Effect of BRMs on Stem and Stromal Cells in Mice with ENU-induced Leukemia

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Abstract

Biological response modifiers (BRMs) can alter interactions between the immune system and cancer cells to boost, direct, or restore the body’s ability to fight disease. Mice with ethylnitrosourea- (ENU) induced leukemia were here used to monitor the therapeutic efficacy of lipopolysaccharide (LPS), Bacillus Calmette Guerin (BCG) and sheep erythrocytes (SRBC). Flow cytometry based CD34+ positivity analysis, clonogenicity, proliferation and ultrastructure studies using scanning electron microscopy (SEM) of stem cells in ENU induced animals with and without BRMs treatment were performed. BRMs improved the stem-stromal relationship structurally and functionally and might have potential for use as an adjunct in human stem cell therapy.

Keywords: Biological response modifiers - ENU-induced leukemia - LPS - BCG - SRBC

Introduction

Within the bone marrow cavity the mystery of HSC health has been found to be dependent on its microenvironment. Studies have revealed that stem cell renewal and maturation requires cytokines/growth factor and chemokines directed guidelines, sequentially. MSCs or Mesenchymal Stem Cells play an important role in this context (Dexter et al., 1977; Dennis & Charbord, 2002). MSCs are thought to be the major source of those specific factors, which interact with HSC in intracellular level (Verfaillie, 1993). Alteration or deviation of bone marrow stromal microenvironment can result in declining or defective hematopoiesis. Disruption of this fine balance between cell proliferation, cell survival and cell death plays a major role in development of several neoplastic diseases, particularly leukemia. Leukemia is a stem cell disease where normal stem cell function and behavior are lost due to transformation events (Li & Li, 2006; Rizo et al., 2006). Leukemia development is a multistep process characterized by progressive cellular transformation of normal hematopoietic stem/progenitor cells into malignant leukemic clones. The functionally abnormal malignant clones become an integral part of the stromal system, selectively stimulating neoplastic cells and inhibiting normal blood cell formation (malignant microenvironment) (Dührsen & Hossfeld, 1996). Although malignant cells have an intrinsic growth and survival advantage, but they also remain dependent on some factors provided by the malignant microenvironment. Several recent studies have found the existence of a leukemic microenvironment (Ayala et al., 2009). In-vivo functional assays from normal and leukemic hematopoiesis have been studied to identify the cytokines; chemokines and adhesion molecules which regulate the development of normal and leukemic stem cells (Meydan et al., 1996; Nagasawa et al., 1996). The immune physiology of stem and stroma in normal and leukemic condition however remain largely unexplored although the defence within the bone marrow cavity is found to remain intact. Several reports using growth factors and BRMs are now taking over for stem cell manipulation and subsequent application for immunotherapy (Law et al., 2001). The functional capability of such cells would however, be considered to be an important factor before cytotherapeutic approaches are made in this aspect. The present work attempted to explore the intimate structure function correlation between the stem and the stromal cells at the ultrastructural level of in-vitro system, in-vivo leukemic experimental model, and that following application of non-specific BRMs.

In this study, leukemia was induced in mice by using a potent carcinogenic agent N-N’ Ethylnitrosourea (ENU). The initiation and progression of leukemia were ascertained by analyzing the blood hemogram profile, peripheral blood and bone marrow smears. The
functional manifestations of both HSC and MSC with an immunological approach revealed that both the categories of the cells are immunologically potent and thereby capable of combating infective procedures, also indicating the presence of immunological defence within the bone marrow compartment. Further, exogenous cytokines, growth factors and stromal supplementation were found to modulate the kinetic relationship between stem and stroma in leukemic condition towards normalcy.

