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Brief communication

Non-erythroid effects of erythropoietin: Are neutrophils a target?

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ABSTRACT

We have previously shown that erythropoietin (EPO) potentiates the immune response. Analysis of various possible cellular mediators was performed on EPO-injected mice and transgenic mice overexpressing human EPO (tg6). Here we present our studies on neutrophils, peritoneal (casein induced), and from the peripheral blood, spleen and bone marrow. Neutrophil counts were elevated in peripheral blood and spleens of the tg6 mice, yet, no other EPO-associated effects were detected in the count and function of the different neutrophil populations. Hence, neutrophils are probably not mediators of the EPO immunological effects, although their counts may be affected by extreme EPO levels.

1. Introduction

Erythropoietin (EPO) is the major cytokine that promotes the proliferation and differentiation of erythroid progenitor cells. Hence, recombinant human EPO (rHuEPO) is a common therapy used for treatment of anemia of various etiologies, including anemia of chronic kidney disease and cancer-related anemia. In addition to the erythroid lineage, EPO receptors (EPO-R) have been identified on a variety of non-erythroid cells [1]. Although our earlier studies have demonstrated EPO effects on the immune system, both on the cellular and on the humoral compartments [2,3], we could not detect EPO-R mRNA transcripts on lymphocytes [3]. We have thus been searching for EPO-R expressing cells that might be the cellular mediators of the EPO immuno-potentiating effects. Our studies indicated that dendritic cells (DCs) have functional EPO-R and that EPO stimulation enhances their survival and functionality [4,5].

Further search for relevant EPO target cells led us to focus on neutrophils, considering that conflicting reports exist concerning direct effects of EPO on these cells. At first, our assessment of human derived neutrophils did not disclose any EPO-R expression or EPO-mediated effects on these cells (data not shown). To determine critically whether EPO has any effect on neutrophils, we thus employed two murine experimental models, EPO injected mice and transgenic mice (tg6) overexpressing human EPO (HuEPO) [6].

2. Materials and methods

2.1. Mice

rHuEPO-injected C57BL/6 female mice, aged 6-8 weeks, were obtained from the Tel-Aviv University Breeding Center. Mice were injected subcutaneously three times every other day during one week, with 180 units of rHuEPO (epoetin alfa, Eprex®) into the back flank, or with the same volume of the diluent as a negative control. Female tg6 mice over-expressing HuEPO, designated tg6, and their wild type (WT) littermates were used at the age of 3–5 months [6]. Mice were used for the experimental procedures conducted according to the Institutional Animal Care and Use Committee of the Tel-Aviv University; permit M-06-109.

2.2. Peripheral blood counts

Peripheral blood was collected using a heparinized capillary tube into ethylenediaminetraacetic acid (EDTA) coated tubes following retro-orbital puncture. Complete blood counts were determined with an ADVIA hematology analyzer through the AML Department of Veterinary in Herzliya Medical Center. Results were further confirmed by light microscopy of Wright-Giemsa-stained cytospins.

2.3. Splenocytes isolation

Spleens were immersed in phosphate buffered saline (PBS) and forced through 200 μm pore-size wire mesh, using the plunger from a 10 mL syringe to produce a single cell suspension. The cells were pelleted by centrifugation, and erythrocytes were lysed by hypotonic shock (10 s in sterile distilled water), followed by the addition of 0.1 volume of 10× 'Hanks' buffered saline solution (HBSS). Splenocytes were centrifuged, washed once in PBS, and then used as indicated.

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2.4. Flow cytometry analysis

Cells were incubated with fluorescence-conjugated antibodies against cell surface markers: Gr-1 (Ly6G) and CD18/CD11b (mac-1) (Miltenyi Biotec Inc. USA) for 30 min at 4 °C and subsequently washed with PBS. Cells were then analyzed on a FACSort flow cytometer (BD). Ten thousand total events were collected and gated for analysis on live cells as determined by forward and side scatter. Results were analyzed using WinMDI software.

