Biochimica et Biophysica Acta 1843 (2014) 2631–2644

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Protease Nexin-1 affects the migration and invasion of C6 glioma cells through the regulation of urokinase Plasminogen Activator and Matrix Metalloproteinase-9/2

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ARTICLE INFO

Article history:
Received 25 March 2014
Received in revised form 26 June 2014
Accepted 17 July 2014
Available online 27 July 2014

Keywords:
Protease Nexin-1 (Serpine2)
Matrix Metalloproteinase-9 (MMP-9)
Matrix Metalloproteinase-2 (MMP-2)
urokinase Plasminogen Activator (uPA)
C6 glioma cell
Cell invasion

ABSTRACT

Protease Nexin-1 (PN-1) or Serpine2 is a physiological regulator of extracellular proteases as thrombin and urokinase (uPA) in the brain. Besides, PN-1 is also implicated in some human cancers and further identified as a substrate for Matrix Metalloproteinase (MMP)-9, a key enzyme in tumor invasiveness. Our aim was to study the role of PN-1 in the migration and invasive potential of glioma cells, using the rat C6 glioma cell line as stable clones transfected with pAVU6 + 27 vector expressing PN-1 short-hairpin RNA. We find that PN-1 knockdown enhanced the *in vitro* migration and invasiveness of C6 cells which also showed a strong gelatinolytic activity by *in situ* zymography. PN-1 silencing did not alter prothrombin whereas increased uPA, MMP-9 and MMP-2 expression levels and gelatinolytic activity in a conditioned medium from stable C6 cells. Selective inhibitors for MMP-9 (Inhibitor I), MMP-2 (Inhibitor III) or exogenous recombinant PN-1 added to the culture medium of C6 silenced cells restored either the migration and invasive ability or gelatinolytic activity thus validating the specificity of PN-1 silencing strategy. Phosphorylation levels of extracellular signal-related kinases (Erk1/2 and p38 MAPK) involved in MMP-9 and MMP-2 signaling were increased in PN-1 silenced cells. This study shows that PN-1 affects glioma cell migration and invasiveness through the regulation of uPA and MMP-9/2 expression levels which contribute to the degradation of extracellular matrix during tumor invasion.

1. Introduction

Protease Nexin-1 (PN-1) or Serpine2, is a 44 kDa serine protease inhibitor that belongs to the serpin super family [1]. While many serpins are found in plasma, PN-1 is present mainly in the extracellular matrix (ECM) and it is secreted by many different cell types, including fibroblasts [2], myoblasts [3], and vascular smooth muscle cells [4]. PN-1 inhibits the enzyme activity of extracellular proteases including thrombin, urokinase, urokinase Plasminogen Activator (uPA), tissue plasminogen activator (tPA) and plasmin [5,6] by a suicide substrate mechanism mediated by the formation of a covalent complex with the target protease [7]. The serpin–protease complex binds to the low-density lipoprotein receptor-related protein (LRP) that mediates its endocytosis and degradation, thus providing a localized mechanism for the inhibition and clearing of the protease from the extracellular environment [6].

PN-1 was first identified as glia-derived nexin (GDN) [5] in the central nervous system (CNS) where it is primarily secreted by astrocytes [8], glial [9], and neuronal [10] cells. In the brain, PN-1 is the main physiological modulator of thrombin-mediated processes such as neurite outgrowth [11], proteolytic damage and apoptosis in brain injury [12,13]. Many evidences have shown that the balance between extra-cellular serine proteases and their cognate serpins play a role in many physiological