Materials and Methods

Animals
Inbred Swiss albino mice of both sexes formed the materials of the animal experimental model. Young mice aging 7-10 days were injected intraperitonially (i.p.) with N-N’-Ethylnitrosourea (ENU) (Sigma Co; USA); at a dose rate of 80mg/kg body weight. 180 days after ENU administration, peripheral blood hemogram of mice demonstrating leukemic disease progression were selected to study the therapeutic efficacy of BRMs. Animals were divided into 6 groups (40 animals per group). Normal control mice (N); ENU treated leukemic mice (E); Leukemic mice treated with (LPS) (Sigma, USA) (E-LPS); Leukemic mice treated with (BCG) (Guindy, India) (E-BCG); Leukemic mice treated with (E-SRBC); Leukemic mice treated with combination of LPS, BCG and SRBC (E-BRM). These groups were maintained for survival study and associated changes under the conditions. BRMs were administered i.p. at a dose of 2.5µg/gm body weight (LPS), 100 particles (BCG) and 0.25ml of 7% (SRBC). They were injected per animal belonging to their respective groups except the normal. Animals were maintained in the animal house facility with normal diet and water ad libitum. This work was approved by the Institutional Ethical Committee for Animal Care, and also by Council of Animal Care, Govt. of West Bengal, India.

Blood hemogram
At 180 days after ENU induction, 200uL of blood was collected from each mouse by tail vein puncture to evaluate certain hematological parameters. White blood cell (WBC) count, reticulocyte count, and percentage of blast cells from differential WBC count were determined using standard laboratory techniques. Peripheral blood and bone marrow smears were prepared and stained with standard Leishman staining.

Bone marrow derived stem cell isolation and culture
180 days after ENU treatment, animals showing progressive disease statuses were sacrificed to isolate the long bones (femur, tibia, and fibula) for the preparation of bone marrow materials. The long bones were cut in both ends by scissors and the red pulp region of the marrow was flushed out with RPMI-1640 media (Sigma Co., USA) supplemented with 10% fetal bovine serum (FBS, Gibco, BRL). Similarly bone marrow from normal, leukemic and BRM treated animals was isolated and maintained as single cell suspension in an aseptic condition. The cells were subjected to centrifugation at 1000 rpm for 20 min on a bi-layered percoll density gradient namely 1.077 at the bottom and 1.050 at the top (Law et al., 2001). Cells obtained from top (i.e low density compartment or LDC) and bottom (i.e high density compartment or HDC) layers were collected separately and washed thrice in PBS followed by culturing in RPMI media.

The LDC and HDC cells were cultured in 75mm culture dish (Corning, USA) containing 4 ml of RPMI-1640 supplemented with 10% FBS, 100 ng/ml recombinant mouse stem cell factor (SCF) (E-Biosciences, USA), 50 ng/ml recombinant mouse interleukin3 (IL3) (BD Biosciences, USA), and 50 ng/ml recombinant mouse granulocyte macrophage colony stimulating factor (GMCSF) (BD Biosciences, USA). The cells were cultured at 37°C in an atmosphere of 5% CO₂. The culture plates were monitored, and cellular growth pattern was observed and photographed under the inverted microscope. Culture subsets with and without stromal supernatant were also studied.

Bone marrow derived stromal cell isolation & culture
Bone marrow-derived cells were suspended in RPMI-1640 media at a concentration of 4x10⁶ cells/plate, supplemented with 30% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin and 0.01% (v/v) 2-mercaptoethanol, as described previously (Beresford & Owen, 1998), for the experimental and control group. At every 72 hours interval, the media was drained off and fresh media supplemented with 30% FBS and 0.01% (v/v) 2-mercaptoethanol added for the maintenance of the culture. The cultures were maintained for 3 weeks to get an adherent stromal population.

Flow cytometry
Bone marrow derived LDC and HDC cells of all groups were taken from 48-hour cultures (4 wells pooled together) and were subjected to flowcytometric analysis for the presence of stem cells (Law et al., 2001). Percentage of CD34+ population in LDC and HDC cells were scanned using FITC conjugated antibody against CD34+ (BD Biosciences, USA). Briefly, LDC and HDC culture groups were washed with PBS, and the final volume adjusted as per the cell yield, and incubated in the dark with FITC conjugated rat anti-mouse CD34 monoclonal antibody (1μg/10⁶ cells) for 30 min in dark. Cells were washed with RPMI medium and fixed with 3% paraformaldehyde. The cells were then further washed with PBS and analyzed in BD-FACS Calibur (Argon Laser, excitation at 488nm, using 530 nm band pass filter for FITC fluorescence), gated properly a total of 10,000 events using Cell Quest software (Becton Dickenson, San Jose, CA).

