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Bacteriophage hyaluronidase effectively inhibits growth, migration and invasion by disrupting hyaluronan-mediated Erk1/2 activation and RhoA expression in human breast carcinoma cells

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ABSTRACT

Aberrant hyaluronan production has been implicated in many types of tumor. In this context, hyaluronidase has been explored as a viable therapeutic approach to reduce tumoral hyaluronan. However, elevated levels of hyaluronan in tumors are often associated with high expression levels of cellular hyaluronidases, which consequently produce various sizes of saturated hyaluronan fragments with divergent pro-tumoral activities. The current study shows that different hyaluronan metabolisms of mammalian and microbial hyaluronidases could elicit distinct alterations in cancer cell behavior. Unlike saturated hyaluronan metabolites, unsaturated hyaluronan oligosaccharides produced by bacteriophage hyaluronidase, HylP, had no biological effect on growth of breast carcinoma cells. More importantly, HylP's metabolic process of hyaluronan into non-detrimental oligosaccharides significantly decreased breast cancer cell proliferation, migration and invasion by disrupting Erk1/2 activation and RhoA expression. Our results suggest that it may be possible to exploit HylP's unique enzymatic activity in suppressing hyaluronan-mediated tumor growth and progression.

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1. Introduction

Elevated levels of hyaluronan (HA) and the resultant accumulation of HA in peritumoral regions are correlated with low survival rates in patients with breast [1], ovarian [2], non-small-cell lung [3] and prostate cancer [4]. HA is a non-sulfated glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with a molecular weight of 10^6–10^7 Da [5]. As a major component of the extracellular matrix, HA contributes to tissue homeostasis and structural integrity [6,7]. In addition, various sizes of HA molecules either generated by hyaluronan synthases or processed by hyaluronidases are known to interact with specific types of hyaladherins (i.e. CD44 and RHAMM), which mediates cellular events including morphogenesis [8], inflammation [9], tissue regeneration [10] and tumor progression [11,12].

The multiple mechanisms of HA in modulating cancer progression has been extensively studied in both *in vitro* and *in vivo* animal models [13–16]. Accumulated HA at the tumor-stroma interface causes poor penetration of

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drugs and induces chemoresistance [17]. The hydrodynamic HA-rich extracellular matrix has also been proposed to diminish contact-mediated inhibition of cell growth [18] and promote tumor-associated tissue remodeling that creates a favorable tumor microenvironment for cancer cell migration and invasion [19]. More importantly, HA activates Ras-Erk1/2 kinase [20–22], PI3K-Akt kinase [19,23] and Rho-GTPase [24] signaling cascades through its interactions with hyaladherins, which consequently promotes cell growth, survival, motility and invasion during the malignant transformation.

Interrupting HA-hyaladherin interactions has been proposed as a viable therapeutic approach in cancer therapy. Studies have demonstrated that treatment with CD44 antibody [25], soluble HA-binding proteins [26,27] and HA oligosaccharides [28] had inhibitory effects on tumor growth and progression by disrupting endogenous HA-cell receptor interactions. On the other hand, there have been attempts to utilize bovine testicular hyaluronidase (BTH) to reduce tumoral HA. Shuster et al. reported that treatment with BTH increased the breast tumor regression *in vivo* [29]. In addition, combined administration of BTH preparations with chemotherapeutics significantly increased the intratumoral drug accumulation in human melanoma mice models [30,31].

BTH generally exists as a complex mixture of three active components: 33-kDa, 58-kDa, and 76-kDa proteins [32]. BTH is enzymatically active on HA at neutral pH, where the 58-kDa BTH fraction (BTH_{58kDa}) dominates the enzyme activity [32,33]. Recent study suggests that the optimal pH for the enzymatic activity of BTH components varies in part depending on whether the methods used to assess the enzyme activity include or exclude BTH's additional transglycosylase activity [33].