Abbreviations: PN-1, Protease Nexin-1; MMP-9 and MMP-2, Matrix Metalloproteinase-9 and -2; uPA, urokinase Plasminogen Activator; shRNA, short hairpin RNA; ECM, extracellular matrix; tPA, tissue plasminogen activator; LRP, low-density lipoprotein receptor-related protein; GDN, glia-derived nexin; CNS, central nervous system; GBM, glioblastoma multiforme; RNAi, RNA interference; Erk1/2, extracellular signal-related kinases 1/2; PAs, plasminogen activators; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffer saline; RT, reverse transcription; qPCR, quantitative real-time polymerase chain reaction; ISZ, *in situ* zymography; FITC, fluorescein isothiocyanate; DAPI, 4′ 6-diamidino-2-phenylindole; Pth, prothrombin; rPN-1, recombinant PN-1; MAPK, mitogen activated protein kinase

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http://dx.doi.org/10.1016/j.bbamcr.2014.07.008
0167-4889/

[14] and pathological processes such as tissue remodeling [15] and cancer progression [16].

Among PN-1 targets, thrombin is also known to regulate tumor cell invasion and metastasis [17] and uPA is critically involved in tumor angiogenesis and metastasis [18]. In addition, uPA controls cell migration through its ability to convert plasminogen into plasmin [19] and is involved in the maturation of other proteins, including Matrix Metalloproteinases (MMPs) and growth factors [20]. Also members of MMP family, especially MMP-2 and MMP-9, known as gelatinases A and B [21], are critically involved in tumor angiogenesis and metastasis and also found overexpressed in malignant tissues [22]. In human tumors, the overexpression of MMP-9 and uPA have been associated with cancer progression [23] and poor prognosis in several cancer types [24].

In the last years, the overexpression of PN-1 mRNA in some cancer cell lines and human cancers, including breast [25] and pancreatic tumors [26], and oral squamous carcinomas [27], has been correlated to metastatic process.

Further, in 2008, Xu et al. [28] reported that PN-1 is a substrate for MMP-9, thus suggesting a new function for this serpin, that may have a regulatory role on Matrix Metalloproteinase/s in the ECM [29]. Several authors explored the functional role of PN-1 in tumor biology and the relationship with MMP-9 and uPA, that are key enzymes in tissue remodeling and cancer progression by promoting the degradation of ECM components [30,31]. However, controversial results have been reported in diverse tumor types and system models.

Up-regulation of PN-1 has been associated with the invasive potential of pancreatic cancer cells S2-028 in a nude mice xenograft model whereas no significant effects were observed using matrigel invasion and zymography assays [26]. In prostate cancer cells DU-145 and PC-3, PN-1 can affect the anti-invasive activity of the serine protease prostasin [32].

Injections in nude mice of 4T1 mammary cancer cells, in which PN-1 had been silenced, did not affect primary tumor outgrowth whereas impaired metastatic potential that could be restored by coexpressing soluble MMP-9 [33]. Thus, these authors postulated a mechanism whereby PN-1, complexed with target proteases, binds the LRP-1 receptor thereby controlling MMP-9 expression and metastatic spread *via* Erk signaling.

In contrast, in 2010, Xu et al. [29] reported that prostate cancer cells, PC3-ML, overexpressing PN-1 or treated with MMP-9 shRNA, had reduced cell invasion in matrigel assay, through the inhibition of uPA activity. In addition, increased PN-1 amounts and inhibition of uPA activity were induced by the downregulation of MMP-9 in PC3-ML cells and in the tissue of MMP-9 deficient mice, consistent with PN-1 degradation by MMP-9 [28]. These authors indicated an anti-invasive function of PN-1 and showed a novel molecular pathway in which MMP-9 regulates uPA activity and tumor cell invasion through the cleavage of PN-1 [28].

