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Research Article

The over-expression of *HAS2*, *Hyal-2* and CD44 is implicated in the invasiveness of breast cancer

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Abstract

Within tumors there appears to be an intricate balance between hyaluronan (HA) synthesis and degradation where the invading edges display increased HA metabolism. The metabolism of HA has not been characterized in breast cancer cell lines; therefore, this study quantitatively identifies and characterizes the enzymes responsible for the synthesis and degradation of HA while correlating gene expression to cancer cell invasiveness and HA receptor status. In ten well-established breast cancer cell lines, the expression of the genes for each hyaluronan synthase (HAS) and hyaluronidase (Hyal) isoform was quantitated using real-time and reverse transcriptase polymerase chain reaction (PCR). The synthesis and degradation rates of hyaluronan were determined by ELISA, while quantitation of HA receptors, CD44 and RHAMM was performed by comparative Western blotting. The molecular weight of HA synthesized by each HAS isoform and the degradation products of each hyaluronidase were characterized by size exclusion chromatography. It was demonstrated that highly invasive cell lines preferentially expressed the *HAS2* and *Hyal-2* isoforms, while less invasive cells expressed *HAS3* and *Hyal-3*. There was a correlation between elevated levels of HA synthesis, CD44 expression and cancer cell migration thereby highlighting the pivotal role that HA metabolism plays in the aggressive breast cancer phenotype.

Keywords: Hyaluronan; Hyaluronidase; Hyaluronan synthase; CD44; RHAMM and breast cancer

Introduction

The extracellular environment of breast tumors consists of a matrix scaffold containing proteins [1,2] and glycosaminoglycans [3]. The major components of the tumoral extracellular matrix (ECM) include laminin, collagen IV, perlecan, entactin, HA, various growth factors and proteases, all of which promote malignancy and/or angiogenesis [4]. The amount and type of these components vary depending on the stage of development and the tissue type. Hyaluronan (HA) is a ubiquitous ECM component of the tumor environment, especially in the stroma where its accumulation can be observed at the invading edge of breast carcinomas [5,6], in the extracellular environment [7] and as an integral component of the cell-associated matrix of aggressive cancer cells [8,9]. Within a tumor, HA appears to be multi-functional where it maintains hydration homeostasis, provides structural integrity and in conjunction with its receptors has been implicated in the intracellular signaling cascades associated with tumor cell proliferation and migration [10–12]. Most malignant solid tumors contain elevated levels of HA [12] where these high levels correlate with poor differentiation and decreased survival [13]. The increased concentration of HA in breast tumors is thought to be the result of fibroblasts being stimulated by the tumor cells to increase HA production [3,14,15]. However, the most tumorigenic and phenotypically aggressive breast carcinoma cell lines also

Abbreviations: Da, Daltons; DX, dextran sulphate; ECM, extracellular matrix; FCS, fetal calf serum; HA, hyaluronan; HABP, HA binding protein; HAS, hyaluronan synthase protein; *HAS*, hyaluronan synthase gene; Hyal, hyaluronidase protein; *Hyal*, hyaluronidase gene; M*r*, molecular weight; PCR, polymerase chain reaction; Conc, concentration.

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synthesize large quantities of HA, unlike the less malignant cell lines [7]. The invasive potential created by the accumulation of HA may be further aggravated by changes in the expression of HA receptors, CD44 and RHAMM, which are both frequently observed in breast cancer cells [16,17]. The increased levels of HA in breast cancer indicate that HA metabolism is altered, and this perturbation of HA synthesis and/or degradation may play an important role in tumor initiation and progression.

Hyaluronan is synthesized by a multi-isoform family of transmembrane glycosyltransferases termed the HA synthases [18] while HA is depolymerized by a combination of enzymic and non-enzymic mechanisms [19]. Hyaluronan polymerization occurs on the inner face of the plasma membrane where it is extruded onto the extracellular surface of the cell [20]. Three eukaryotic HAS isoforms have been identified, termed HAS1, HAS2 and HAS3. Sequence data of the HAS isoforms suggest that they contain seven membrane-associated regions and a central cytoplasmic domain possessing several consensus sequences that are substrates for phosphorylation by protein kinase C [18,21]. The catalytic rate for each HAS isoform is reported to be different [22]. HAS1 is supposedly the least active and drives the synthesis of high molecular weight (Mr) HA (2000 kDa), suggesting low constitutive levels of HA synthesis. HAS2 is more catalytically active and is associated with synthesis of high Mr HA (2000 kDa). HAS2 is implicated in developmental processes involving tissue expansion and growth. HAS3, the most active, drives the synthesis of short (100–1000 kDa) HA chains. HAS3 expression may be activated to produce large amounts of low Mr HA to contribute to the pericellular matrix or may interact with cell surface HA receptors, triggering signaling cascades and profound changes in cell behavior [22]. Within the tumor environment, the production of high Mr HA is thought to provide a hydrated matrix which forces gaps in the ECM, enabling tumor cells to migrate and metastasize to other tissues.

