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Nitric oxide depletion alters hematopoietic stem cell commitment toward immunogenic dendritic cells

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ABSTRACT

Background: NO• is a key molecule involved in the regulation of cell survival, proliferation and differentiation in many cell types. In this study we investigated the contribution of NO• during the differentiation of human peripheral blood hemopoietic stem cells ($CD34^+$HSCs) toward immunogenic dendritic cells (i-DCs).
Methods: We depleted autocrine NO• production, using N^G-monomethyl-L-arginine monoacetate (L-NMMA) and paracrine NO•, using oxy-hemoglobin (HbO_2) as a NO• scavenger during *in vitro* differentiation of $CD34^+$HSCs to i-DCs. We monitored the NO• level, cell proliferation, phenotype and differentiation potential.
Results: We found that the depletion of paracrine or autocrine NO• correlated with (I) an active proliferation state at the end of differentiation, when control cells were not proliferating; (II) a significant reduction in the expression levels of differentiative markers (CD1a and HLA-DR) with a parallel high expression of the CD34 marker (III) with a retrieved clonogenic ability compared to control cells.
Conclusions: On the whole, our data indicate that the depletion of NO• during the *commitment* stage blocks $CD34^+$HSC differentiation into i-DCs and maintains an undifferentiated, highly proliferating cell population, indicating/revealing a novel role for NO• in the *commitment* of $CD34^+$HSCs into i-DCs.
General significance: The essential finding of the present study is that NO•, produced in HSCs by NOS enzymes, may act as autocrine and paracrine effectors regulating the *in vitro* differentiation process of $CD34^+$-HSCs toward i-DCs.

1. Introduction

Nitric oxide (NO•) is a free radical endogenously synthesized from L-arginine by a group of enzymes known as nitric oxide synthases (NOSs), which exist as 3 different isoforms known as neuronal (NOS1, nNOS), inducible (NOS2, iNOS) and endothelial NOS (NOS3, eNOS) [1,2].

NO• operates as an endogenous messenger in most cells in both autocrine and paracrine signaling modes. At high concentrations it interacts with reactive oxygen species forming peroxinitrites that modify proteins and induce short and long term signaling cascades [3–7]. The main physiological target of NO• is the cyclic GMP (cGMP)-synthesizing enzyme referred to as soluble guanylyl cyclase [8–11]. NO• is a key molecule that regulates vasodilatation, immunity, and neurotransmission and is also involved in the regulation of cell survival and proliferation in many cell types [4,5,12,13], as well as in the proliferation and differentiation balance along several developmental and differentiation settings [14–17]. It is reported that within the hematopoietic system, NO• is involved in the regulation of hematopoietic stem (HSCs) and progenitor cells in bone marrow [18–22]. In the immune system NO•, synthesized by inducible NO• synthase (iNOS), is necessary for the immunostimulatory function of murine and human monocyte-derived dendritic cells (DCs). In this regard, specific inhibitors of NOS (N-nitro-L-arginine methyl ester) have been seen to prevent DC maturation and preserve an immature phenotype, indicating a potential autocrine effect of nNOS-derived NO• on human DC maturation [23–26]. Over the last few years we have been studying the molecular events occurring during the commitment process of human peripheral blood $CD34^+$HSCs toward i-DCs [27–30].

In this paper we have investigated the role of NO• during this biological process. We found a peak of NO• production at the time of stem cell *commitment* after which it decreased through the differentiation process with the lowest level found at complete differentiation of i-DCs. We found that the inhibition of NOS with a chemical compound (N^G-monomethyl-L-arginine, monoacetate) or the depletion of paracrine NO• with the use of a NO• scavenger

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(oxy-hemoglobin) blocked the differentiation process of i-DCs from $CD34^+$HSCs and preserved the stem cell commitment stage for a long time in culture.

2. Materials and methods

2.1. Isolation of human HSCs

Blood samples were collected from healthy volunteers who provided informed consent. Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Paque™ PLUS (GE Healthcare), after which $CD34^+$cells were purified through immunomagnetic selection using the mini-magnetic-activated cell sorter (MACS) system (Miltenyi Biotec). The purity of the $CD34^+$-enriched population was determined by immuno-labeling the cells with a monoclonal antibody anti-CD34 FITC-conjugated (Miltenyi Biotec Gmbh, Germany), that reacted with an epitope other than the antibody used for separation. After two Mini-MACS column separations, cells were 90% to 95% $CD34^+$.

Following this purification, to deplete monocytes that are $CD4^{low}$ positive PBMCs were cultured in RPMI supplemented with FCS (10%), 100 U/ml pen/strep and 2 mM L-glutamine, for 2 h at 37 °C in a humidified incubator. Then $CD4^+$ T lymphocytes were purified by immunomagnetic selection by MACS technologies using the anti CD4-microbeads monoclonal antibody (Miltenyi Biotec Gmbh, Germany).

2.2. Hemoglobin purification

Human oxy-hemoglobin (HbO_2) was purified from fresh hemolysate and purified as previously described [31,32]. Briefly red blood cells were lysed with hypotonic phosphate buffer and centrifuged to eliminate the ghosts at 14,000 $g \times 10$ min using a MiniSpin plus (Eppendorf). Hemoglobin was purified by gel filtration using a Sephadex G-25 column (60 × 2 cm) equilibrated with PBS, pH 7.4 at 4 °C and analyzed by light absorption from 300 to 750 nm using a Cary 50 spectrophotometer (Varian Analytical Instrument Inc.).

