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Research Article

Epigenetic control of endothelin-1 axis affects invasiveness of breast carcinoma cells with bone tropism

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ABSTRACT

Here, we report a complex regulation of endothelin-1 (ET-1) axis driven by epigenetic reactions in 1833-bone metastatic cells, emphasizing the importance in skeletal metastasis from breast carcinoma. Inhibitors of histone deacetylases, trichostatin A (TSA), and of DNA methylases, 5′-Azacytidine (Aza), caused, respectively, reduction and increase in 1833 cell invasiveness, without affecting the basal migration of parental MDA-MB231 cells. Of note, in the two cell lines exposed to Aza the blockade of the ET-1 receptor ET_AR with BQ-123 oppositely changed invasive properties. Even if in MDA-MB231 cells the ET-1 axis was scarcely influenced by epigenetic reactions, ET_AR remarkably decreased after Aza. In contrast, in 1833 cells Aza exposure enhanced ET-1 coupled to ET_AR wild type, being also ET_AR truncated form increased, and invasiveness was stimulated. Under demethylation, the increase in ET-1 steady state protein level in 1833 clone seemed regulated at transcriptional level principally via Ets1 transcription factor. In fact, actinomycin D almost completely prevented ET-1 mRNA induction due to Aza. Only in 1833 cells, TSA exposure inactivated ET-1 axis, with reduction of the expression of ET-1 and ET_AR mutated form, in agreement with Matrigel invasion decrease. This treatment favoured the ET-1 repressional control, taking place at the level of mRNA stability due to the 3′-untranslated region in the ET-1 gene, and also decreased transcription via NF-*k*B. Environmental conditions that alter the balance between epigenetic reactions might, therefore, affect metastasis migratory mode influencing ET-1 axis.

Introduction

Mounting evidence indicates that epigenetic mechanisms influence tumor growth and metastasis phenotype [1,2], but their role in the control of endothelin axis in bone metastatic cells is scarcely known.

Endothelin-1 (ET-1) binds to G-protein-coupled receptors ET_AR and ET_BR. The activation of ET_AR triggers signaling networks involved in cell proliferation, new vessel formation, invasion, inflammation and metastatic spread [3–6]. Stroma remodeling via matrix metalloproteases 2 and 9 (MMP2 and 9), critical for metastasis, is influenced by ET_AR silencing [7]. A truncated form of

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ET_AR, that does not bind ET-1, is present in Ewing sarcoma, choriocarcinoma and melanoma cells [8–10]. ET-1 axis is frequently activated in a variety of human malignancy, including breast cancer, and sustained autocrine ET-1/ET_AR signaling contributes to epithelial to mesenchymal transition [11]. ET-1 production is stimulated by cytokines and growth factors, hypoxia and shear stress [3], leading to suppose that tumor-microenvironment stimuli might influence ET-1 expression also through epigenetic mechanisms. The epigenetic control of gene expression seems deterministic rather than stochastic [12].

The balance of acetylation/deacetylation as well as methylation state of chromatin affect the expression of the genes also through the control of transcription-factor activities [13,14], influencing metastasis plasticity [1,15,16]. Histone deacetylases (HDACs) are emerging as critical regulators of cell growth, differentiation and apoptosis. HDAC activity in human tumors leads to conformational changes within the nucleosome, which result in the transcriptional repression of genes during migration and metastasis. Changes of HDAC 1, 2 and 3 expressions are associated with prognosis in patients with invasive breast cancer [17]. HDAC6 modulates migration and metastasis by interacting with cortactin [18], and by blocking breast cancer metastasis suppressor 1 [19]. Hypermethylation includes promoters of tumor suppressor genes whose expression becomes repressed, thereby facilitating cancer formation [20]. Conversely, extensive blockade of methylation reactions partially hampers bone metastasis [1].

In the present paper we study how ET-1 expression and cellular invasiveness are modulated by acetylation and demethylation state in 1833 cells with bone tropism, and in parental breast carcinoma MDA-MB231 cells. HDACs were blocked with trichostatin A (TSA), and methylases inhibition was carried out with 5′-Azacytidine (Aza). Little is known about potential regulatory mechanisms controlling ET-1 expression at epigenetic level in metastatic cells. Molecular analysis of the sequences upstream of exon 1 in the human ET-1 gene reveals the putative binding sites for transcription factors, which are important for cell type control of ET-1 gene expression in response to different stimuli [21,22]. However, ET-1 might undergo not only transcriptional but also post-transcriptional regulation via mRNA degradation exerted by a genetic element present in the 3′-untranslated region (3′-UTR) of the gene [23].

