Relative Proportions of Reticulocytes in Splenectomized Condition

Reticulocyte proportion in blood was estimated by staining with anti-mouse CD71 (transferrin receptor) monoclonal

Fig. 1 Modulation of erythrocytes turnover in splenectomized mice. Mice were labeled according to double in vivo biotinylation (DIB) protocol. Briefly, mice were administered intravenously three daily doses of 1 mg biotin-X-NHS Ester (BXN) (first biotinylation step). After 5 days rest, a single additional dose of 0.6 mg BXN was administered intravenously (second biotinylation step). Blood samples were collected at different time points (2, 7, 14, 21, 28, 35 and 42 days of second biotinylation step) and different aged groups of erythrocyte were estimated by distribution of biotin label on erythrocytes by staining the cells with streptavidin-APC and flow cytometric analysis. Box-Y in **a** and **b** contain erythrocytes that entered the circulation between the first and second biotinylation step

in control and splenectomized mice, respectively. The erythrocytes younger than Y are in box-X (biotin^{negative} population). The box-Z (biotin^{high} population) represents the erythrocytes population older than the population in box-Y. Dot-plot **a** and **b** shows the distribution of all three different aged groups of erythrocytes in control and splenectomized group after 14 days of second biotinylation step. **c** and **d** show the survival kinetics of biotin^{negative} (young aged population) and biotin^{high} (old aged populations) of erythrocytes in blood of control and splenectomized group. Values in parentheses represent percentage of cells. Data is represented as mean $\pm$ SEM; $n = 5$ in control and 5–6 in splenectomized groups. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$

antibodies. Reticulocytes express transferrin receptor that disappears with the maturation of reticulocytes to mature erythrocytes. Flow cytometric histograms in Fig. 2 suggest that reticulocytes proportion increased from 3.98% in control to 5.49% in splenectomized mice. Cumulative data obtained from six mice show that there was a significant enhancement in reticulocytes proportion on day 2 (1.5 fold) and 7 (1.2 fold) in splenectomized mice (Fig. 2c).

Random Destruction of Erythrocytes in Splenectomized Mice

It was of interest to determine whether the killing of circulating erythrocytes in splenectomized mice was random or was restricted to any specific age group of erythrocyte populations in circulation. DIB technique was used to track the age defined erythrocyte cohort in the blood circulation of control and splenectomized mice. Results in Fig. 3 show

that there was a progressive decline in DEC (biotin^{low} erythrocytes) population in splenectomized mice. Erythrocyte cohort that entered the blood circulation at zero time points decreases about 29% in 42 days in control mice (0.69% per day) as compared to splenectomized mice where the depletion was 44% (1.05% per day). These results suggest that intermediate aged erythrocytes seem to undergo significant elimination in splenectomized mice. These results clearly indicate that splenectomy alters the erythrocyte turnover and the random component of elimination of erythrocytes in splenectomized mice was increased.

Splenectomy Induces the Level of ROS Production and CD147 Expression

The expression levels of ROS and CD147 expression on erythrocytes were examined by staining with

Fig. 2 Changes in relative proportions of reticulocytes in splenectomized mice. Blood samples were collected at indicated time points and proportion of reticulocytes at different time points was determined by staining with the anti-mouse CD71 antibody followed by flow cytometric analysis. Histograms in **a** (control) and **b** (splenectomized) show the changes in proportions of reticulocytes after 2 days

of resting period. Box-X and Y in **a** and **b** represents erythrocytes and reticulocytes in control and splenectomized mice, respectively. Bar graph in **c** shows the cumulative changes in reticulocytes proportion at different time points. Data is represented as mean $\pm$ SEM; $n = 5$ in control and 6–8 in splenectomized group. * $p < 0.05$ and ** $p < 0.005$

CMH₂DCFDA and anti-mouse CD147 antibody. There was a significant increase in the ROS level in the erythrocytes in splenectomized mice. Representative histograms in Fig. 4a, b depicts that MFI of ROS production in control and splenectomized mice were 69.91 and 97.26, respectively. Time kinetics of ROS production have been shown in Fig. 4e. The ROS level was significantly higher (15% to 41% at different time points) on erythrocytes in splenectomized mice. The pattern of CD147 expression was almost similar to ROS accumulation. The MFI of CD147 staining was 131.25 in control mice that increased to 183.56 in splenectomized mice (Fig. 4b, d). Further, time kinetics of CD147 expression indicate that the level of CD147 expression was significantly enhanced (15–47%)

from day 2 to day 28 of observation (Fig. 4f). The expression level of CD147 also normalized on later time points in splenectomized mice.

