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EFFECT OF SYNTHETIC CXCL12 SUPPLEMENTATION ON PROGENITOR HEMATOPOIETIC POPULATION OF IRRADIATED MICE

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ABSTRACT

CXCL12 is a primordial chemokine that have been cited as one of the most important homing factors to hematopoietic progenitor cells (HPC`s) at its proliferation and/or differentiation sites (i.e. in mice, bone marrow and spleen). In this work, we tested the effects of supplementation of CXCL12 (i.p., 1μg/animal/day) (0-4th day post irradiation) in C57Bl/6j mice irradiated at non-lethal (4Gy) and sublethal (8Gy) doses. Groups were analyzed on 2nd, 4th and 7th days post-irradiation events. Blood samples (2μL) were collected by tail puncture to perform manual counts of total erythrocytes and platelets. Mice were killed by respiratory anaesthesia (CO_2) and spleen and bone marrow suspensions were prepared and fixed (EtOH 70%) to perform later flow cytometry analysis using $CD34^+$ surface expression as major parameter. Animals treated with CXCL12 supplementation presented remarkable increase (~ 4-fold) of $CD34^+$ cell count in bone marrow of mice irradiated at 4Gy on day 4th, one day after rapidly income of circulating platelet counting. Flow cytometry analysis of spleen polimorfonuclear fraction showed increase in $CD34^+$ (~2-fold) on day 7th, prior to a second slight increase of platelet count. These data show a close relationship between CXCL12 and mieloid progenitor cells, and may indicate future use of this chemokine in treatment of acute radiation syndrome.

1. INTRODUCTION

Ionizing radiation (IR) events promotes very well known systemic syndromes, depending upon dose, dose rate and linear transfer energy (LET). Hematopoietic syndrome, leading to blood-forming failure and subsequently immunity failure is very prevalent in these cases. The very high increase in the number and magnification of power of meteorological disasters may be leading public and private sectors to discuss even more consistent use of nuclear energy sources. Beside this fact, the increasing use of radiactive isotopes in medicine (new-brand diagnostics and treatment) are factors that may contribute to augment the risk of exposure of patients, workers and general public to ionizing radiation, despite all recommendations and already well-stablished procedures.

CXCL12 was primarily described as a ligand to the LESTR-Fusin receptor, known to be a invasion route of HIV virus particles in lymphocytes [1], and later as a potent pro-inflammatory chemoattractant [2], but its main function is known as an important homing factor of HSC to the extracellular matrix of sinusoid capillaries via CXCR4-CXCL12-Heparan sulphate axis [3]. Its use on hematopoietic reconstitution, solely or concomitant with

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another factors [4] has been suggested in the last years. Here we evaluated the use of a primordial chemokine (CXCL12), known as a lymphocyte chemoattractant to inflammatory sites and a potent and one of the most important homing factors of hematopoietic stem-cells (HSC) to hematopoietic sites, as an adjuvant factor to hematopoietic recovery after radiation exposure.

2. MATERIALS AND METHODS

2.1. Experimental groups and irradiation procedures

All procedures were performed according to principles of animal welfare elsewhere described [5]. Groups of male six-week old C57Bl/6j mice obtained from our colony, (Centro de Bioterismo da Fac.Medicina da Universidade de São Paulo – USP) and maintained at our own facilities, were γ-irradiated in a ^{60}Co panoramic source (Yoshizawa Kiko Co.) at the Center of Radiation Technology (IPEN/CNEN – SP) at 0, 4 or 8Gy (rate: 11,24 kGy/h), at 70cm from source and behind a 70% attenuator. Animals were immobilized in PVC capsules (6cm length, 2cm diameter) during irradiation events to prevent from heterogeneous exposure due to movimentation of mice during the event. Irradiated (4 and 8Gy) and non-irradiated (0Gy) groups received i.p. 1μg/day of synthetic CXCL12 (Dr. Nobutaka Fujii, Kyoto University) diluted in saline daily, from day 0 to 4th post-irradiation on days 0-4th. Non-irradiated groups (0Gy) were similarly mantained like described above, subdivided in treated and non-treated groups. On days 2nd, 4th and 8th of experiment, tail blood was collected from specimens to perform counts (hematocytometer chamber) of total number of red blood cells and platelets, and mice were sacrificed by CO_2 inhalation to provide bone marrow and spleen cell suspensions.

