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The expression of endothelin-1 in chronic lymphocytic leukemia is controlled by epigenetic mechanisms and extracellular stimuli

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ABSTRACT

Endothelin-1 (ET-1) is a hormone peptide widely expressed and is involved in several biological processes, important not only for normal cell function but also for tumor development, including cell proliferation, invasion, metastasis, angiogenesis and osteogenesis. In accordance, ET-1 was already shown to contribute to the growth and progression of many different solid cancers. We recently demonstrated that ET-1 has a role in the pathogenesis of chronic lymphocytic leukemia (CLL) where it is abnormally expressed. In the context of this malignancy, ET-1 is able to mediate survival, drug-resistance and growth signals in leukemic cells. Previous studies, not conducted in CLL, have shown that ET-1 regulatory mechanisms are numerous and cell specific. Here, we valued the expression of ET-1 in CLL, in relation to DNA methylation but also in response to stimulation of some important pathways for the dialogue between CLL and microenvironment. We found that a high methylation of ET-1 first intron affects the basal expression of ET-1 in CLL. Moreover, we showed that the activation of CD40 or Toll-like receptor (TLR) by extracellular stimuli produces an augment of ET-1 level in CLL cells. Finally, we demonstrated the fundamental role of NF-kB signalling pathway in promoting and maintaining ET-1 expression in CLL cells, both in basal conditions and after CD40 activation.

1. Introduction

Endothelin-1 (ET-1) is the most abundantly and widely expressed member of the endothelin family of proteins [1]. Transcription of ET-1 gene gives a 2.8-kb mRNA that encodes the preproET-1 [2]. A leader sequence targets the preproET-1 to the endoplasmic reticulum where it enters the secretory pathway [3]. Prior to exocytosis, furin-like proteases cleave the preproET-1 to big ET-1 [4]. Finally, endothelin-converting enzymes cleave the big ET-1 into active ET-1 [5]. Although all these post-translational processing steps, transcriptional regulation is thought to be the major mechanism controlling ET-1 bioavailability. From previous studies, ET-1 gene is known to be regulated by more than twenty different stimuli, each acting in selected tissues. In response to those stimuli, at least ten different transcription factors bind to the promoter proximal region [6]. In specific cell contexts, ET-1 expression can also be modulated by DNA methylation or histone modification. In particular, several CpG located in the first ET-1 intron are subject to methylation. In fibroblasts expressing low ET-1 amounts, this region was found to be hypermethylated, whereas in renal collecting duct cells with high ET-1 levels, the gene resulted to be hypomethylated [7]. In renal epithelial cells the methylation of histone H3 lysine 4 residues was associated with ET-1 induction by aldosterone [8].

Alterations of ET-1 expression have been documented in various human diseases including atherosclerosis, cardiomyopathy and cancer [9–11]. In several tumor cells, the ET-1 axis was shown to be relevant in leading to autocrine/paracrine feedback loops, which promote the development and progression of cancer. Such processes include cell proliferation, escape from apoptosis, angiogenesis, invasion, metastatic dissemination and aberrant osteogenesis [12]. Our research group has recently showed that ET-1 is abnormally expressed in chronic lymphocytic leukemia (CLL) cells. We observed an enhanced resistance to spontaneous apopto-

Abbreviations: ET-1, Endothelin-1; CLL, Chronic Lymphocytic Leukemia; ETAR, Endothelin receptor type A; TLR, Toll-like receptor; PBMC, peripheral blood mononuclear cell; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MSP, Methylation Specific PCR; FBS, Fetal bovine serum; DAC, 5-aza-2′-deoxycytidine; IL-4, Interleukin 4; IL-2, Interleukin 2; AKT, Protein kinase B; MEK1, Mitogen-activated protein kinase kinase; NF-kB, Nuclear Factor kappa-light-chain-enhancer of activated B cells; MFI, Mean Fluorescence Intensity; SDS, Sodium dodecyl sulfate; TCL-1, T-cell leukemia 1; ANG-2, Angiopoietin 2; HDAC, Histone deacetylase; PI3 K, PhosphatidylInositol 3-Kinase; ERK, Extracellular signal–regulated kinase; TK, Tyrosin kinase.

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sis in CLL cells cultured with recombinant ET-1, due to the activation of survival pathways throughout ETAR. We also found that ET-1 reduces the cytotoxic effect of fludarabine on CLL cells cultured alone or on endothelial layers [13]. Since these data suggest an important role of ET-1 in CLL pathogenesis, we looked for possible biological mechanisms responsible for its abnormal production, which have not been explored yet in this leukemia. Previous studies in CLL have revealed a number of dysregulated molecules because of aberrant gene methylation [14–17]. Interestingly, several overexpressed genes important for leukemia pathogenesis were found to be hypomethylated [18–21]. On the other hand, CLL expression profile is strongly influenced by extracellular signals, coming from microenvironment and involving for example the pathways of CD40 and Toll-like receptor (TLR). CD40 is expressed on B-lymphocytes, dendritic cells, macrophages and hematopoietic progenitor cells [22]. Its ligand (CD40L) is found on activated T lymphocytes and the interaction CD40/CD40L induces B-cell activation and proliferation, Ig secretion, memory cell formation, and isotype switching [23]. In CLL, the triggering of CD40 by anti-CD40 antibody was found to induce survival molecules and inhibit the apoptosis by fludarabine [24–26]. Several TLRs are expressed by memory B cells at constitutively high levels [27] and CLL cells are characterized by a similar set, including TLR-1, TLR-2, TLR-6, TLR-7 and TLR-9 [28]. CLL stimulation with TLR agonists was previously found to promote the proliferation of leukemic blasts [29]. Considering these evidences, in the present study we searched for an eventual role of DNA methylation and/or stimulation of CD40 and TLR signalling pathways in the regulation of ET-1 expression in CLL, and we demonstrated that both mechanisms are someway involved.

