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Coordinate regulation of microenvironmental stimuli and role of methylation in bone metastasis from breast carcinoma

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ABSTRACT

The pathogenesis of bone metastasis is unclear, and much focus in metastatic biology and therapy relays on epigenetic alterations. Since DNA-methyltransferase blockade with 5-aza-2′-deoxycytidine (dAza) counteracts tumour growth, here we utilized dAza to clarify whether molecular events undergoing epigenetic control were critical for bone metastatization. In particular, we investigated the patterns of secreted-protein acidic and rich in cysteine (SPARC) and of Endothelin 1, affected by DNA methyltransferases in tumours, with the hypothesis that in bone metastasis a coordinate function of SPARC and Endothelin 1, if any occurs, was orchestrated by DNA methylation. To this purpose, we prepared a xenograft model with the clone 1833, derived from human-MDA-MB231 cells, and dAza administration slowed-down metastasis outgrowth. This seemed consequent to the reductions of SPARC and Endothelin 1 at invasive front and in the bone marrow, mostly due to loss of Twist. In the metastasis bulk Snail, partly reduced by dAza, might sustain Endothelin 1-SPARC cooperativity. Both SPARC and Endothelin 1 underwent post-translational control by miRNAs, a molecular mechanism that might explain the *in vivo* data. Ectopic miR29a reduced SPARC expression also under long-term dAza exposure, while Endothelin 1 down-regulation occurred in the presence of endogenous-miR98 expression. Notably, dAza effects differed depending on *in vivo* and *in vitro* conditions. In 1833 cells exposed to 30-days dAza, SPARC-protein level was practically unaffected, while Endothelin 1 induction depended on the 3′-UTR functionality. The blockade of methyltransferases leading to SPARC reduction *in vivo*, might represent a promising strategy to hamper early steps of the metastatic process affecting the osteogenic niche.

1. Introduction

The molecular events underlying the tropism for the secondary organs represent an unresolved problem of the metastatic process. Metastasis to bone is one of the most common and devastating complication in patients with advanced cancers of the breast, prostate or lung [1]. Osteomimetic properties contribute to the preference of breast-carcinoma metastasis for the bone, as exemplified by Endothelin 1 axis that orchestrates signalling pathways including Runt-related transcription factor 2 (Runx2) [2]. Also, the tumour-stroma interaction and the composition of bone microenvironment are critical for skeleton metastatization, favouring specific adhesion/recognition and invasion [1,3–5]. The bone marrow is not conditioned by osseous metastatic cells as extensively as stroma in pulmonary metastasis, and the same mechanisms that govern the homing of hemopoietic stem cells in healthy individuals are co-opted by tumour cells [1].

Secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein, is associated with bone remodelling, repair, development, cell turnover, mineralization process, and collagen fibril assembly [3]. The SPARC function in tumorigenesis and tumour progression is still controversial and not fully understood, and the different expression and activity of SPARC depend on cancer type [6]. These diverse patterns of SPARC would be influenced by tumour microenvironment in terms of local composition of matrix, molecules, cytokines and protease profile, while some inconsistencies are due to SPARC proteolytic products (peptide fragments corresponding to different regions of SPARC) [3].

There are only few studies regarding prostate and breast cancer, that try to disclose the roles of SPARC in bone metastasis [3]. Prostate carcinoma PC3 cells on wild type matrices containing SPARC, show decrease of cell proliferation and resistance to radiation-induced cell death, with an increase in cell spreading [7]. SPARC gene is an early marker of poorly differentiated phenotype, and high SPARC expression at the time of prostatectomy is associated with development of metastasis [8]. Data obtained with breast carcinoma MDA-MB231 cells, showing low SPARC level [9] and added exogenous SPARC, lead to suppose its

Abbreviations: Runx2, Runt-related transcription factor 2; SPARC, secreted protein acidic and rich in cysteine; dAza, 5-aza-2′-deoxycytidine; PPARγ, Peroxisome proliferator activated receptor γ; ROI, regions of interest; PTEN, phosphatase and tensin homolog; ME, mice bearing bone metastasis; CHX, cycloheximide.