2.2. Hematopoietic cell suspensions used to Flow Cytometry Analysis

Spleens were asseptically removed and dissociated by mechanical disruption and sucessive, but gentle pippeting in PBS + 10% Fetal Bovine Serum (FBS) + 5mM EDTA (pH 7,4). Cell suspensions were passed through a Ficoll® cushion during centrifugation step (700 x g, 30min, RT) to collect polimorfonuclear fractions and mantained in same saline solution, in plastic tubes placed in ice until processing. Femurs were removed and internally flushed with PBS + FBS 10% + 5mM EDTA to dettach stem cells from bone cavitities. All obtained cell suspensions (spleen and bone marrow) were centrifuged as described above. Cells from all samples (spleen and bone marrow) were counted and adjusted to 4x10^5 cells/mL in a total volume of 200μL, and reacted against monoclonal rat anti-mouse CD34 – phycoerythrin (MEC 14.7) for 30 minutes on ice in dark. After this step, cells were suspended with cold EtOH 70% (800μL) and mantained at -20ºC until flow cytometry experiments. Acquisition of 50000 events was performed using FACSCalibur (BD Biosciences) and analysis using Summit Software (DAKO Cytomation). CD34$^+$ counts were acquired using gates shown in Fig.1. The positive capture gate was delineated considering side scatter and log of fluorescence, using a modified version of the Milan-Mulhouse protocol elsewhere described [6] and briefly detailed in Fig.1.

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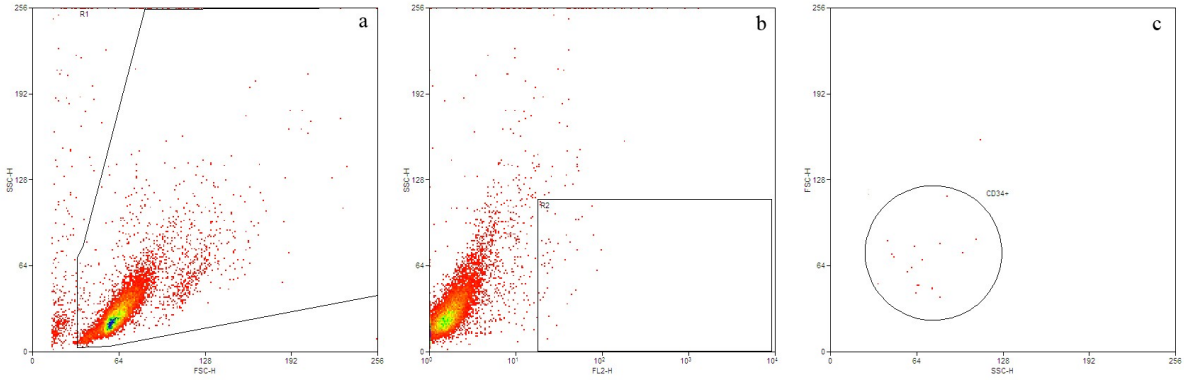

Figure 1.: Example of used gates. (a) R1 containing integer cells, excluding debris. (b) R2 containing cells with lower side-scater values and higher fluorescence log values. (c) Final result, indicating positive cell population. The figure shows sample from a non-irradiated control.

3. RESULTS

Blood cell counts (red cells and platelets) of treated and untreated groups were distributed by dose and shown in Fig. 2 (4Gy) and 3 (8Gy).

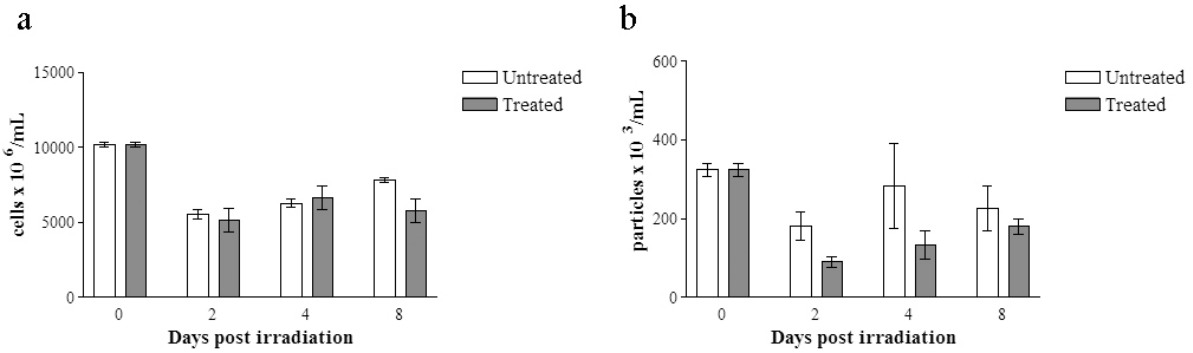

Figure 2.: Variation of number of erythrocytes (a) and platelets (b) of peripheral blood in treated and untreated groups irradiated at 4Gy.

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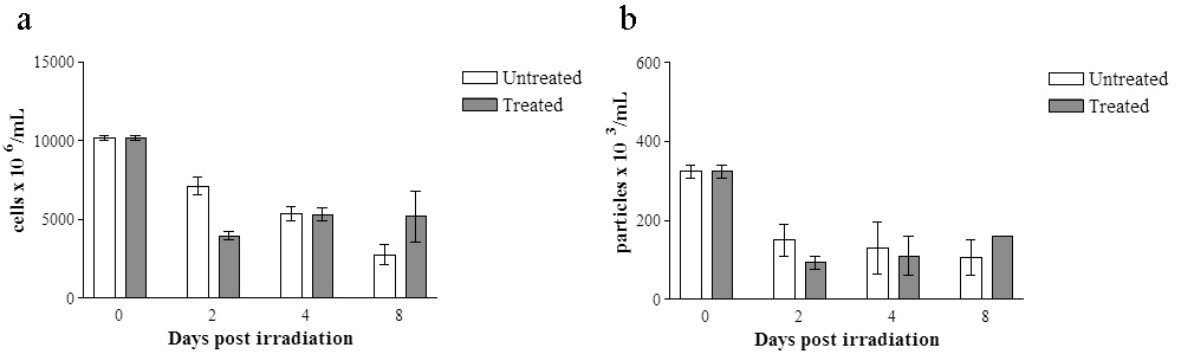

Figure 3.: Variation of number of erythrocytes (a) and platelets (b) of peripheral blood in treated and untreated groups irradiated at 8Gy.

Relative results of $CD34^+$ were given in percentage proportional to the absolute number of events acquired using controls. Fig. 4 shows results on spleen cells and Fig. 5 on bone marrow suspensions.

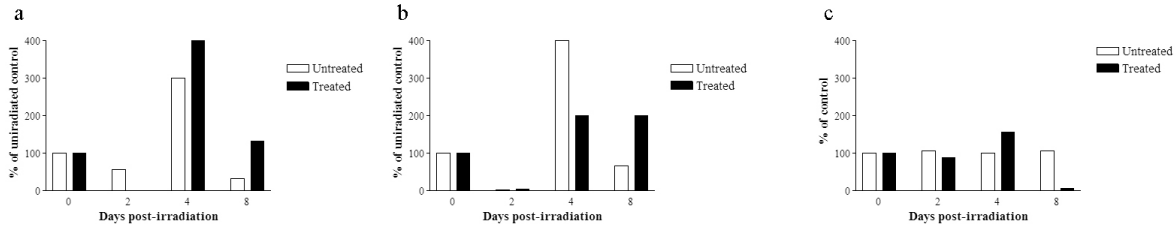

Figure 4.: Variation of relative number of $CD34^+$ cells in spleen cells. (a) 4Gy. (b) 8Gy. (c) Non-irradiated (NI) group.

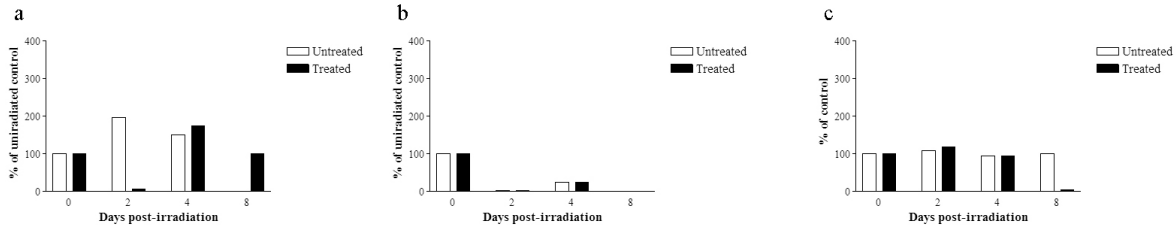

Figure 5.: Variation of relative number of $CD34^+$ cells in bone marrow cells (a) 4Gy. (b) 8Gy. (c) Non-irradiated (NI) group.