Ultrastructural studies
Cultured cells were collected and washed in PBS to remove the medium. They were then processed through graded alcohol dehydration (50% to 100%) and finally kept in acetone. Cells were then critically point dried (CPD), fixed on small cover glasses and subjected to gold coating in an ion sputter coater. Finally, the cells were scanned under scanning electron microscopy (SEM, JSM-5200 Jeol, Japan) and photographed.
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Studies on cell mediated immune parameters.

The functional efficacy of the cells isolated as above has been carried on in each group as follows, cytotoxicity essay: Cytotoxic efficacy by fluorochrome (HO-33342) release assay (Law et al., 2001). This consists of lysing the HO-33342 labelled target tumor cells (Dalton’s lymphoma) by effector bone marrow cells (LDC and HDC) at a ratio of 10:1 (T: E) and measuring the fluorochrome released in the supernatant following 18 hr of incubation at 37°C and 5% CO² atmosphere.

Phagocytosis assay: Phagocytic burst by Nitro Blue Tetrazolium chloride (NBT) salt reduction assay was carried using Dalton’s lymphoma cells as the target in the presence of NBT in the reaction media with an effector: target ratio of 10:1.

Statistical analysis

All Statistical analysis of results performed for survival study, cytotoxic efficacy, and phagocytosis are represented as mean ± standard deviation. Student’s t-test was performed for inter-group comparison. All results were evaluated statistically by applying the SPSS-PC Package (Version 9.0, SPSS, Chicago, Illinois, USA). A probability of P < 0.05 was considered statistically significant.

Results

Leukemia induction, BRM application and survival study

N-N’- Ethylnitrosourea induced a mixed type of leukemia, which was predominantly lymphoblastic within a period of 4-7 months following ENU application. Peripheral blood (Figure. 1a-1b) and bone marrow smears (Figure. 1c) showed leukemic blasts which are mostly lymphoblastic in nature although occasional myeloblasts were also found. Out of these animals, 5% died due to the acute toxicity (A) within a period of 8 days, 12% died due to secondary infections (s) within 42 days and 65% showed a progressive leukemia status and died at around 192 days if kept untreated (E). ENU induced leukemic animals treated (180 days post ENU administration) individually with LPS (ELPS), BCG (EBCG) and SRBC (ESRBC) showed significant benefit with increased survival but combination of three BRMs exhibited the maximum benefit (EBRMs) (Figure. 1d).

Bone marrow derived CD34+ cell: Density specific compartmentalization

Cells isolated from the bone marrow through densitometric centrifugation technique yielded two distinct categories of cells. One at a lower density (1.050), the low-density compartment (LDC), and the other at a higher density (1.077), the high-density compartment (HDC). Cells thus harvested were subjected for CD34 positivity in cell sorter showing a higher enrichment at the LDC level than the HDC. In normal mice the LDC and HDC layer showed an enrichment of 37.02% (Figure. 2a) at LDC and 23.39% (Figure. 2b) at HDC in comparison to the leukemic mice where the Bone marrow cells (BMCs) showed an enrichment of 14.20% at LDC(Fig-2c) and 18.64% at HDC (Figure. 2d). In combined BRMs treated leukemic mice the LDC and HDC layer of BMC showed an enrichment of 48.07% at LDC (Figure. 2e) and 31.65% at HDC (Figure. 2f).

Cellular morphological changes

Cells isolated as LDC from normal group of mice showed normal morphology with active signs of proliferation and growth characteristics after 24 hours (Figure. 3a-b). In leukemic group, multiple cell growth

Figure 1. Characterization of Peripheral Blood, Bone Marrow Biopsy and Survival. Peripheral blood (a & b) and bone marrow smear (c) of mice showing leukemic blasts 6 to 7 months following neonatal (7-10 days) ENU administration. The cells are mostly lymphoblastic in nature although occasional myeloblasts were also found. Survival curve, of different groups of mice (including normal) with experimental leukemia and that following application of BRMs (LPS, BCG and SRBC) (Fig-1d). LPS, BCG & SRBC when administered separately in leukemic mice showed beneficial effects over their survival period. Further combination of three BRMs showed a more significant (p<0.05) benefit in comparison to the leukemic group.