2.5. Isolation of neutrophils from the peritoneum

To induce nonspecific inflammatory exudates, mice were injected intraperitoneally (i.p.) with 2 ml of 0.2% casein (C5890, Sigma). Mice were sacrificed 3 h post casein injection and total intraperitoneal cells were obtained by injecting 10 ml of ice-cold PBS containing 1 mM EDTA into the peritoneal cavity. After gentle massage, the fluid was harvested and the cells were centrifuged and washed before further use.

2.6. Migration of neutrophils to the peritoneal cavity

Recruitment of neutrophils was assessed at 3 h post casein injection, when neutrophils counts routinely peak. Cells were collected from the peritoneal cavity, stained with Trypan Blue and counted in a light microscope.

2.7. Phagocytosis of fluorescently labeled E. coli by peritoneal neutrophils

Peritoneal neutrophils were harvested as previously described and seeded in 96-well plates (0.5×10^6 cells/well). *E. coli* was heat-killed by incubation at 65 °C for 1 h and labeled with 0.2 mg of FITC (Sigma) per ml in 0.1 M $NaHCO_3$ (pH 9.0) for 1 h at 37 °C. FITC-labeled *E. coli* (5×10^7 CFU) were added to the cells (100 bacteria per neutrophil). After incubation for increasing times at 37 °C or at 4 °C (to determine fluorescence background), phagocytosis was stopped by immediate transfer of the cells to 4 °C and washing with ice-cold PBS. Cells were treated with 0.1% crystal violet to quench extracellular fluorescence, and analyzed using a FACSort flow cytometer (BD). Neutrophils were gated as based on forward light scatter. Results are represented as phagocytosis index, defined as the percentage of cells with internalized *E. coli* minus the background fluorescence, multiplied by the mean fluorescence intensity.

2.8. Reactive oxygen species (ROS) production

Superoxide-generating activity was evaluated by cytochrome *c* reduction assay. The assay mixtures consisted of 1×10^5 peritoneal neutrophils, 150 mmol/l cytochrome c (C2506, from equine heart; Sigma) and 0.2 μM PMA as a stimulant, in a total volume of 1 ml HBSS (containing 1.3 mmol/l $CaCl_2$ and 0.4 mmol/L $MgSO_4$). Samples containing 20 μg/ml of the inhibitor superoxide dismutase (SOD; S2515, Sigma) served as an additional control (data not shown). Baseline O_2^- production was assessed by examining tubes containing cells with no stimulant. After incubation at 37 °C for 10 min, the reactions were terminated by cooling the samples on ice. The samples were centrifuged at 10,000 × *g* for 5 min, and the supernatants were aliquoted in triplicates on a 96-well plate (100 μl/well). Absorbance of the supernatant at OD_{550} was determined.

2.9. Statistical analysis

Data are presented as mean values ± standard error of the mean (S.E.M.); *p* values were calculated using an unpaired 2-tailed Student *t*-test. *p* value of less than 0.05 was defined as statistically significant.

3. Results and discussion

Neutrophil levels and morphology in peripheral blood, spleen and bone marrow were assessed. Neutrophils derived from EPO-injected and from tg6 mice constitutively overexpressing EPO showed normal morphology (data not shown), and were indistinguishable from their respective controls. EPO-injected mice displayed peripheral and splenic neutrophil counts (assessed by $Gr1^+/CD11b^+$ labeling) similar to the diluent injected mice. The levels of bone-marrow neutrophils were similar in the tg6 and in the control mice (data not shown). In the tg6 mice, both peripheral blood counts (tg6: $2.37 \pm 0.5 \times 10^3/\mu l$ versus WT: $0.8 \pm 0.16 \times 10^3/\mu l$, $n = 5$ in each group, $p < 0.05$) and splenic neutrophil counts (tg6: $15.25 \pm 2.93\%$ versus WT: $5.5 \pm 0.91\%$) were increased compared to their normal WT littermate counterparts (Fig. 1). In addition to EPO expected effects on the red blood cell compartment, we also observed a decrease in the platelet counts in the tg6 mice ($652.8 \pm 115 \times 10^3/\mu l$ versus WT: $1314.2 \pm 122 \times 10^3/\mu l$, $n = 5$ in each group, $p < 0.01$), consistent with previous reports. Total white blood cell counts ($26.2 \pm 1.81 \times 10^3/\mu l$ versus WT: $10.60 \pm 0.76 \times 10^3/\mu l$, $n = 5$ in each group, $p < 0.01$) as well as lymphocyte counts ($22 \pm 1.56 \times 10^3/\mu l$ versus WT: $8.95 \pm 1.03 \times 10^3/\mu l$, $n = 5$ in each group, $p < 0.01$) were increased in the tg6 mice. The elevated neutrophil counts in the peripheral blood and in the spleens of the tg6 mice may be related to the long term exposure of progenitor cells to high plasma levels of EPO *in-vivo*. The effect of EPO may be exerted on the microenvironment of the splenic progenitor cells in the tg6 mice, thus leading to increased neutrophil and lymphocyte production. However, these differences were not translated into either altered neutrophil morphology or function in the tg6 mice.

To address inflammation-associated neutrophils, rHuEPO-injected mice, tg6 mice and their respective controls were injected i.p. with 2 ml of 0.2% casein (C5890, Sigma). Three hours after the injection mice were sacrificed and cells present in the peritoneal cavity were harvested by introducing 10 ml of PBS containing 1 mM EDTA. We could not detect any EPO-R mRNA transcripts in these neutrophils (data not shown). We then assessed basic functions of the inflammatory neutrophils, yet, the results did not disclose any effect of EPO on the neutrophils. The assays included the following. (a) Migration to the inflammation *foci in-vivo* (Fig. 2A); (b) Superoxide production in response to *in-vitro* stimuli (Fig. 2B); (c) *In-vitro* phagocytosis (Fig. 2C); (d) IL-6 secretion (data not shown); and (e) Kinetics of migration to the inflammation *foci* (data not shown). Our results thus support the notion that EPO does not affect neutrophil function, either directly or indirectly. Importantly, results showing that EPO has no effect on neutrophils supports the safety profile of EPO, especially considering the recent concerns that were raised

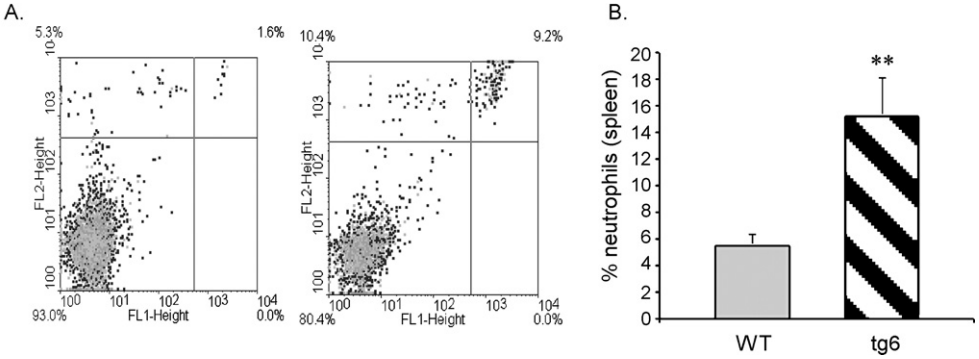

Fig. 1. tg6 mice exhibit a higher percentage of splenic neutrophils. Splenocytes were collected and stained with anti-Gr-1 and anti-CD11b antibodies conjugated to Fluorescein Isothiocyanate (FITC) and Phycoenythrin (PE), respectively. (A) Spleen samples were analyzed by flow cytometry, and the percentage of total $Gr\text{-}1^+/CD11b^+$ cells (top right quadrant) is indicated. Results are representative of 3 independent experiments ($n = 6$ for WT and tg6). (B) Summary of the percentage of $Gr\text{-}1^+/CD11b^+$ positive splenocytes, representing the mean ± S.E.M of 3 independent experiments, each depicting 2 female mice from each genotype, $^{**}p < 0.01$ comparing WT with tg6.