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favouring role for metastasis to bone, by stimulating indirectly the motility and chemoattraction towards vitronectin [10]. Adenovirally produced SPARC, however, inhibits *in vivo* metastasis of MDA-MB231 cells into lung and bone, reducing the aggregation of tumour cells with platelets [11].

While normal mammary tissue has undetectable or lightly detectable amounts of SPARC, and benign breast lesions are weakly positive, 75% of both *in situ* and invasive breast carcinomas are strongly positive for SPARC in stromal cells (CD-34-negative, α-SMA-positive) [12,13]; breast carcinoma cells also show SPARC signal [14]. In various carcinomas, high SPARC expression is associated with significantly poorer outcomes compared with low SPARC expression [15].

Much focus in cancer biology and therapy deals with epigenetic alterations that are drivers in neoplastic progression, and targeting the molecular events regulated by DNA methylation may be an useful approach for chemoprevention [16,17]. Even if SPARC is methylated in a 300 bp CpG island spanning from exon 1 to intron 1 in many tumours [18], the knowledge of the biological function and regulation of SPARC by methylation in bone metastasis from breast carcinoma is scarce.

The aim of the present paper was to examine whether endogenous SPARC plays a role in the metastatic process of breast carcinoma with tropism for the skeleton, and whether SPARC is regulated by mechanisms dependent on DNA-methyltransferases, to clarify the relevance of methylation for metastasis outgrowth. To this end, a xenograft model was prepared with the metastatic clone 1833 with bone tropism- derived from invasive MDA-MB231 cells [19]- blocking DNA methyltransferases with 5-aza-2′-deoxycytidine (decitabine, dAza). dAza is a chemotherapic directly incorporated in the newly synthesized DNA strands, that is in phase III trial for myeloid monocyte chronic leukaemia, colangiocarcinoma and colon carcinoma [16,20]. Of note, dAza has never been used to fight bone metastasis from breast carcinoma. The significance of our study *in vivo* would be to identify the network of molecular events implicated in SPARC expression both in metastatic cells and microenvironment under DNA methyltransferases, that might include the transcription factors Twist and Snail, and the biological stimulus Endothelin 1. The additional *in vitro* studies were performed to examine the molecular mechanisms of SPARC expression at transcriptional, translational and post-translational levels, with miRNAs involvement: they would clarify the *in vivo* data. The post-translational mechanism was investigated since SPARC as well as Endothelin 1 are genes containing 3′-UTR regulatory sequences [21,22], which bind miR29a and miR89 [23,24]. The miR29a is enhanced by Peroxisome proliferator activated receptor (PPAR)γ activity [24], which is elevated in 1833 cells [25]. By binding to the 3′-UTR, miRNAs may control mRNA degradation or the translational inhibition of cancer associated protein coding genes [26].

We found that SPARC was highly expressed in bone metastasis and in the bone-marrow cells, and the blockade of DNA methyltransferases slowed-down metastasis outgrowth. Notably, dAza reduced not only SPARC but also Endothelin 1 at the invasive front of bone metastasis and in supportive cells of the bone marrow. The transcription factor Twist seemed to be especially involved in the methylation-controlled signalling pathway for SPARC expression *in vivo* and *in vitro*. In 1833 cells exposed to dAza, exogenous miR29a reduced SPARC expression, and Endothelin 1 transactivation depended on the 3′-UTR. Endothelin 1 protein level was reduced under PPARγ activity, being present miR98 in metastatic cells. These features of DNA methylation pertaining to SPARC in bone metastasis from breast carcinoma might have functional and therapeutic relevance.