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On Fig. 2, results showed no significant variation of erythrocyte counts of animals irradiated at 4Gy (2a), but platelet counts showed increase on 2nd and 4th days in untreated group (2b) comparing with treated group. Irradiation at 8Gy (Fig.3) caused decrease of RBC counts on day 2nd and increase on day 8th (3a). Platelet counts (3b) showed no significant variation during the course of experiment. Fig. 4 shows variation of relative number of CD34-positive cells during experiments in spleens of treated and untreated animals, using non-irradiated and non-treated groups as parameter. Treated group showed remarkable increase on day 4th (4Gy)(4a) and day 8th (8Gy)(4b) than untreated group, and a slight increase on day 4th in non-irradiated controls (4c). Fig.5 shows same parameters in bone marrow samples. In 5a (4Gy), treated group presented large decrease of $CD34^+$ cells on day 2nd and a significant increase on day 8th . In 8Gy-irradiated mice (5b) there were no significant variation between treated and non-treated groups. Treated non-irradiated mice showed large decrease of HSC count on day 8 post-irradiation, comparing with those non-treated.

4. CONCLUSION

CXCL12 is a homing chemokine highly associated with hematopoietic stem cells [7], whose phenotype include the expression of CD34 syalomucin on cell surface [8] beside its specific receptor, CXCR4. Its use on non-irradiated (NI) animals caused high decrease on $CD34^+$ counts on mice main hematopoietic tissues after treatment, probably due to mobilization phenomena [9]. As like G-CSF (Granulocyte-Colony Stimulating Factor), CXCL12 can mobilize HSC`s to peripheral blood [10]. After a total-body-irradiation (TBI) event using non-lethal dose (4Gy), spleens of suplemmented-mice were more responsive than controls. CXCL12 seems to have sinergistic effect with compensation mechanisms that may act together in order to restablish normal hematopoiesis at this point. Although treatment did not showed effectiveness on day 2nd in any of used doses, 8Gy-irradiated mice showed similar counts of HSC`s on days 4th and 8th in spleens, confirming the homeostatic role of CXCL12 [11]. On bone marrow tissues, treatment showed some effectiveness only on day 8th, and discrete increase of $CD34^+$ counts on day 4th. Bone marrow, as a major hematopoietic organ [12] have a large number of mitotic-active cells, augmenting he probability of radiation-induced death [13].

CXCL12 was poorly effective probably due to extensive destruction of tissue, creating an inflammatory environment that leads HSC`s to death by necrosis and/or apoptosis [14]. The results cannot allow to exclude a removal effect on HSC`s induced by chemokine homing character. In this way, CXCL12 could not attract cells that were in this state. By another hand, CXCL12 seems to attract HSC`s to spleen, probably due to i.p. infiltration and with its biodistribution directed to spleen. Previous results from our group [15] showed maximum CXCL12 mRNA expression in these tissues on day 5th post-irradiation, falling to undetectable levels on day 8th in bone marrow, what also could explain increment of HSC in spleen at this point (4Gy). This data also shows that on day 2nd, chemokine expression reaches its minimum in both organs. The 4th day might be also correlated with the higher count of platelets on 4Gy-irradiated mice, what may lead us to understand if CXCL12 production is a potent stimulatory factor of pro-mieloid cells, and if this phenomena could be useful in bone marrow reconstitution protocols, altough its use only affects HSC proliferation after 12 days after infusion [16] after radiation-induced myeloablation.

CXCL12 common producers in bone marrow are known as mesenchymal cells, that are

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relatively radioresistent and constitute a important population in hematopoiesis, releasing chemokines. The intensive knowledge on how CXCL12 can coordinate migration of HSC`s between hematopoietic organs can be a promising key factor in the protocols cited above, specially if further experiments can show how this chemokine could redirect this migration leading stem cells away from inflammation and hemorrhagic sites, induced after radiation exposure [17] and also aggressive to undifferentiated cells.

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