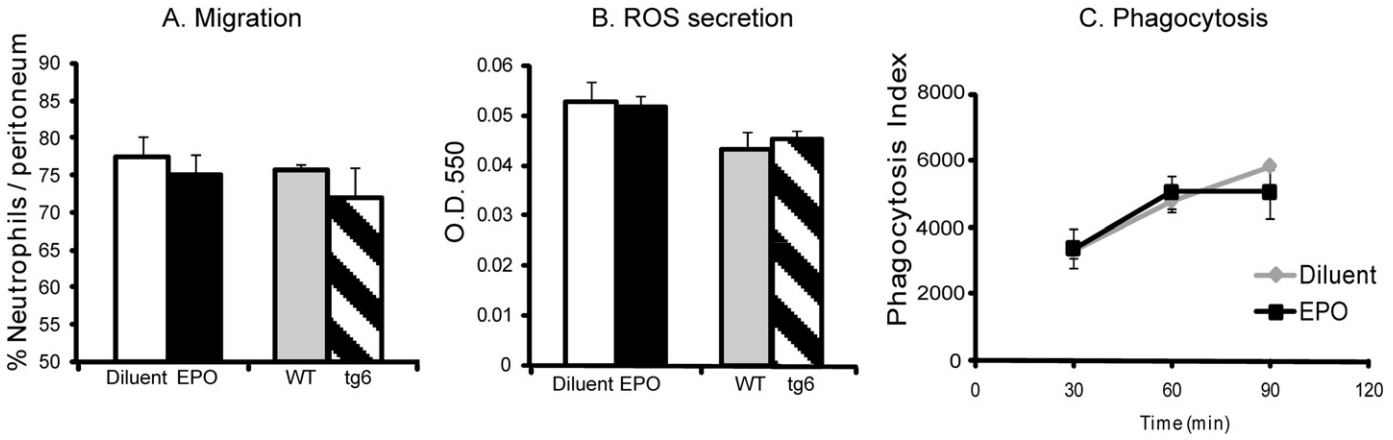

Fig. 2. EPO does not affect the basic functions of peritoneal neutrophils. (A) 3 h following i.p. injection of 0.2% casein, peritoneal cells were collected from C57BL/6 female mice (6–8 weeks old), injected three times with 180U rHuEPO every other day during a week, or with diluent (control), and from female tg6 mice and their C57BL/6 WT littermates (aged 3–5 months). Peritoneal cells were stained with anti-Gr-1 and anti-CD11b antibodies conjugated to FITC and PE, respectively, and analyzed by flow cytometry. The percentage of Gr-1$^+$/CD11b$^+$ cells is indicated. Results are expressed as the mean ± S.E.M of 3 independent experiments (n = 7 mice in each group for EPO and diluent injected C57BL/6 mice, and n = 6 mice for tg6 and WT mice). (B) Peritoneal cells (at least 75% neutrophils) harvested from EPO or diluent-injected Balb/c mice, or from tg6 mice or their C57BL/6 littermates, were cultured *ex-vivo* with or without 0.2 μM phorbol 12-myristate 13-acetate (PMA) for 10 min at 37 °C. Superoxide release was measured by colorimetric detection of cytochrome *c* (horse heart; Sigma) reduction in a spectrophotometer at OD_{550}. Samples were run in triplicates and the results of a representative experiment are presented as mean ± S.E.M (n = 4 for EPO injected and diluent injected mice and n = 2 for WT and tg6 mice). (C) Peritoneal neutrophils were incubated *ex-vivo* with FITC-labeled, heat-killed inactivated *E. coli* for the indicated time periods. Crystal-violet was used for quenching surface-bound bacteria. Uptake of FITC-labeled *E. coli* was determined by FACS analysis on a gated neutrophil population. Results are expressed as the phagocytosis index, defined as the percentage of cells with internalized *E. coli* multiplied by the mean fluorescence intensity. The Graph represents the mean ± S.E.M of 3 independent experiments, each depicting 2 Balb/c mice per experimental group.

regarding the use of the hormone in the treatment of several types of tumors [7].

Taken together, the current results reinforce our recent demonstration that DCs but not neutrophils are the target cells of the immunomodulatory function of EPO [4,5], as previously observed in hemodialysis [1] and multiple myeloma patients [8].

Conflict of interest

None

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