2. Materials and methods

2.1. Materials

dAza was from Cayman Chemical (Ann Arbor, MI, USA). Human Endothelin 1 Calbiochem® was from Merck Chemicals Ltd. (Nottingham, UK). Anti-SPARC (H-90), anti-Endothelin 1/2/3 (H-38), anti-Twist 1/2 (H-81) and anti-Akt 1/2/3 (H-136) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphoAkt (pAkt, Ser 473) antibody was from Cell Signaling Technology (Beverly, MA, USA). Anti-Snail 1/2 antibody (ab53519) was from Abcam (Cambridge, UK).

2.2. Cell lines

The parental breast carcinoma MDA-MB231 cells, the derived 1833-bone metastatic clone and the 1833 cells, retrovirally transfected with HSV1-tk/GFP/firefly luciferase (1833/TGL), were kindly given by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York). The comparative study of the transcriptomic profile of the two cell lines identifies a gene set whose expression pattern is associated with, and promotes the formation of metastasis to bone [19]. 1833 and MDA-MB231 cells were authenticated with the method of short-tandem repeat profiling (STR) of nine highly polymorphic STR loci plus amelogenin on September 2014 (Cell Service from IRCCS-Azienda Ospedaliera Universitaria San Martino-IST-Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy). The cells, routinely maintained in DMEM containing 10% (*v*/*v*) FBS, were used after 2 or 3 passages in culture [27].

2.3. Western blot analysis

We used total extracts (100 μg of protein) from cells exposed to dAza, Endothelin 1, or troglitazone. dAza (1 μM) was added to the cells at the time of seeding (1 day) and after 24 h (2 days). For 30 days treatment, dAza (0.1 μM) was added in concomitance with cell splitting (every 4 days) [28]: for comparison some cells were cultured for 30 days (c30) without treatments. When present, cycloheximide (100 μg/ml) was added to 1-day or 30-days dAza exposed cells, and to the respective controls. Then, the protein extracts were prepared over a 24-h time course. Endothelin 1 (50 ng/ml) was added to starved cells [2]. Troglitazone was used at the final working concentration of 20 μM [25]. Some cells were transfected with 25 nM siRNASnail (ON-TARGETplus Human SNAI1, SMART pool) or siRNA control (ON-TARGETplus Non targeting Pool) (Dharmacon, Lafayette, CO, USA) [29], with 400 ng/ml of the expression vectors for Twist (pCMV-TWIST) and for Snail, or with 30 nM miR29a-3p mimic or miRcontrol (Ambion/Life Technologies, Carlsbad, CA, USA) [30]. The antibodies used for immunoblotting were: anti-SPARC (1:200), anti-Akt (1 μg/ml), anti-pAkt (1:1000), anti-Endothelin 1/2/3 (1:200), anti-Twist (1:200) and anti-Snail 1/2 (1:500). SPARC level was also evaluated on the conditioned medium: 20 x 10^6 cells were seeded in T75 flasks, were treated or not for 30 days with dAza, were maintained without serum for 48 h, and the supernatants from two flasks was pooled [2]. The conditioned medium was harvested, lyophilized and, then, reconstituted in the loading buffer.

The densitometric analysis of Western blots was performed after reaction with ECL plus chemiluminescence kit from Thermo Fisher Scientific (RockFord, IL, USA).

2.4. Plasmids, siRNA and miRNA transfection, and luciferase activity assay

The cells seeded in 24-multiwell plates, were transfected with 400 ng/ml of the gene reporter for the Endothelin 1 promoter 650(+UTR)Luc (Dr. F. Rodriguez-Pascual, Madrid, Spain), for the Endothelin 1 promoter 650Luc [31], or for the SPARC promoter (SPARCLuc, GoClone n° 32001, Active Motif, La Hulpe, Belgium). The cells transfected with the gene reporters for Endothelin 1, were co-transfected with *Renilla* luciferase plasmid, and Firefly/*Renilla* luciferase activity ratios were calculated by the software. The SPARCLuc activity was evaluated by following the manufacturer's protocol. Cells transfected with SPARCLuc in 96 multiwell, were co-transfected with 2 mg/ml of the dominant negative for Runx2 (ΔRunx2), Ets1 (ΔEts1,