Manipulation of the HAS genes has enabled the over-expression or inhibition of the different HA synthase isoforms which has provided a preliminary insight into the role of HA synthesis in cancer. The upregulation of *HAS1* in HA-deficient mouse carcinoma cells restored metastatic ability [23] while transfection of fibroblasts did not increase anchorage-independent growth or the rate of formation of a subcutaneous mass [24]. The over-expression or inhibition of HAS2 has generated more profound results where several studies demonstrated that in a variety of cancer cells, HAS2 is responsible for the generation of an HA pericellular coat, anchorage-independent growth and tumor formation [24–26]. The contrary has also been found where very high levels of HAS2 expression can inhibit tumor growth [26]. Similarly, the induction of HAS3 expression resulted in the formation of an HA pericellular coat and promoted the growth of TSU human prostate cancer cells without inducing a metastatic phenotype [27].

Hyaluronan is degraded by a group of enzymes known as the hyaluronidases where they exist in several isoforms, namely, Hyal-1, 2, 3 and PH-20 (for a review, see [19]). *Hyal-1* and *Hyal-2* are widely distributed and in collaboration with CD44 degrade high Mr HA [28]. It has been suggested that high Mr HA binds to CD44 and in cooperation with the GPI-anchored Hyal-2 [29]. The HA is internalized and degraded to 20-kDa HA fragments within unique acid endocytic vesicles [30]. The fragments are transported intracellularly and further digested by Hyal-1 together with two β-exoglycosidases, β-glucuronidase and β-*N*-acetyl glucosaminidase resulting in very low Mr oligosaccharides [29]. The bone-marrow-associated *Hyal-3* has not been fully characterized. The sperm-associated HAase, PH-20, plays an important role in fertilization and differs from the other HAases by exhibiting enzymic activity at neutral pH [31].

Regulated uptake of tumor-associated HA via a CD44 receptor-mediated endocytosis pathway and subsequent degradation by HYAL-2 may be important for tumor growth and progression where it may play two roles; (i) induction of angiogenesis through the generation of small HA fragments (3–25 disaccharide units) that have been shown to promote angiogenesis, cell migration and differentiation of capillary endothelial cells [32,33]; and/or (ii) degradation of HA around blood vessels may also enhance tumor metastasis by enabling tumor cells to enter the circulation more readily [34]. There are contradictory reports about the role of hyaluronidases in cancer, where elevated levels of PH-20 are found in human melanoma, glioblastoma and colon cancer cell lines and in tumor biopsies from colorectal and laryngeal cancer [35,36]. An elevated level of *Hyal-1* has been found in prostate cancer [37,38] even though *Hyal-1* has been identified as a candidate tumor suppressor [38,39]. The expression of *Hyal-1* has been demonstrated to suppress the growth of colon cancer [40] even though it enhances extravasation and metastasis of prostate cancer cells [37]. *Hyal-2* has the ability to act as an oncogene where its over-expression in murine astrocytoma cells accelerated tumor formation [41]. *Hyal-3* has not been implicated in cancer and to date very little is known about its activity or function.

Despite the extensive evidence associating HA with cancer through its ability to promote experimental tumor progression, no direct relationship between the levels of HA synthesis and degradation has been established with respect to the invasiveness of the malignant phenotype, more specifically breast cancer. This study aims at identifying and establishing a relationship between the intricate and continual balance that may occur between the synthesis and degradation of HA in breast cancer while correlating this to the differential expression of the HAS and Hyal isoforms. Through establishing these relationships, it may be possible to highlight the causal role that HA metabolism may play in the initiation and progression of human breast cancer.