Figure 2. FACS Scan of CD34+ Cells in Normal and Leukemic Mice. In normal mice, the LDC and HDC layer of BMC shows an enrichment of 37.02% at LDC (2a) and 23.39% at HDC (2b). Whereas, in leukemic mice the LDC and HDC layer of BMC shows a decrease enrichment of 14.20% at LDC (2c) and 18.64% at HDC (2d). Administration of BRMs showed increased enrichment of 48.07% at LDC (2e) and 31.65% at HDC (2f).
and accumulation of undifferentiated blasts after 24 hours of culture was observed (Figure. 3c-3d). In combined BRM treated leukemic group a prospective result was observed showing reduction in undifferentiated blasts and reappearance of normal cellularity and growth kinetics (Figure 3e-3f).

Morphological changes in stromal cells

Generation of healthy stromal precursors was observed in normal mice (Figure 4a-4b). In ENU induced leukemic mice the numbers of stromal precursors are minimized due to leukemic inhibition (Figure. 4c). In ENU induced leukemic mice stromal generation is seen to be disrupted. The stromal architecture was found to be strained and devoid of cell generation (Figure. 4d). In BRMs treated leukemic mice the number of stromal cell generation is improved. (Figure. 4e-4f).

SEM features of the bone marrow cells

SEM features of normal LDC of the BMC showed normal morphology. The cells cling to the stromal cord like structure prior to its release (Figure. 5a-5b). LDC of ENU induced leukemic mice showed distortion with tortuous surface architecture and discontinuous stromal support (Figure. 5c-5d). SEM features of combined BRM treated LDC showed close proximity between stem-stromal contact points followed by active signs of regeneration (Figure 5e-5f).

Figure 3. Characterization of Cell Growth and Proliferative Potentials of Bone Marrow Cells from Normal and Leukemic Mice with and Without BRM Treatment. Cells isolated as low density cells (LDC) from normal group of animals seeded at ‘0’ hour (3a). Cells isolated as low density cells (LDC) from normal group of animals after 24 hours of culture showing active sign of proliferation and growth characteristics (3b). LDC of ENU induced leukemic group showing multiple cell growth and accumulation of undifferentiated blasts (3c). LDC of ENU induced leukemic group showing multiple cell growth and accumulation of undifferentiated blasts after 24 hours of culture (3d). LDC cells of BRM treated leukemic group showing considerable reduction in undifferentiated blasts and appearance of healthy cells after 24 hours of culture (3e). LDC cells of BRM treated leukemic group producing the best result with appearance of healthy normal cellularity and growth kinetics (3f).

Figure 4. Generation of Stromal Cells in Normal and Leukemic Mice with or Without BRM Treatment. Healthy stromal cells from the underneath layer of culture flask in normal mice (4a-4b). In ENU induced leukemic mice the numbers of stromal precursors are minimized due to leukemic inhibition (Figure. 4c). In ENU induced leukemic mice stromal generation is seen to be disrupted. The stromal architecture was found to be strained and devoid of cell generation (Figure. 4d). In BRMs treated leukemic mice the number of stromal cell generation is improved. (Figure. 4e-4f).

Figure 5. Scanning Electron Microscopy (SEM) Features of Normal and Experimental Bone Marrow Cells. SEM features of normal bone marrow cells showing that these cells cling to a cord like structure and remains engraved as flowers in a garland (5a). SEM features of normal bone marrow cells showing its gradual release. The cells form clumps with associated heterogeneous cells (5b). SEM features of ENU induced leukemic bone marrow cell shows ghost like appearance, distortion with tortuous surface architecture (5c) and discontinuous stromal support (5d).SEM features of BRM treated animals shows a close proximity between stem-stromal contact point (5e) followed by active signs of regeneration (5f).
Functional efficacy of the bone marrow cells at the two compartments