Discussion

The primary aim of this study was to investigate whether the removal of circulating erythrocytes in splenectomized mice was random or restricted to any specific age group of erythrocyte populations in circulation. We have utilized the DIB technique to analyze the survival kinetics of different aged erythrocytes [26–28]. This technique provides an easy way to tag a cohort of erythrocytes generated during a

Fig. 3 Survival kinetics of intermediate aged erythrocytes cohort in splenectomized mice. Mice were labeled according to DIB protocol as described earlier. Blood samples were collected from DIB labeled splenectomized and control mice at different time points of second biotin dose. The proportion of erythrocytes cohort (biotin^{low} Intermediate aged erythrocytes) as percentage of all erythrocytes was determined after staining with streptavidin–APC followed by flow cytometric analysis. Proportions of biotin^{low} cohort of erythrocytes in blood for control and splenectomized are shown. Data is represented as mean $\pm$ SEM; n = 5 in control and 5–6 in splenectomized groups. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0005$

defined time window and then to assess the changes in this labeled population as it ages in circulation. Erythrocytes survive about 60 days in peripheral blood circulation and removed by the reticulo-endothelial system in spleen and liver. The survival kinetics of erythrocytes have shown that freshly released erythrocytes in blood are stable till 12 days of age and all aged groups of cells survive within this time period [17]. From day 12 to 40, erythrocytes are removed at an average rate of 1.3% per day. The removal rate further increases up to 2.3% from day 40 to 60 in normal conditions. Since spleen plays an important role in removal of older erythrocytes, it was interesting to study the kinetics of erythrocytes removal in splenectomized conditions. Our results show that there was a rapid turnover of erythrocytes in splenectomized condition; circulating erythrocytes are removed at a much faster rate in splenectomized mice than the control. We observed random destruction of erythrocytes and intermediate as well as old aged erythrocytes were randomly eliminated in splenectomized mice.

We observed a steep fall in intermediate aged erythrocytes proportions (20% lesser than control) on day 14 and day 21. The data suggest that the overall reduction in intermediate aged population in control and splenectomized mice was 29% (0.69% per day) and 44% (1.05% per day), respectively during the study period. The relative proportions of younger erythrocytes were increased in splenectomized mice. This increment is due to a marked compensatory efflux of reticulocytes that occurs in the splenectomized condition. These results clearly indicate that the resistivity of erythrocytes, a feature of young erythrocytes in control mice is lost in splenectomized condition. The random elimination of erythrocytes may be coupled with the fact that spleen not only remove senescent erythrocytes but also maintains the erythrocytes healthy [30]. The ROS is one of the major regulator of erythropoiesis and erythrocyte turnover [31]. The random loss of erythrocytes in blood circulation could be due to enhanced ROS production in asplenic mice. We estimated the ROS level on erythrocytes by staining with CMH₂DCFDA dye. Our results show that ROS production on erythrocytes was significantly enhanced in splenectomized condition. The increment in ROS coupled with the fact that erythrocytes are enucleated and lack strong self-repair mechanism. The enhanced ROS level could be a possible contributing factor in the random loss of erythrocytes in circulation.

During oxidative stress condition, cellular metabolism shifted to anaerobic glycolysis and production of lactate increased. Lactate efflux is mediated by the functional lactate transporters which are composed of catalytic unit monocarboxylate transporters (MCT) and an accessory subunit CD147 [32]. CD147 is expressed on mature erythrocytes and various erythroid precursor and play an important role in regulation of lactate transport [18]. Since splenectomized mice have the increased level of ROS production, we have also examined the level of CD147 expression on erythrocytes. Our data suggest that splenectomy induced the level of the CD147 expression on erythrocytes. These observations correlate well with the increased level of ROS on erythrocytes. However the mechanism of increment in CD147 expression on erythrocytes is not known. We have earlier shown that stress induced erythroid cells have an increased level of CD147 expression on its surface [16]. We may speculate that splenectomy mediated stress induced the ROS as well as CD147 expression on erythroid cells in bone marrow progenitors. These stressed bone marrow erythroid progenitors transformed into mature erythrocytes with higher ROS level and CD147 expression. These results further indicate that oxidative stress modulated the lactate metabolism and induced CD147 expression on erythrocytes. Taken together, we can conclude that erythrocyte turnover is significantly modulated in splenectomized mice. The

Fig. 4 Time kinetics of changes in ROS and CD147 expression on erythrocytes in splenectomized mice. Blood samples were collected at indicated time point. The expression levels of ROS and CD147 were measured by staining with CMH₂DCFDA and anti-mouse CD147-FITC monoclonal antibody followed by flow cytometric analysis. Representative Histograms in **a**, **c** and **b**, **d** show the expression level

of ROS and CD147 in control and splenectomized mice, respectively. Summarized time kinetics data from 6 mice showing the relative level of ROS and CD147 in erythrocytes have been shown in **e** and **f**. The relative expression of ROS and CD147 measured by considering the expression in control erythrocytes as 100. Data is represented as mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.005$

enhanced level of ROS and CD147 expression may be a possible cause to increase erythrocyte removal in splenectomized mice.

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Author Contributions NB designed, performed, analyzed and drafted the manuscript. AS provided important discussion on data analysis and help in drafting the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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