Erythropoietin and primed leukocyte: modulation of gene expression profile

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Introduction

Anaemia is an inevitable condition that develops early in the course of chronic kidney disease (CKD) and increases in frequency during the further decline of glomerular filtration rate [1]. With the advent of erythropoiesis-stimulating agents (ESAs) in the 1990s, great improvements were achieved in the treatment of anaemia in the majority of patients with end-stage renal disease, although about 10% of all treated patients remain anaemic despite large ESA doses; those patients are known as resistant to ESA treatment [2,3]. A number of factors have been implicated as contributors to ESA resistance, though persistent inflammation with higher levels of pro-inflammatory cytokines is increasingly being appreciated as the major cause of EPO hyporesponsiveness[4,5,6,7]. Pro-inflammatory cytokines can, indeed, cause erythropoietin (EPO)- resistance, inhibiting the in vitro growth of erythroid progenitors [8,9,10] and interfering with iron homeostasis. The bond between EPO-α and EPO receptor (EPOR) in PBMCs leads to a reduction in inflammation [11,12]. However, EPO administration was also found to further augment the LPS-induced increase in plasma levels of TNF-a and IL-6 and to attenuate the decrease in lymphocyte number [13]. These findings suggest that the effects of EPO treatment on inflammatory mediators are strictly dependent on the phase and intensity of the inflammatory condition present during drug administration.

Materials and Methods

In the present study we applied gene expression microarray technology to identify the differentially expressed genes in PBMCs, primed or not, when exposed to human recombinant erythropoietin-α (EPO-α). Buffy coats were collected from the blood of 10 healthy individual donors. PBMCs obtained from each subject were prepared under identical experimental procedures and were divided into four samples for microarray experiment: (1) control cells; (2) cells stimulated with TNF-α (10 ng/mL, 30 min); (3) cells stimulated with EPO-α (20 U/mL, 15 min); (4) cells pre-stimulated with TNF-α (10 ng/mL, 15 min) and then treated with EPO-α (20 U/mL, 15 min). Real-time PCR experiments were used to validate expression of the molecular targets considered as most relevant to inflammation.

Table I. Sequences of human primers used for real-time PCR analysis.

Species	Gene name	Reference sequence	Primers
Homo sapiens	GADPH	NM_002046	For GCGCCCAATACGACCAA
			Rev GCTCTCTGCTCCTCCTGTT
	IL1A	NM_000575.3	For TCCCAATCTCCATTCCCAAAC
			Rev CTCTACCAAGGACCAGAGAGAA
	IL1B	NM_000576.2	For CAAAGGCGGCCAGGATATAA
			Rev CTAGGGATTGAGTCCACATTCAG
	CXCL1	NM_001511.3	For GGAACAGAAGAGGAAAGAGAGAC
			Rev TAGGACAGTGTGCAGGTAGA
	CXCL5	NM_002994.3	For CCTGAAGAACGGGAAGGAAA
			Rev CTGCTGAAGACTGGGAAACT
	IL8	NM_000584.3	For CTTGGCAGCCTTCCTGATTT
			Rev GGGTGGAAAGGTTTGGAGTATG
	CXCR2	NM_001557.3	For CTCGTGATGCTGGTCATCTTAT
			Rev CAAGGTCAGGGCAAAGAGTAG
	CCL8	NM_005623.2	For TCATTGTTCTCCCTCCTACCT
			Rev GCACTGATTGCCAAAGAATACC

Quantitative measurements of human cytokines were assayed using specific ELISA development systems to detect IL-1β and IL-8.

Results

The results obtained by microarray analysis showed that the biological functions modulated by treatment with each stimulus either alone or in combination, involved key functions of immune system regulation. The alteration of gene expression is mainly implicated in regulation of cellular development, though also in PBMCs dynamic processes, such as cell movement and cell to cell interaction. These genes encode for chemokines, soluble mediators of inflammation CXCL1, CXCL5 and CCL8. Table 2 shows the values of fold change (FC) for the above-mentioned genes and for the β isoform of IL-1.

Table II. Expression variations for key genes involved in inflammation obtained in microarray analysis.

*significant genes considering the FDR=0%; ns=not significant.