Like other mammalian hyaluronidases, BTH hydrolyze HA of high molecular mass into *saturated* HA fragments that are known to modulate various cellular events [34,35]. Particularly, substantial evidence indicates that various sizes of HA fragments produced by mammalian hyaluronidases elicit divergent pro-tumoral activities. Sugahara et al. revealed that low-molecular-weight HA fragments (6–36-mers) isolated from the digestions of sheep testicular hyaluronidase [36] and tumor-produced Hyal-1 and Hyal-2 [37] promote cell migration of human pancreatic carcinoma cells (MIA PaCa-2) by enhancing CD44-cleavage. Smaller HA fragments, including oligosaccharides, also have been shown to stimulate endothelial-cell proliferation, motility and tubule formation, and induce angiogenesis [38–42]. In addition, human melanoma (SMMU-2) and colon carcinoma (VACO5) cells expressing human testicular hyaluronidase (PH-20) were reported to induce angiogenesis *in vivo* [43]. In cancer metastasis, LMW-HAs (36-mer) promote integrin-mediated colon carcinoma cell interaction with intercellular adhesion molecule-1 of endothelial cells [44]. On the other hand, recent study demonstrated that HA oligosaccharides (3–10-mers) inhibit the anchorage-independent growth of tumor cells [28].

In contrast to mammalian hyaluronidases, microbial hyaluronidases (or hyaluronan lyases) cleave HA by a β-elimination reaction yielding *unsaturated* HA oligosaccharides [45,46]. The hyaluronidase of *Streptococcus pyogenes* bacteriophage H4489A (HylP) used in this study is a 39.5-kDa enzyme [46]. The biological function of HylP is predicted to degrade a HA-rich matrix which encapsulates *S. pyogenes*, thereby facilitating bacteriophage infection to host strains [46]. To date, three different bacteriophage hyaluronidase genes *HylP, HylP1* and *HylP2* have been identified [47–49], where HylP has an additional 30 amino-acid sequence that resembles collagen-like Gly-X-Y repeats [48]. HylP does not share sequence homology with bacterial or mammalian hyaluronidases [50].

Although it is a well-known fact that HA receptor-mediated cellular alterations are associated with saturated HA fragments produced by mammalian hyaluronidases, the cellular effects of microbial hyaluronidases and their unsaturated HA metabolites are not well characterized. To our knowledge, this study shows for the first time that BTH_{58kDa} and its saturated HA metabolites promote the growth of MDA-MB-231 and MCF-7 breast carcinoma cells by enhancing Erk1/2 kinase activation, whereas unsaturated HA oligosaccharides released by HylP have no significant effect on cell proliferation as well as invasive properties. Furthermore, HylP's metabolism of cancer-associated HA into unsaturated HA oligosaccharides effectively disrupts the activation of Erk1/2 kinases and expression of Rho GTPases, which consequently attenuates proliferation, migration and invasion of Hs578T, MDA-MB-231 and MCF-7 cells.

2. Materials and methods

2.1. Cell lines

Three breast carcinoma cell lines, Hs578T, MDA-MB-231, and MCF-7, were chosen based on their HA-production levels [15]. These cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Hs578T cells were grown in DMEM (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS, Gibco, Ontario, Canada), 4 mM L-glutamine (Sigma), 4.5 g/L glucose (Sigma), and 0.01 mg/ml bovine insulin (Sigma). MDA-MB-231 cells were grown in Leibovitz's L-15 with 10% FBS, and 2 mM L-glutamine. MCF-7 cells were grown in MEM with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), and 0.01 mg/ml bovine insulin.

2.2. Expression and purification of HylP

BL21(DE3)/pSD102 *Escherichia coli* cells harboring the *HylP* gene of M-type 49 strain of *S. pyogenes* bacteriophage H4489A [46] were grown overnight at 37 °C in Terrific Broth medium (American Bioanalytical, Natick, MA) and induced at an $OD_{600} \geqslant 0.6$ with 1 mM Isopropyl-D-thiogalactopyranoside (IPTG, American Bioanalytical) at 30 °C for 5 h. Induced cells were collected and resuspended in a buffer containing 25 mM HEPES pH 7.5, 2 mM EDTA and protease inhibitors (Roche). Soluble cell lysate was prepared by sonication (Fisher Scientific Sonic Dismembrator 500), and purification of HylP enzyme was carried out

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