DNA Repair xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

DNA Repair

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

Nicotinamide phosphoribosyltransferase can affect metastatic activity and cell adhesive functions by regulating integrins in breast cancer

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ARTICLE INFO

Article history:
Received 20 April 2014
Received in revised form 25 June 2014
Accepted 28 August 2014
Available online xxx

Keywords:
NAMPT
NAD+ salvage pathway
Integrins
αvβ3
Breast cancer
Metastasis

ABSTRACT

NAD+ metabolism is an essential regulator of cellular redox reactions, energy pathways, and a substrate provider for NAD+ consuming enzymes. We recently demonstrated that enhancement of NAD+/NADH levels in breast cancer cells with impaired mitochondrial NADH dehydrogenase activity, through augmentation of complex I or by supplementing tumor cell nutrients with NAD+ precursors, inhibits tumorigenicity and metastasis. To more fully understand how aberrantly low NAD+ levels promote tumor cell dissemination, we here asked whether inhibition of NAD+ salvage pathway activity by reduction in nicotinamide phosphoribosyltransferase (NAMPT) expression can impact metastasis and tumor cell adhesive functions. We show that knockdown of NAMPT, the enzyme catalyzing the rate-limiting step of the NAD+ salvage pathway, enhances metastatic aggressiveness in human breast cancer cells and involves modulation of integrin expression and function. Reduction in NAMPT expression is associated with upregulation of select adhesion receptors, particularly αvβ3 and β1 integrins, and results in increased breast cancer cell attachment to extracellular matrix proteins, a key function in tumor cell dissemination. Interestingly, NAMPT downregulation prompts expression of integrin αvβ3 in a high affinity conformation, known to promote tumor cell adhesive interactions during hematogenous metastasis. NAMPT has been selected as a therapeutic target for cancer therapy based on the essential functions of this enzyme in NAD+ metabolism, cellular redox, DNA repair and energy pathways. Notably, our results indicate that incomplete inhibition of NAMPT, which impedes NAD+ metabolism but does not kill a tumor cell can alter its phenotype to be more aggressive and metastatic. This phenomenon could promote cancer recurrence, even if NAMPT inhibition initially reduces tumor growth.

1. Introduction

Nicotinamide adenine dinucleotide (NAD+) metabolism is an essential regulator of cellular redox reactions, energy pathways, and a substrate provider for NAD+ consuming enzymes [1–3]. These include ADP-ribose transferases, poly(ADP-ribose) polymerases (PARPs), and NADases such as CD38 and sirtuins. While NAD+ consuming enzymes affect NAD+ availability and typically degrade it to nicotinamide, redox reactions involve reduction of NAD+ to NADH, which can be converted back to NAD+ by NADH dehydrogenase activity of mitochondrial complex I [1–9].

NAD+ can be synthesized de novo from tryptophan, nicotinamide, nicotinic acid or nicotinamide riboside, or be derived via the salvage pathway. The initial and rate-limiting step within the NAD+ salvage pathway is mediated by nicotinamide phosphoribosyltransferase (NAMPT), the enzyme that catalyzes conversion of nicotinamide to nicotinamide mononucleotide (NMN+) using

Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; BRCA1, breast cancer 1; BRCA2, breast cancer 2; eNAMPT, extracellular nicotinamide phosphoribosyltransferase; ERK, extracellular signal-regulated kinases; iNAMPT, intracellular nicotinamide phosphoribosyltransferase; MMTV-PyMT, mouse mammary tumor virus – polyoma middle T antigen; mTORC1, mammalian target of rapamycin complex 1; MMP, matrix metalloproteinases; NAD+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NaMN, nicotinic acid mononucleotide; NAMPT, nicotinamide phosphoribosyltransferase; NaR, nicotinic acid riboside; Ndi1, NADH-ubiquinone reductase (H(+)-translocating; NMN+, nicotinamide mononucleotide; NMNAT, nicotinamide nucleotide adenylyltransferases; NR, nicotinamide riboside; NRK, nicotinamide riboside kinases; PARPs, poly (adenosine diphosphate-ribose) polymerases; SCID, severe combined immunodeficiency; shRNA, small hairpin RNA; uPAR, urokinase plasminogen activator receptor; VEGF, vascular endothelial growth factor.