Aza treatment of 1833 clone increased ET-1 axis, with induction of ET-1 and ET_AR protein levels, leading to invasiveness enhancement. ET-1 protein level depended on the amount of mRNA, undergoing transcriptional regulation. The presence of the 3′-UTR in ET-1 gene did not seem to counteract the Aza-dependent enhancement of transactivation. Oppositely, TSA favored the known repressional control exerted by the 3′-UTR in ET-1 gene. HDACs1 and 3 but not HDAC6 reverted the TSA inhibitory effect on ET-1 transactivation, indicating a specific involvement of HDACs. TSA or Aza did not affect invasiveness of MDA-MB231 cells consistent with their scarce partial effect on ET-1 axis.

Materials and methods

Reagents and plasmids

TSA, Aza and actinomycin D were from Sigma-Aldrich (St. Louis, MO). Anti-HDAC1 and anti-ET_AR were from Abcam (Cambridge, UK); anti-HDAC3 was from Cell Signaling (Beverly, MA); anti-HDAC6 was from Millipore (Billerica, MA). BQ-123 was purchased from Enzo Life Sciences (Lausen, Switzerland). Anti-ET-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). SN50 was from Alexis (Lausen, Switzerland). The gene reporters were: Endothelin-1 promoter (−3580/+560, kindly given by S. Li, Pittsburg, PA); Endothelin-1-promoter fragment (−650/+173) with 3′-UTR (kindly given by F. Rodriguez-Pascual, Madrid, Spain); Endothelin-1-promoter fragment (−650/+173) without (3′-UTR), that was prepared in our laboratory by removing the 3′UTR with XbaI and subcloning into pGL3-Basic vector; the constructs containing the multimer for Hypoxia-responsive element (6-HRE, pGL3PGK6TKp) and for NF*k*B-binding site (3-NFkB) were kindly given by P.J. Ratcliffe, Wellcome Trust Centre for Human Genetics, Oxford, U.K. and M. Hung, Anderson Cancer Center, Holcombe, Houston, TX [24]; Ets1Luc containing 5 Ets1 consensus sequences was prepared from p600Luc construct [25]. The expression vectors were: HDAC1 and HDAC3 (from E. Seto, Tampa, FL); pRC-CMV-HDAC6 (from S. Hayashi, Sendai); the dominant negative form of ARNT/HIF-1β (pcDNA3ARNTdelta_b, ΔARNT) was from M. Schwartz, Tübingen, Germany [26]. 1833 cells were obtained from R. Gomis (IRB, Barcelona, Spain), and MDA-MB231 cells were kindly given by J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY).

Transient transfection assay

Cells, seeded in 24-multiwell plates, were transfected using Fugene 6-Roche with 200 ng/well of the gene reporters or the expression vectors for HDACs, or equivalent amounts of the corresponding empty vectors, and exposed to 2.5 μM TSA for 1 or 2 days, or to 1 μM Aza for 2 days: the treatments were performed daily. Some cells treated for 2 days with TSA or Aza were concomitantly exposed to 50 μg/ml SN50, (NF-*k*B) inhibitor [24], or co-transfected with 1 μg/well of ΔARNT. Cells treated with 0.1 μM Aza for 30 days were transfected during the last 24 h [1]. For normalization the cells were transfected with pRL-TK (*Renilla* luciferase) vector, and Firefly/*Renilla* luciferase activity ratios were calculated by the software [1].

Evaluation of ET-1 mRNA transcriptional regulation

1833 cells seeded into 6 multiwell plates, were treated with 5 μg/ml actinomycin D [27] together with the first 1 μM Aza exposure that was repeated 16 h apart. Also control 1833 cells, exposed to the vehicle, were concomitantly treated with actinomycin D. All the cells were harvested at various times thereafter, and the RNA was extracted using RNeasy mini kit (Qiagen, Milan Italy). The retrotranscription of 1 μg of RNA was performed and PCR analysis was performed using TaqMan® Gene Expression Assays for EDN1 (endothelin-1) (Assay ID Hs00174961_m1, Life Technologies, Carlsbad, CA). Relative mRNA levels in cells exposed to Aza, Aza plus actinomycin D, and actinomycin D alone were calculated vs. control sample at time 0, set as 1, after normalization for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels (Assay ID Rn01775763_g1, Life Technologies).

Western blot assay

Samples of total extracts (100 μg of proteins) from cells untreated, inhibitor treated or transfected with the expression vectors for HDACs were analyzed [1]. The antibody dilutions were: HDAC1 (1:1000), HDAC3 (1:2000), HDAC6 (1:1000), ET-1 (1:200), and ET_AR (1:500).