	TNF-α FC (mean ± SD)	EPO-α FC (mean ± SD)	TNF-α + EPO-α FC (mean ± SD)
IL1A	ns	ns	5.68 ± 1.35*
IL1B	2.86 ± 2.53	ns	7.87 ± 6.08
IL8	3.19 ± 1.27*	ns	ns
CXCL1	ns	ns	3.19 ± 0.40*
CXCL5	ns	ns	3.34 ± 0.55*
CCL8	2.26 ± 1.19*	ns	6.12 ± 1.65*
CXCR2	ns	ns	-4.54 ± 1.72*
FES	ns	ns	-1.71 ± 0.16*

The anti-inflammatory action of EPO-α transpires from the normalization of the expression on the transcript of pro-inflammatory cytokine IL-8 and the down-regulation of gene coding for its receptor CXCR2. Also, the gene encoding for kinase Feline Sarcoma Oncogene (FES), involved in regulating the hematopoietic process and chemotaxis, is significantly down-regulated in cells treated with both stimuli. In order to quantitatively evaluate the changes to gene expressions obtained by microarray, we performed qPCR experiments which show similar trends of expression obtained in microarray. In particular, the expression of IL-1β that is significantly induced by TNF-α further increases upon treating primed PBMCs with EPO-a. Since our data suggested some controversial effects by EPO-a on pro-inflammatory mediators, particularly. on TNF-α up-regulation of IL-1β and IL-8, we further investigated the mechanisms involved in this process, analyzing the expression of MAPK p38a and its relative phosphorylated isoform [14,15].

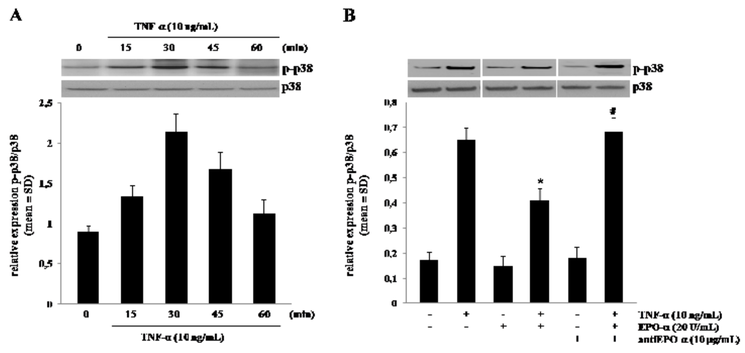

Fig. 1. Effects of TNF-α and EPO-α treatment on p-p38 expression. A) The expression of p-p38a(Thr180/Tyr182) and p38a proteins in representative time course Western blot experiments (top). At the bottom, relative expression of p-p38a/p38a (mean SD, n=3) in PBMCs primed with TNF-α. B) Western blotting representative image of p-p38a and p38a proteins expression in PBMCs treated or not with TNF-α (10 ng/mL, 30 min), and EPO-α and/or antiEPO-α (top).

Discussion and Conclusions

We focused our attention on expression modulation as exerted by EPO-α on primed PBMCs, and started functional analysis of the kinases involved at a post-receptor level. Microarray data showed genes strictly related to cytokine signaling,. We have confirmed that pro-inflammatory cytokines of the IL-1 family exert various EPO-α effects on PBMCs, whether primed or not. The cytokines IL-1α and β have already been strongly associated with establishment and maintenance of the anaemic state in CKD patients, Again, we propose the genes of chemokines CXCL1, CXCL5 and CCL8 be considered as potential new biomarkers for EPO-resistance. These chemokines are involved in the chemoattraction of leukocytes to inflammatory sites and strongly contribute to chronic inflammation [16]. On the other hand, we observed negative modulation in the case of important molecules involved both in the early stages and in the continuation of inflammation, confirming the anti-inflammatory effect of EPO previously described in vivo [11,12], in particular the expression and secretion of IL-8 and the gene coding for its receptor CXCR2 significantly down-regulated upon applying both stimuli. All together, our results show that the effects of EPO-α on inflammatory mediator expression are somewhat controversial. To clarify the mechanisms underling these processes, we started by considering the post-receptor level, investigating the expression of MAPK p38. We noted that potent and selective inhibition of MAPK p38 significantly increases IL-1β mRNA expression and secretion in primed cells treated with EPO-α. The exact mechanisms of EPO-α treatment on primed cells remain to be clarified. To this end, we aim in future to further integrate our expression data with functional analysis of the kinases. We also intend to measure in vivo the molecular targets we have highlighted, in order to shed light on any additional markers in patients hypo-responsive to EPO.

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DOI: 10.3252/pso.eu.51era.2014