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http://dx.doi.org/10.1016/j.dnarep.2014.08.006
1568-7864/

phosphoribosylpyrophosphate as a co-substrate. NMN^+ is then converted to NAD^+ by nicotinamide nucleotide adenylyltransferases (NMNAT) [1,2,10–12].

Cancer cells, especially highly proliferative cells in fast growing tumors such as triple negative breast cancers, generally accumulate high levels of DNA damage and genomic instability [13–16]. These cells can have increased NAD^+ degrading PARP activity for DNA damage repair, and thus a high need for NAD^+ to maintain cell viability. Therefore, fast growing cells often have low NAD^+ levels which sensitize them to further NAD^+ reduction [1,17,18]. Thus, it has been suggested that high NAMPT expression should enhance tumor cell survival by elevating NAD^+ levels [19–21], while chemical inhibition of NAMPT to reduce cellular NAD^+ levels should inhibit tumor cell viability, especially when used in combination with PARP inhibitors. This strategy has been proposed as a therapeutic approach against breast cancer [11,20,22,23].

In contrast to the concept that inhibition of NAMPT activity might eliminate tumor cells, we showed earlier that impaired NAD^+ metabolism activity and resulting decrease in NAD^+/NADH redox levels in human breast cancer cells can actually significantly stimulate their metastatic properties [7]. Mechanisms underlying the enhanced metastatic aggressiveness were found associated with aberrant mitochondrial complex I and poor NADH dehydrogenase activity. Metastatic aggressiveness could be suppressed by enhancing complex I function through expression of yeast NADH dehydrogenase Ndi1 in the tumor cells. Augmentation of complex I activity enhanced the cellular NAD^+/NADH balance and supported autophagy, while suppressing the mTORC1 pathway [7]. To more fully understand how reduced NAD^+ levels promote tumor cell dissemination, we here asked whether inhibition of NAD^+ salvage pathway activity by reduction in NAMPT expression can impact tumor cell adhesive properties. Aberrant cell adhesion supports the metastatic process by mediating tumor cell interaction with vascular cells or the lymphatic system, as well as with tissues and matrices in target organs of metastasis [24,25]. As major adhesive, migratory and invasive tumor cell functions are mediated by adhesion receptors, we here asked whether interference with NAMPT affects adhesion receptor expression and function.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 human breast cancer cells and their variants were stably transduced with Firefly luciferase (F-luc) using lentiviral expression vector pTacoma (CMV promoter) (BE Torbett, TSRI) to analyze metastasis by non-invasive bioluminescence imaging (55). Cells were grown in EMEM supplemented with nonessential amino acids, vitamins, 2 mM L-glutamine, 1 mM pyruvate, and 10% FBS.

2.2. NAMPT expression and knockdown

Lentiviral vector containing small hairpin RNA (shRNA) against NAMPT (shNAMPT) (TRCN0000116180) or non-mammalian targeting control shRNA (SHC0002) (shCT) were from Sigma–Aldrich, MO. Knockdown efficiency was quantified by real time PCR using FastStart Universal SYBR Green Master (Rox) (Roche) and the following primers: human NAMPT-F (GCCAGCAGGGAATTTTGTTA), human NAMPT-R (TGATGTGCTGCTTCCAGTTC), human GAPDH-F (GGGAAGGTGAAGGTCGGAGT), and human GAPDH-R (TCCACTTTACCAGAGTTAAAAGCAG). The same primers were used to analyze NAMPT gene expression in lung metastases developing in SCID mice after i.v. injecting shNAMPT versus shRNA control cells. Data were recorded and analyzed using an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) and Sequence Detector Software (SDS v2.0). Reduction of NAMPT protein expression in shNAMPT knockdown cells was confirmed by Western blot using cells lysed in Laemmli's buffer and analyzing 20 μg of total protein, probed with anti-NAMPT (ab45890, abcam, MA) or anti β-tubulin (Sigma–Aldrich, MO), and followed with secondary antibodies conjugated to IRDye 800 (NAMPT) or IRDye 680 (β-tubulin) using an Odyssey infrared imaging system (LI-COR Biosciences). Data were analyzed and quantified using Odyssey infrared imaging system application software v3.0.