The functional efficacy of HDC and LDC groups of cells in terms of cytotoxicity was determined. The HDC group of cells showed a significantly (p<0.05) (Figure. 6a) greater cytotoxic efficacy against the tumor compared to the LDC counterpart (Figure. 6b). In leukemic group of animals a significant decrease (p<0.05) in cytotoxic efficacy was noticed in both LDC and HDC compared to the normal group. BRMs when administered separately elevated the cytotoxicity against the tumor with little or no variation in between the different groups. The maximum benefit was, however, observed when the animals were treated with LPS, BCG and SRBC in a combined dose (p<0.05) indicating the additive effect of each BRM under the particular events.

The Phagocytic burst by the LDC and HDC of the BMCs as determined through NBT assay demonstrated the phagocytic efficacy of all categories of pluripotent BMCs under different experimental conditions (Figure. 6c-6d). In leukemic mice both LDC (Figure. 6c) and HDC (Figure. 6d) showed a significant decrease (p<0.05) in phagocytic burst compared to the normal group, the LDC suffered the most than the HDC. In leukemic group treated with LPS, BCG and SRBC, a reversal of functional depression resulted in a significantly elevated phagocytic burst (p<0.05) was noticed both in LDC and HDC. The BRMs when administered in combination, however, did not show a highly significant improvement compared to the individual effects. The recovery was, however, significantly beneficial (p<0.05) which showed that the effects are not straight way additive.

Discussion

Hematopoiesis is a complex process of proliferative and differentiative signalling cascades responsible for the generation of healthy mature blood cells from a small population of hematopoietic stem/progenitor cells throughout the lifetime of an individual. The blood cell formation from single stem/progenitor cells requires continuous stimulation of various cytokines and growth factors secreted from its surrounding micro environmental accessory cells or the niche components (Can, 2007). Normal hematopoiesis is critically balanced by several cell proliferation, differentiation, and death signalling pathways. Alteration in one or more of these signalling pathways may ultimately shift the finely tuned equilibrium to either hypoproliferative or hyperproliferative disease condition. Under the condition of neoplastic progression such as leukemia, the normal hematopoietic cells can be transformed into leukemic cells. The resulting accumulative alterations in the leukemic cells shift the balance between cell proliferation and cell death towards the former, thus leading to a continuous increase of leukemic cells within the bone marrow microenvironment (Najman et al., 1991). In this present study, we have used the mice model of leukemia developed by injecting ENU (Chatterjee et al., 2009), a potent carcinogenic agent (Justice et al., 1999), to evaluate the changes in the characteristics of leukemia and deviation in the behavior of normal bone marrow microenvironment in leukemic disease progression. The bone marrow cells isolated from normal mice showed a higher CD34 positivity (37.02%) at low-density compartment (LDC) than the high-density compartment-HDC (23.39%). Whereas, bone marrow of mice with ENU induced leukemia demonstrated a significant decrease in CD34+ cell population both at LDC (14.20%) and HDC (18.64%) level. Decreased CD34 receptor expression on leukemic bone marrow cells hints at the decreased primitive hematopoietic stem cells and suppression of normal haematopoiesis in leukemia. Such depression may be due to ENU induced inhibition or tumorigenic inhibition in the animals concerned. Under most circumstances such inhibitions are caused by molecular dysregulation in course of differentiation through signals transduced. This further indicates that LDC contain more immature stem cells than the HDC which is possibly due to the migration and differentiation of stem cells in HDC in a matured form; the cells under the circumstances might have lost CD34 receptors following subsequent differentiation and maturation. Studies have revealed that at stable phase in individuals, compartmental mobilization of the bone marrow cells under different physiological/pathological trigger is a routine phenomenon (Potten & Loeffler, 1990).