2.3. Generation of i-DCs from $CD34^+$HSCs

To generate immunogenic dendritic cells, CD34-enriched cells were transferred to 96 wells at a density of 10^5 cells/ml and cultured for 14 days in RPMI-1640 medium (Euroclone) containing 10% fetal calf serum (Euroclone), 100 U/ml pen/strep, 2 mM L-glutamine and a cytokine cocktail composed of human recombinant Flt3L (50 ng/ml), GM-CSF (50 ng/ml), IL-4 (10 ng/ml), and TNF-α (2.5 ng/ml) (PeproTech EC), named basal cocktail (BC). Every three days, half of the culture medium was replaced with fresh medium supplemented with the same cocktail. Cells were analyzed after 3, 7 and 14 days of cytokine induction to evaluate the expression of specific differentiation markers [27–30].

2.4. $NO^\bullet$ inhibitor treatment

CD34-enriched cells (3×10^4) were cultured in 96 wells in 300 μL of RPMI-1640 + 100 U/ml pen/strep, 2 mM L-glutamine *plus* BC cytokines for 14 days in the presence of 5 mM N^G-monomethyl-L-arginine, monoacetate (L-NMMA) as NOS inhibitor (Sigma-Aldrich) [33], or 10 μM oxy-hemoglobin (HbO_2) as $NO^\bullet$ scavenger [34], as well as in the presence of both L-NMMA *plus* HbO_2. A fresh solution of L-NMMA was prepared immediately before use. L-NMMA and HbO_2 were added on the first day and replaced every three days together with a cytokine enriched medium.

2.5. $NO^\bullet$ donor treatment

To increase the $NO^\bullet$ concentration sodium nitroprusside (SNP) was used as a source. Briefly, CD34-enriched cells were cultured in 96 wells at a density of 10^5 cells/ml and cultured for 14 days with a cytokine enriched medium as described above, in the presence of 50 nM SNP. A SNP (Sigma-Aldrich) solution was made immediately before use and added directly to the cell culture medium.

2.6. Real time RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). Reverse transcription was carried out using 1 μg of total RNA in the presence of 200 U of SuperScript® II Reverse Transcriptase and random hexamers (Invitrogen). Real-time RT-PCRs were performed using 5 ng total RNA cDNA converted and primers for NOS1, NOS2 and NOS3 genes were from Sigma (see Table 1 for sequences) and Brilliant® II SYBR® Green QPCR Master Mix (Agilent Technologies). A MX 3000P™ Sequence Detector System (Stratagene) was used to collect the signals. Relative quantification of gene expression was determined using GAPDH (see Table 1 for sequences) as an endogenous control [35].

2.7. Electrochemical assay detection of $NO^\bullet$ metabolites

$NO^\bullet$ present in culture media was determined by means of its auto-oxidation derivative nitrite (NO_2^-) using an electrochemical apparatus [36–38] consisting of a reaction vessel (5 ml) containing 2 ml of 0.1 M $CuCl_2$ and 0.1 M Cys, equipped with an injector, maintained at a constant temperature (25 °C), and supplied with a flow of 5 ml/min of N_2. Cell culture medium was subjected to centrifugation with a Centricon filter device (1 h at 10,000 ×*g*, cut off of 10 kDa) before being subjected to the determination of nitrite by the electrochemical assay. Aliquots of the biological samples (20–50 μl) were injected into the reaction vessel of the electrochemical apparatus for chemical reduction. The $NO^\bullet$ formed in the vessel was carried by N_2 current to the amperometric sensor used as an electrochemical detector of the apparatus. The apparatus was calibrated by injecting known amounts of standard nitrite solution into the reaction vessel.

2.8. Analysis of in vitro proliferation by BrdU incorporation

BrdU (5-bromo-2′-deoxyuridine) incorporation was performed after 14 days in stem cells grown on basal cocktail in the presence/absence of HbO_2, L-NMMA or HbO_2 *plus* L-NMMA. Cells were pulsed with 10 μM BrdU for 5 h at 37 °C in a humidified incubator with 5% CO_2. The procedure was carried out according to the BD Pharmingen™ manufacturer. Briefly, cells were resuspended in PBS and centrifuged using a Heraeus-Christ Digifuge-GL cytospin at 700 rpm for 7 min. Samples were fixed in 4% paraformaldehyde for 30 min, washed with PBS, treated with blocking solution (10% FBS, 0.1% Triton X-100 in PBS) and incubated overnight at 4 °C with a primary antibody. Fixed cells were permeabilized and blocked in PBS, 0.5% Tween 20 (Sigma)

Table 1
Primers used for real time-PCR amplification.

Gene	Sequences (5′–3′)	Accession number	Tm (°C)
NOS1 sense primer	TCGGAAGCTAGTTACCAT	NM_000620	54.7
NOS1 anti-sense primer	CTTGAAGAGACGAACAGAA		55.4
NOS2 sense primer	CCAGATAAGTGACATAAGTGA	NM_000625	54.2
NOS2 anti-sense primer	GGTGGAATTTGGTCTTGA		58.4
NOS3 sense primer	CAAGGAGACGAAGAGAAC	NM_000603	54.6
NOS3 anti-sense primer	TCTAAGGTTTTAGCCTGTAA		53.4
GAPDH sense primer	TAACTCTGGTAAAGTGGATATTGT	NM_002046	57.4
GAPDH anti-sense primer	GATCTCGCTCCTGGAAGA		60.7