Moreover, NAMPT Knockdown was confirmed by western blot. Cells were lysed with Laemmli's buffer and 20 μG of total protein was loaded in a SDS-page gel. Western blots were incubated with antibodies against: NAMPT (ab45890, abcam, MA) and β-tubulin (Sigma–Aldrich, MO). Antibody binding was detected following incubation with secondary antibodies conjugated to IRDye 680 (β-tubulin) 800 (NAMPT), using an Odyssey infrared imaging system (LI-COR Biosciences). Data were analyzed and quantified using Odyssey infrared imaging system application software v3.0.

2.3. NAD^+/NADH measurement

NAD^+ and NADH were analyzed independently in extracts of whole cells (1×10^6) prepared as previously described [7]. Concentrations were determined using a fluorescence NAD^+/NADH detection kit according to the manufacturer's protocol (Cell Technology, Inc.).

2.4. Metastatic activity

For experimental metastasis, female 6–8 week-old C.B-17/SCID mice were injected intravenously (i.v.) with F-luc–tagged cancer cells: 2.5×10^5 MDA-MB-231 shCT or MDA-MB-231 shNAMPT cells. Mice were imaged weekly (IVIS 200, Xenogen) 10 min after i.p. injection of D-luciferin (100 mg/kg). Bioluminescence was quantified as photons/second/cm^2 in defined regions of interest using Living Image software. Animal work complied with National Institutes of Health and institutional guidelines (TSRI is AAALAC accredited).

2.5. Cell-adhesion assay

Cell-adhesion assays were performed in 96-well plates coated with 50 μl of one of the following matrix proteins: human vitronectin (10 μg/ml), fibrinogen (20 μg/ml), fibronectin (10 μg/ml) or collagen I (10 μg/ml), in PBS overnight at 4 °C. The plates were washed twice with TBS and blocked with 2% BSA in TBS overnight at 4 °C. Cells were harvested with versene and washed with adhesion buffer (serum free EMEM medium containing 0.5% BSA) and then pre-incubated with or without 10 μg/ml function blocking monoclonal anti-integrin antibodies: β3 (M21.3), αv (HB8448), αvβ5 (P1F6), β1 (P5D2) and α5 (P1D6) [26–29] for 15 min at RT. Cells were then seeded at 4×10^4/well in the presence of the antibodies. Adhesion time was 35 min at 37 °C. Non-adhered cells were removed by adding floatation medium (0.9% NaCl, 80% Percoll) as described previously [30]. Attached cells were fixed with 3.3% glutaraldehyde and 20% Percoll. The attached cells were stained with crystal violet, lysed with 0.5% Triton X-100 in water, and quantified by measuring optical absorbance at 585 nm.

2.6. Integrin expression

Surface integrin expression was analyzed by flow cytometry as follows: cells were harvested with versene, washed with PBS, and resuspended in ice cold binding buffer (1% BSA in TBS pH 7.4). 50 μl containing 10^5 cells were then incubated with 30 μg/ml of anti-integrin antibodies: αvβ3 (VNR1), αvβ5(P1F6), α5 (P1D6)

Please cite this article in press as: A.F. Santidrian, et al., Nicotinamide phosphoribosyltransferase can affect metastatic activity and cell adhesive functions by regulating integrins in breast cancer, DNA Repair (2014), http://dx.doi.org/10.1016/j.dnarep.2014.08.006