The mechanism of cellular inhibition in ENU induced leukemic group animals both at LDC and HDC of bone

Figure 6. Functional Efficacy of the Bone Marrow Cells at the Two Compartments. Cytotoxic efficacy of LDC of bone marrow cells against Dalton’s Lymphoma (target) as measured by HO-33342 release assay in ENU induced leukemic group showed reduced cytotoxicity compared to the normal which upon application of BRMs in combination (LPS+BCG+SRBC) showed highly significant increase in cytotoxic capacity. HDC in ENU induced leukemic group also showed diminished cytotoxic efficacy compared to the normal; BRMs either in individual or in combination showed significant improvement of cytotoxic efficacy (6a & b). Phagocytic capacity of bone marrow cells against Dalton’s Lymphoma (Target) as measured by NBT reduction assay showed decreased phagocytic capacity in LDC of ENU induced leukemic group compared to the normal, which upon application of BRMs either alone or in combination represented significant increase in phagocytic capacity. HDC following leukemic induction also showed reduced phagocytic capacity compared to the normal. Application of BRMs either alone or in combination significantly increased the phagocytic capacity (6c & d).
marrow appears to be due to two separate reasons: First, the role of ENU perse in the animals concerned which in one way is a carcinogen (Chen et al., 2011) on the other an agent which can sufficiently cause immuno-suppression (Vaqero et al., 1998). Leukemogenesis is a function of ENU (Basak et al., 2010), which also causes induction of brain tumors (Briancon-Marjollet et al., 2010) and reproductive tumors (Stoica, 2002). Simultaneously, it is an inhibitor of cellular kinetics with certain limitations; mostly dose dependent activity. But, the residual effect of ENU mediated inhibition after long period of 6-8 months is less likely, rather than the tumorigenic inhibition. Like many other instances selective suppression of the bone marrow derived cells due to tumor burden has amply been recorded. This is an instance of “struggle for existence” for the tumor cells themselves in order to make way for their own maintenance and growth at the expense of the remaining pluripotent cells at particular stages. Therefore, tumor cells predominates, whereas, the pluripotent bone marrow cells subsides.

At the therapeutic level, LPS, BCG and SRBC all showed beneficial effects towards the survival period of the leukemic animals, either alone or in combination. Moreover, BRMs stimulated a greater purity or renewal of CD34+ cells in both LDC (48.07%) and HDC (31.65%). The mechanisms by which the non-specific BRMs trigger a large number of CD34+ cell generation or release are yet to be established and such protective effect may be diverse depending largely on the cytokine and chemokine network. The role of BRMs within the bone marrow compartment has not been discussed before. The context that stem/progenitor cells can also be influenced by the biomodulators as mentioned is far from conjecture and, as such, can be considered as general immunopotentiation. Several reports have indicated that T11TS, a glycopeptide derived from SRBC surface could modify the immune status of tumor bearing animals and even in humans (Mukherjee et al., 2005). HSC express toll like receptors (TLRs) which recognize bacterial or viral proteins (Nagai et al., 2006), induce proliferation along with immune cells mediated induction in proliferation (Baldridge et al., 2010). Recent studies have shown that injection of the bacterial component LPS increased the proliferation and self-renewal capacity of HSCs (Takizawa et al., 2011). BCG on the other hand has been found to help in generation of self-renewing immature dendritic cells from mouse spleen that can take up mycobacteria and present antigens to T cells (Corocleau, 2008).

The intricate mechanism might involve the microenvironment within the bone marrow leading to complete differentiation and maturation of the associated stromal cells and finally help the signalling pathway leading to stem cell regeneration and steady maintenance. Our results demonstrated that normal LDC showed intimate adhesion with the stromal cells or cord. ENU induced leukemic group showed evidence of cellular damage, dysregulation of clonogeneity and loss of stem-stromal association whereas, BRMs projected to be beneficial with respect to surface ultrastructure, stromal association, clonogeneity and confluence. BRMs were found to protect the carcinogenic effect of ENU in one hand and tumorigenic inhibition at the other. The stroma appears to be capable of patho-physiological responses to hematopoietic requirements and abnormalities. The BRMs might have played an important regulatory role in combating the leukemic status by stimulating the stromal microenvironment. The present work aimed to delineate the hematopoietic stem cell biology, structure and functions in health and after development of experimental leukemia in mice and finally, those following non-specific BRMs can improve the overall hematopoietic regeneration organ system for a successful immunotherapy.

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