2. Materials and methods

2.1. Patients and healthy samples

Peripheral blood mononuclear cells (PBMCs) of 25 untreated CLL cases (Table 1) diagnosed at Hematology Division of Modena and 4 age-matched healthy blood donors (HD) afferent to Transfusional Division of Modena were collected and preserved in liquid nitrogen. All people provided informed consent in accordance with local institutional review board requirements and the Declaration of Helsinki Principles. To purify CLL or normal B lymphocytes, PBMCs were thawed, incubated with CD19-specific Microbeads (Miltenyi Biotech) and separated by AutoMACS (Miltenyi Biotech), obtaining a purity >99%.

2.2. Study of mRNA expression

Total RNA was extracted using RNeasy Mini Kit (Qiagen) and reverse-transcribed with random primers. Ten nanograms of cDNA were analyzed in Real-Time PCR on LightCycler 480 v.2 (Roche) using SYBR Green Master Mix (Applied Biosystems) and specific primers. The relative expression of ET-1 to the house-keeping gene GAPDH was calculated using the ΔCt method and normalized to a calibrator sample (Universal Human Reference RNA; Stratagene).

2.3. DNA methylation analysis

Genomic DNA was extracted using QIAamp DNA mini kit (Qiagen) and bisulfite-converted with EpiTect Bisulfite Kit (Qiagen). We designed sets of primers for Methylation Specific PCR (MSP) and bisulfite sequencing on ET-1 first gene intron by www.urogene.org/methprimer (Suppl. Fig. 1). MSP for the methylated or unmethylated DNA sequence was performed on converted DNA samples and results were confirmed by bisulfite sequencing. Commercial fully methylated or unmethylated converted DNA samples (EpiTect PCR control DNA set; Qiagen) were used as controls.

Table 1
The characteristics of CLL cohort.

Features		
Age	years	
Median	69	
Range	32–81	
Follow up	months	
Median	77	
Range	25–1330	
TTFT	months	
Median	50	
Range	1–1316	
	N° patients	%
Sex		
Male	17/25	68,0
Female	8/25	32,0
Binet stage		
A	19/23	82,6
B–C	4/23	17,4
IGHV mutational status		
Mutated (<98%)	9/25	36,0
Unmutated (≥98%)	16/25	64,0
CD38 expression		
CD38 negative (<30%)	16/22	72,7
CD38 positive (≥30%)	6/22	27,3
ZAP70 expression		
ZAP70 negative (<20%)	10/20	50,0
ZAP70 positive (≥20%)	10/20	50,0
FISH stratification		
Low risk	18/22	81,8
Intermediate/high risk	4/22	18,2
Treatment		
Yes	18/25	72,0
No	7/25	28,0
Death censored		
Yes	5/25	20,0
No	20/25	80,0

Low FISH risk = no abnormalities or del(13q); Intermediate/high FISH risk = del(11q), del(17p) or trisomy 12. Abbreviations: FISH = fluorescence in situ hybridization; IGHV = immunoglobulin heavy chain variable genes; TTFT = time to first treatment.

2.4. In vitro *treatment with DNA methylation inhibitor*

RAMOS cells were plated in RPMI medium with 20% fetal bovine serum (FBS; Sigma-Aldrich) in a 24-well plate. The following day, cells were treated with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (DAC; Sigma-Aldrich) 1 μM, 5 μM or 10 μM, by refreshing DAC-supplemented medium every day. In parallel control cells were cultured without drug. After 48 h, cells were collected for analysis.

2.5. In vitro *CLL stimulation and drug treatments*

CLL cells were resuspended in RPMI + 10% FBS, plated in a 24-well plate and stimulated with CD40L 100 ng/ml (PeproTech) + IL-4 20 ng/ml (PeproTech) for CD40 activation or Type B CpG oligonucleotides 1 μg/ml (ODN 2006; InvivoGen) + IL-2 100U/ml (PeproTech) for TLR activation. Control cells were cultured in parallel without stimulations. After 4 h, cells were collected for evaluation. In some experiments, before CD40 activation, cells were treated for 30 min with wortmannin 150 nM for AKT inhibition (Sigma-Aldrich) or PD98059 50 μM for MEK1 inhibition (Sigma-Aldrich) or BAY 11-7085 5 μM and 10 μM for NF-kB inhibition (Selleckchem).

2.6. Immunofluorescence

CLL cells were plated in RPMI + 10% FBS on coverslips in a 24-well plate and the following day, they were stimulated/blocked as described in paragraph 2.5. After 4 h, cells were fixed/permeabilized on coverslips. After washes, anti ET-1 anti-