Gliomas are among the most common type of human primary brain tumors with an infaust prognosis [34]. Since no studies are available on PN-1 functions in glioma tumors, this study was designed to investigate the role of PN-1 on migration and invasion ability on cells derived from rat glioma. The rat C6 glioma cell line [35] was chosen as an *in vitro* model system because these cells maintain many characteristics of "glioblastoma multiforme" (GBM) [36] and constitutively secrete PN-1 [9]. We used a vector-based (pAVU6 + 27) [37] RNA interference (RNAi) approach [38] to silence PN-1 expression in C6 cells. Selected clones were tested for migration and invasion ability using migration and matrigel invasion assays and the results were compared to control unsilenced cells, transfected with the empty vector. We have analyzed the effects of PN-1 silencing on expression levels of its target proteases, such as thrombin, uPA and MMP-9. Moreover, our study also focused on the gelatinase A, MMP-2, which is the metalloproteinase closely related to MMP-9 for structure and biological functions. Thrombin, uPA, MMP-9 and MMP-2 protein and mRNA expression levels were evaluated either by western blotting or semiquantitative RT-PCR analysis, respectively. MMP-9, MMP-2 and uPA enzyme activities were assessed by SDS-PAGE zymography analysis. Our results demonstrated a correlation between the enhancement of migration and invasion ability and up-regulation of uPA, MMP-9 and MMP-2 expression levels triggered by PN-1 silencing in C6 glioma cells. In addition, we determined the phosphorylation levels of the extracellular signal-related kinases, Erk1/2 and p38 MAPK [39], known to be implicated in the migration and invasion ability of various cell types, through the activation of plasminogen activators (PAs) and MMPs [33,40,41].

2. Materials and methods

2.1. Construction of pAVU6 +27-siPN-1 expression vectors

Three siRNA sequences targeting rat PN-1 were selected using the Ambion and Genescript web-based (www.ambion.com; www.genescript.com) criteria. The selection was performed by identifying target sites along the open reading frame of rat PN-1 gene sequence (NCBI, Reference Sequence: NM_019197.1; Supplementary Fig. 1A). The RNAi sequences were the following:

D31: 5′-AGGAACCAUGAAUUGGCAU-3′, spanning nucleotides 118 to 136;
2G: 5′-CCACAGUGACUUUAUCCUC-3′, spanning nucleotides 156 to 174;
A7: 5′-GUCACUCGAGGAACUAGGC-3′ spanning nucleotides 199 to 216.

The corresponding DNA sequences were chemically synthesized as a couple of complementary oligos (CEINGE, Naples, Italy) containing also a *Sal*I at the 5′ end and *Xba*I at the 3′ end (Supplementary Fig. 1B) restriction sites, used for cloning in the pAVU6 + 27 vector [37] that contains one U6 promoter cassette and the first 27 nucleotides of human U6 RNA (Supplementary Fig. 2A). The RNAi sequences and the secondary structure of hairpin oligos are reported in Supplementary Fig. 2B.

For the cloning procedure, the two oligos of which above were incubated at 95 °C for 30 min with annealing buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl), gradually cooled to room temperature to anneal shDNAs. These were then inserted into the *Sal*I and *Xba*I restriction sites of pAVU6 + 27 vector previously digested with these endonucleases.

The resulting shRNA expression vectors, named pAVU6 + PN1-D31, pAVU6 + PN1-2G and pAVU6 + PN1-A7 were transformed into *Escherichia coli* strain DH5α. Transformants were grown overnight and purified plasmids were first subjected to 1.5% agarose gel electrophoresis for restriction analysis prior DNA sequence determination.

2.2. Cell culture, DNA transfection and generation of C6 stable cell clones

The C6 rat glioblastoma cell line [35] (American Type Culture Collection, ATCC, CCL-107) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1.5 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin. Human glioblastoma (A 172), glioblastoma–astrocytoma (U-87 MG, U-373 MG) and astrocytoma (SW-1088) cell lines were provided by the cell culture facility and biobank of CEINGE-Biotecnologie Avanzate, Naples, Italy. A 172 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin; U-87 MG and U-373 MG cells in Eagle's minimal essential medium (EMEM) with 10% FBS, 2 mM L-glutamine; SW-1088 cells were grown in Leibovitz's L-15 medium with 10% FBS, 2 mM L-glutamine. The cells were maintained at 37 °C in a 5% CO_2 atmosphere and sub-confluent cultures (70–80%) were splitted 1:3 to 1:6 twice a