Hyaluronan modulates growth factor induced mammary gland branching in a size dependent manner

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Abstract

Mammary gland morphogenesis begins during fetal development but expansion of the mammary tree occurs postnatally in response to hormones, growth factors and extracellular matrix. Hyaluronan (HA) is an extracellular matrix polysaccharide that has been shown to modulate growth factor-induced branching *in culture.* Neither the physiological relevance of HA to mammary gland morphogenesis nor the role that HA receptors play in these responses are currently well understood. We show that HA synthase (HAS2) is expressed in both ductal epithelia and stromal cells but HA primarily accumulates in the stroma. HA accumulation and expression of the HA receptors CD44 and RHAMM are highest during gestation when gland remodeling, lateral branch infilling and lobulo-alveoli formation is active. Molecular weight analyses show that approximately 98% of HA at all stages of morphogenesis is >300 kDa. Low levels of 7–114 kDa HA fragments are also detected and in particular the accumulation of 7–21 kDa HA fragments are significantly higher during gestation than other morphogenetic stages ($p < 0.05$). Using these *in vivo* results as a guide, *in culture* analyses of mammary epithelial cell lines (EpH4 and NMuMG) were performed to determine the roles of high molecular weight, 7–21 kDa (10 kDa MWavg) and HA receptors in EGF-induced branching morphogenesis. Results of these assays show that while HA synthesis is required for branching and 10 kDa HA fragments strongly stimulate branching, the activity of HA decreases with increasing molecular weight and 500 kDa HA strongly inhibits this morphogenetic process. The response to 10 kDa HA requires RHAMM function and genetic deletion of RHAMM transiently blunts lateral branching *in vivo*. Collectively, these results reveal distinct roles for HA polymer size in modulating growth factor induced mammary gland branching and implicates these polymers in both the expansion and sculpting of the mammary tree during gestation.

Introduction

Branching morphogenesis is a central mechanism for establishing the tissue architecture of many glandular and non-glandular organs of mammals and lower organisms, and results from fractal patterns of domain branching, planar bifurcation and orthogonal bifurcations [1,2]. The localized production and concentration of growth factors such as FGF10, TGFß-1 or EGF control branch initiation in mammary glands [3–5]. Stromal cells [6] contribute to the spatial localization of these factors by producing and organizing extracellular matrix that sequester these components [7–10]. The tissue polysaccharide hyaluronan (HA) is one example of an extracellular matrix component that regulates growth factor receptor responses [11–14] and that is required for epithelial branching and sprouting [15].

HA is a polydisperse polysaccharide composed of repeating units of *N*-acetyl β-D-glucosamine and β-D-glucuronic acid synthesized by three tissue specific and plasma membrane-associated HA synthases [HAS1–3] [16,17]. HAS2 is the major synthase expressed in mammary glands. Native HA polymers are large (*e.g.* > 200–300 kDa) and are subject to fragmentation by hyaluronidases, mechanical shearing and reactive oxygen/nitrogen species. HA fragments increase in remodeling, stressed, injured or diseased tissues and have different biological properties than native HA. For example, HA fragments bind to cellular HA receptors [17–20], which then associate with growth factor receptors to coordinate pro-survival, proliferation and migration signaling [6,21,22]. Different sized HA fragments appear to cluster receptor complexes to varying degrees thus affecting the strength and duration of signal activation and accounting for the often-specific effects of different HA fragment sizes [14,23–28]. Little is known about either the amounts of native and fragmented HA or their functions in tissues *in vivo*. However, *in culture* analyses show that HA fragments promote tubulogenesis and sprouting of endothelial cells and kidney epithelia [29]. We were motivated to initiate analyses of the "hyaluronome" during mammary gland morphogenesis because to our knowledge few reports have documented HA production during gland branching and because aberrant HA production and HA receptor expression are key factors in the progression of breast cancer [18,21,30].

Here, we analyzed mammary glands at varying stages of branching morphogenesis *in vivo* for evidence of HAS expression, HA production and fragmentation and HA receptor expression. For HA receptor analysis, we focused upon CD44 since this HA receptor has previously been shown to be required for post-partum milk production and RHAMM because it frequently partners with CD44 to regulate activation of signaling pathways implicated in mammary gland morphogenesis [31–33]. Using the *in vivo* results as a guide, we then assessed the consequences of both adding native *vs.* fragmented HA and HA receptor function blocking on EGF-initiated branching of mammary ductal epithelial cells in 3D cultures. We show a role for specific sizes of HA fragments in stimulating branching through RHAMM. We also demonstrate that higher molecular weight HA polymers antagonize these stimulatory effects and conclude that HA polymers play important roles in sculpting the mammary tree during post-pubescence and pregnancy.

Materials and methods

Materials

Tissue sections of paraffin embedded CD1 mouse mammary glands were generously provided by Dr. J. Fata. Oligo HA MW avg. 10 kDa was kindly provided by Dr. F Winnik (U. Montreal CA) [34]. Briefly, 10 kDa HA was prepared from medical grade 240 kDa HA (Hyal Pharma, Mississauga) using testicular hyaluronidase. The digest was heat inactivated and tested for lack of endotoxin then analyzed for molecular weight distribution as described previously [34]. High molecular weight (HMW) HA (avg. MW 200–240 and 500 kDa) and 50 kDa HA fragments were purchased from Lifecore. Lifecore HA is manufactured as GMP, pharmaceutical and injectable grade lots. Quality control includes testing for impurities such as bioburden, endotoxin, alcohols, acetic acid, metals, and nucleic acids. If not stated otherwise, chemicals were purchased from Sigma, Aldrich. DIG-RNA Labeling Kit (SP6/T7), blocking Reagent, ribonuclease A, goat anti-DIG alkaline phosphatase conjugate, 4-nitro-blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate were purchased from Roche. Yeast tRNA was purchased from Invitrogen, ECL kit from Amersham Pharmacia Biotech. 4-Methylumbelliferone (4MU) and Benzonase were purchased from Sigma, Aldrich. Rhamm blocking antibodies were raised in rabbits against antigenic RHAMM peptides [35]. These antibodies specifically block migration of Rhamm expressing cells but have no effect on migration of Rh−/− cells. CD44 antibodies were purchased from Invitrogen.

Mice and cell lines

RHAMM−/− and RHAMM:CD44−/− were on a C57BL6/N background and have previously been described [35]. These mice were housed in the London Health Sciences Center animal facility and maintained according to animal ethics outlined in University of Western Ontario protocol # (2009-060). EpH4 cells and NMuMG cells were purchased from ATCC.

In situ hybridization

The cDNA for mHas2 was amplified by RT-PCR as previously described [36]. The primers used for RT-PCR: sense primer: 5′-CTGTGAAAAGGCTGACC TAC-3′, anti-sense primer: 5′-TCAGTAAGGCACT TGGACCG-3′. The amplified product was then used as templates for the second PCR using sense primer: 5′ GGAAAGCTTAACTCAGACGACGACC-3′, and anti-sense primer: 5′-CGTCACCAAAACTGCATTGG-3′. The PCR product was then cloned into vector pSPT18 using *Hin*dIII and *Eco*RI. DIG-labeled anti-sense/sense RNA probes were generated using DIG-RNA Labeling Kit (SP6/T7) (Roche).

The *in situ* hybridization procedure was modified from protocols described previously [36]. Tissue sections were de-paraffinized in xylene 2 times 10 min and re-hydrated in ethanol series (100%, 95%, 70%, 5 min each). Proteinase K treatment: