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Involvement of the MEKK1 Signaling Pathway in the Regulation of Epicardial Cell Behavior by Hyaluronan

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Abstract

During embryonic development, cells comprising the outermost layer of the heart or epicardium play a critical role in the formation of the coronary vasculature. Thus, uncovering the molecular mechanisms that govern epicardial cell behavior is imperative to better understand the etiology of cardiovascular diseases. In this study, we investigated the function of hyaluronan (HA), a major component of the extracellular matrix, in the modulation of epicardial signaling. We show that stimulation of epicardial cells with high molecular weight HA (HMW-HA) promotes the association of MEKK1 with the HA receptor CD44 and induces MEKK1 phosphorylation. This leads to the activation of two distinct pathways, one ERK-dependent and another NFκB-dependent. Furthermore, HMW-HA stimulates epicardial cells to differentiate and invade, as suggested by increased vimentin expression and enhanced invasion through a collagen matrix. Blockade of CD44, transfection with a kinase inactive MEKK1 construct or the use of ERK1/2 and NFκB inhibitors significantly abrogates the invasive response to HMW-HA. Together, these findings suggest an important role for HA in the regulation of epicardial cell fate via activation of MEKK1 signaling cascades.

Keywords

epicardial cells; hyaluronan; MEKK1; cellular invasion; differentiation

1. Introduction

In early embryogenesis, the heart is composed of only two layers, the endocardium and the myocardium. However, as development progresses, an extracardiac cluster of mesothelial cells known as the proepicardium, migrates and adheres to the surface of the primitive cardiac tube [1]. The resulting epithelial outer layer that extends throughout the heart and covers the myocardium is termed the epicardium [2]. Some epicardial cells then undergo epithelial to mesenchymal transition (EMT), a complex process in which cells detach, differentiate and invade the subepicardial space, between the epicardium and myocardium [3]. Epicardial EMT is crucial for the generation of coronary vascular components such as perivascular fibroblasts,

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endothelial and smooth muscle cells [4]. However, many questions remain unanswered about the mechanisms that regulate this important process.

In the developing heart, EMT is not unique to the epicardium, as it also occurs in the endocardial cushions during the formation of cardiac valves and septum [5]. Here, the extracellular matrix component hyaluronan (HA) is abundant and its disruption severely compromises proper cardiac morphogenesis. This has been demonstrated in mice lacking hyaluronan synthase 2 (Has2), the enzyme responsible for the production of HA. Has2 knock out embryos contain very little HA, fail to undergo endocardial cushion EMT, and as a result exhibit severe cardiovascular defects, leading to death by embryonic day E 9.5 [6]. However, because these HA-deficient mice die before the formation of the epicardium at around day E10.5 [7], it is not known whether HA also plays a role in epicardial EMT.

Interestingly, HA is highly abundant around the newly formed epicardium, with its presence decreasing towards the end of gestation [8]. In embryonic as well as in cancer cells, HA has been shown to initiate signals by binding to cell surface receptors such as CD44 [9], RHAMM [10] and LYVE-1 [11], with CD44 being the most widely expressed [12]. Several studies also indicate that HA activates intracellular signal mediators, such as nuclear factor kappa B (NFkB) [13], c-Src kinase [14], PI3K [15], and the extracellular signal regulated kinases 1 and 2 (ERK1/2) [9]. Nevertheless, the exact mechanism of activation remains unclear, as HA receptors do not possess kinase activity, and thus are not able to directly phosphorylate downstream proteins. Coincidentally, several of these HA-activated molecules, namely NFkB and ERK1/2, are highly expressed in the developing heart [16,17] and are induced in migratory cells by members of the mitogen-activated protein kinase (MAPK) cascade, such as MEK kinase 1 (MEKK1) [18,19].

Although mice lacking MEKK1 do not exhibit life-threatening congenital defects, MEKK1 disruption results in the impairment of embryonic epithelial cell motility both in vivo and in vitro [20,21], suggesting that MEKK1 is an important contributor in the regulation of epithelial cell biology during development.

The aim of this study was to define the potential role of HA in the activation of the MEKK1 pathway and determine the relevance of these interactions in the induction of epicardial EMT. Here we demonstrate that high molecular weight HA (HMW-HA), but not low molecular weight HA (LMW-HA), induces the formation of a CD44/MEKK1 complex with subsequent activation of the ERK1/2 and NFkB pathways. Mobilization of these signaling cascades by HMW-HA results in epicardial cell invasion and differentiation, which highlights a previously unknown HA-mediated mechanism to exert biological responses in the epicardium.

2. Materials and Methods

2.1 Cell culture

The inducible immortalized epicardial cell line was generated as previously described [22]. Briefly, embryonic hearts from transgenic mice harboring a thermolabile SV40 TAg gene were harvested at E11.5 and placed dorsal side down on collagen culture dishes. Hearts were then incubated for 15 hrs at 37°C to allow for epicardial monolayers to form at the collagen coated surface. The resulting monolayers were propagated at 33°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), antibiotics, insulin-transferrin-selenium (Invitrogen, Carlsbad, CA) and mouse gamma interferon (10 units/mL, R&D systems, Minneapolis, MN).

2.2 Reagents

High molecular weight hyaluronan (HA) (~980 kDa) and low molecular weight HA (~31 kDa) were purchased from R&D systems. Rat anti-CD44 blocking antibody (clone KM201), which directly inhibits binding of CD44 to HA [23], was obtained from Southern Biotech (Birmingham, AL) and used at a concentration of 2 ng/mL. The cell permeable NFκB inhibitor SN50 (2 μM) and ERK activation inhibitor peptide (500 nM) were purchased from Calbiochem (San Diego, CA). Antibodies against MEKK1, CD44 and NFκB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) while those against hemagglutinin and β-actin were from Roche Applied Science (Indianapolis, IN) and Affinity Bio Reagents (Rockford, IL), respectively. Antibodies recognizing histone 3, ERK1/2, Snail2 and vimentin and the MEK inhibitor U0126 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against phospho-ERK1/2 and phospho-MEKK1 were obtained from Sigma (St Louis, MO) and Abgent (San Diego, CA).

2.3 Expression constructs

The hemagglutinin-tagged kinase-inactive MEKK1 construct (K1253M) was kindly provided by Dr. Richard Vaillancourt (University of Arizona, Tucson, AZ). Vectors pNFκB-SEAP, pSRE-SEAP, p-CMV-βgal, pTAL-SEAP and pSEAP2 were purchased from Clontech (Mountain View, CA).

2.4 SEAP reporter Assays

NFκB activity was determined by transfecting cells with a pNFκB-SEAP reporter vector, as previously described [13]. The induction of the serum response element (SRE) was monitored using the pSRE-SEAP construct, which expresses a secreted form of serum embryonic alkaline phosphatase (SEAP) driven by three tandem copies of the SRE consensus sequence. SEAP activity was normalized for transfection efficiency using a vector coding for β-galactosidase. After treatments and a 24 hr incubation period, SEAP and β-galactosidase expression levels were detected using the Great EscAPe™ SEAP chemiluminescence kit and the luminescent β-gal detection kit, respectively, as indicated by the manufacturer (Clontech).

2.5 Immunoprecipitation and Western Blotting

Epicardial cells were serum starved overnight and left untreated or treated with CD44 blocking antibody and/or HA for various time intervals. Subsequently, cells were lysed and diluted in TNEN buffer (1M Tris base, 5M NaCl, 0.5M EDTA and NP40). Samples were then incubated with anti-CD44 for 1 hr at 4°C and rotated with protein G agarose beads for 2 hrs. Next, beads were centrifuged, washed with TNEN buffer three times and boiled with SDS sample buffer for 5 min.

In some cases, cells were treated with various inhibitors and/or HA, lysed and solubilized in SDS sample buffer. To obtain nuclear extracts, cells were lysed using NE PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). Total cellular lysates, nuclear lysates, or immunoprecipitates were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride membrane. After blocking in 3% BSA, membranes were probed with different primary and secondary antibodies. Detection was performed using Super Signal West Pico substrate (Pierce). Quantification by densitometry was performed with the ImageJ program (NIH, Bethesda, MD).

2.6 Immunofluorescence

Cells grown and treated on glass coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100, and blocked with BSA. Cells were then incubated with anti-MEKK1 and anti-CD44 antibodies for 3 hours and fluorescently labeled secondary antibodies (Molecular

Probes, Eugene, OR) for 1 hour at room temperature. Fluorescent images were taken with the Leica DMLB fluorescence microscope (Leica, Bannockburn, Ill) using the Image ProPlus software (Media Cybernetics, Bethesda, Md).

2.7 FACE assays

Cell-based ELISAs were performed using the FACE assay system according to the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, cells were seeded into 96-well plates and treated as indicated. Cells were then fixed with formaldehyde and assayed for the presence of total or di-phosphorylated ERK using a colorimetric reaction. Results are expressed as the ratio of di-phosphorylated ERK to total ERK.

2.8 Real-time PCR

After the indicated treatments, Total RNA was isolated from cells using the RNA-STAT60 reagent (Tel-test). cDNA synthesis was conducted using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions.

Quantitative real-time PCR was performed as previously described [13]. The gene RPS7 was used for normalization. The following primer sequences were employed: *RPS7*: AGCACGTGGTCTTCATTGCT, CTGTCAGGGTACGGCTTCTG; *Snail1*: ACCTGCTCCGGTCTCAGTC, TTGTCAAGGCTGAACCAATG; and *Snail2*, GATCTGTGGCAAGGCTTTCT, ATTGCA GTGAGGGCAAGAGA.

2.9 Proliferation Assays

Cells were seeded into 96-well culture plates at a density of 1.8×10^4 cells/well and routinely cultured overnight. Next, cells were serum starved for 1 hour, followed by 30 minute treatment with HA at 0-300 μ g/mL and a 24 hour incubation period. Cell proliferation was assessed using the Vybrant® MTT cell proliferation assay kit (Molecular Probes) as indicated by the manufacturer.

2.10 Invasion Assays

The invasive potential of cells upon HA stimulation was determined by using a transwell chamber system, as previously described [13]. Briefly, fluorescently labeled cells were plated in the upper chambers of a transwell 96-well plate containing collagen gels. After incubation to allow for adherence, cells were treated with different inhibitors and/or HA for 30 min, washed with PBS and incubated in low serum media for 24 hours. Invasion was quantified by measuring fluorescently labeled cells that crossed into the lower chamber of the transwell system. Fluorescence was determined at 538 nm with a plate reader (Spectramax Gemini, Molecular Devices, Sunnyvale, CA).

2.11 siRNA Experiments

Small interfering RNA (siRNA) against Snail2 and control siRNA (siRNA-A) were purchased from Santa Cruz Biotechnology. Cells grown to 50% confluence in 6-well plates were transfected with either Snail2 siRNA or control siRNA using XtremeGene siRNA transfection reagent according to the manufacturer's instructions (Roche Molecular Systems). Transfected cells were incubated for 48 hours in medium containing 10% FBS, then harvested by trypsin treatment and used for invasion assays.

2.12 Statistical Analysis

Quantitative data were analyzed using two sample Student's t tests. The quantitative data displayed represent the means $\pm$ S.D. of 2 independent experiments performed in triplicate,

except for invasion assays, in which samples were analyzed in sets of 6. The level of significance was established at $P < 0.05$.

3. Results

3.1 HMW-HA induces CD44-MEKK1 interaction and activation of MEKK1

Although the activation of MEKK1 and interactions between HA and the CD44 receptor have been shown to play an important role in epithelial cell motility, no functional connection between these molecules has been reported to date [24-26]. Thus, we sought to determine whether MEKK1 physically associates with CD44 in epicardial cells following HA stimulation. To address this question, we treated epicardial cells with 300 $\mu\text{g/mL}$ of HMW-HA for various time points (0-15 min) followed by immunoprecipitation with an antibody against CD44. Subsequent Western blotting was performed to detect MEKK1 and CD44. As shown in Figure 1A, stimulation with HMW-HA for 5 minutes induces recruitment of MEKK1 to CD44. Similarly, immunostaining studies demonstrate that CD44 and MEKK1 colocalize in the cell membrane in response to HMW-HA (Fig. 1B). Cells treated with LMW-HA or pretreated with the CD44 blocking antibody KM201 followed by stimulation with HMW-HA also fail to show CD44/MEKK1 association (Fig. 1C). This suggests that HMW-HA physically binds CD44 in order to induce the interaction of this receptor with MEKK1.

We next assessed whether HMW-HA plays a role in the activation of MEKK1. As shown in Figure 1D, cells stimulated with HMW-HA, but not with LMW-HA, for up to 10 minutes show increased phosphorylation of MEKK1 on threonine 1383, which is located within the kinase domain. These findings suggest that HA promotes binding of MEKK1 to CD44 in a size-dependent manner and also induces MEKK1 kinase activity.

3.2 HMW-HA promotes ERK phosphorylation and SRE activation via CD44 and MEKK1

Both HA and MEKK1 have been involved in the activation of the ERK1/2 signaling pathway during cancer metastasis [9,27]. Therefore, we investigated whether ERK1/2 is activated in response to HMW-HA in epicardial cells and whether CD44 and MEKK1 play a role in the transduction of these signals. ERK1/2 phosphorylation status was determined via Western blotting and a fast activated cell enzyme-linked immunosorbent assay (FACE). We found that HMW-HA significantly stimulates the phosphorylation of ERK1/2 in a dose-dependent manner (Fig. 2A). However, treatment with LMW-HA does not affect ERK1/2 phosphorylation status, indicating specific response to the HMW-HA size (Fig. 2B). Furthermore, pretreatment with CD44 blocking antibody, transfection with KI-MEKK1 or pretreatment with U0126 followed by stimulation with HMW-HA significantly decreases ERK1/2 phosphorylation as compared to treatments with HMW-HA alone (Fig. 2B). These data indicate that CD44, MEKK1 and MEK1/2 are important for the activation of ERK1/2 by HMW-HA, although other molecules may also be involved.

One of the mechanisms through which ERK is known to collaborate in the regulation of cell motility is by increasing gene transcription under the control of the serum response element (SRE) [28]. Thus, we examined whether HA-induced ERK1/2 activation could be correlated with increased SRE activity. For this, we transfected cells with a reporter construct consisting of serum embryonic alkaline phosphatase (SEAP) driven by three copies of the SRE consensus sequence. As shown in Figure 2C, stimulation with HMW-HA induces a significant activation of the SRE, which is coincident with the reported ERK1/2 phosphorylation pattern. This induction of SRE activity is not observed in the presence of LMW-HA and is greatly decreased when cells are treated with a CD44 blocking antibody, the MEK1/2 inhibitor U0126, an ERK inhibitor peptide or transfected with KI-MEKK1 previous to the addition of HMW-HA (Fig.

2D). These results demonstrate that HMW-HA induces a CD44/MEKK1/MEK/ERK cascade to regulate SRE activity.

3.3 HMW-HA regulates NF κ B activity and Snail2 expression in a CD44 and MEKK1-dependent but ERK-independent manner

HMW-HA has been shown to induce the transcription of the zinc finger protein Snail2, but not the related Snail1 factor, in a mechanism involving NF κ B in mesenchymal cells [13]. Thus, we investigated whether this pathway is also activated in epicardial cells which are epithelial in phenotype. NF κ B activity was assessed by transfecting cells with a NF κ B-SEAP reporter construct and measuring SEAP secretion after stimulation with HA. As shown in Figure 3A, HMW-HA significantly induces NF κ B activity in a dose-dependent manner. These results were verified by preparing nuclear extracts from cells stimulated with HA and performing Western blots to detect NF κ B. Figure 3B shows that HMW-HA, but not LMW-HA, induces NF κ B translocation into the nucleus and that the NF κ B inhibitor SN50 successfully blocks this response.

Next, NF κ B-SEAP reporter assays were performed in the presence of specific inhibitors to CD44, MEKK1, MEK1/2 and ERK1/2 to determine the role of these molecules in the activation of NF κ B by HMW-HA. While pretreatment with CD44 blocking antibody or expression of KI-MEKK1 significantly abrogates the ability of HMW-HA to activate NF κ B, incubation with a MEK or ERK inhibitor does not appear to disrupt these HA-mediated signals (Fig. 3C). These observations suggest that HMW-HA induces NF κ B activity through CD44 and MEKK1 in a manner that is independent of ERK1/2 activation.

The role of HA in the regulation of Snail2 was investigated by performing RT-PCR and Western blotting to detect mRNA and protein levels, respectively. Here we show that HMW-HA induces the expression of Snail2 at the mRNA (Fig. 3D) and protein levels (Fig. 3E) in a dose-dependent manner. However, Snail2 mRNA is unaffected by LMW-HA treatments. Incubation with CD44 antibody, KI-MEKK1 or SN50, but not the MEK or ERK inhibitor, effectively inhibits the induction of Snail2 by HMW-HA (Fig. 3E). Together, these data indicate that a NF κ B/Snail2 pathway is activated in response to HMW-HA and that these signals are transduced via CD44 and MEKK1 but not ERK1/2.

3.4 Epicardial cells invade through a collagen matrix in response to HMW-HA

HA has been shown to regulate the motility of several cardiac cell types during embryonic development [6,29]. Thus, we tested whether HA affects epicardial cell motility in vitro by performing a modified Boyden chamber assay. As shown in Figure 4A, stimulation with HMW-HA, but not LMW-HA, significantly induces epicardial cell invasion after 24 hrs. This increased invasion is not the result of increased cell number, as MTT assays do not show increased cell proliferation in response to HA (Fig. 4B). Interestingly, stimulation with HMW-HA, but not LMW-HA, increases the expression of vimentin, a mesenchymal cell marker, in a manner that correlates with the increased invasive potential (Fig. 4C). This suggests that the enhanced epicardial cell motility in response to HMW-HA may be due to differentiation into a mesenchymal phenotype.

3.5 CD44 and MEKK1 are important for the induction of cellular invasion and differentiation by HA

As we have demonstrated that both CD44 and MEKK1 play a role in the regulation of epicardial signaling by HA, we next sought to determine whether these molecules are downstream of HA during the induction of epicardial EMT. Incubation of cells with a CD44 blocking antibody or expression of a KI-MEKK1 construct followed by treatments with HMW-HA significantly decreases HA-mediated cellular invasion (Fig. 5A) and vimentin expression (Fig. 5B) but does

not completely abrogate it. Thus, CD44 and MEKK1 participate in the transduction of HA signals to promote cellular invasion and differentiation but other compensatory mechanisms also participate in this response.

3.6 Both the ERK and NF κ B pathway mediate HA-induced EMT

We next studied the contribution of ERK and NF κ B effectors during HA-mediated cell motility by treating cells with HMW-HA in the presence or absence of specific inhibitors. As shown in Figure 6A, transfection with control siRNA (lane 3) or Snail2 siRNA (lane 4) or treatment with SN50 (lane 5) does not significantly affect the invasive response to HA. In contrast, incubation with the ERK inhibitor (Fig. 6A, lane 6) significantly decreases HA-stimulated invasion while addition of both the ERK and NF κ B inhibitors (Figure 6A, lane 7) completely blocks this biological response. Similarly, only pretreatments with ERK inhibitor alone or a combination of ERK and NF κ B inhibitors were able to abrogate HA-mediated increases in vimentin expression (Figure 6B). These observations indicate that the ERK and NF κ B cascades work synergistically under the control of HMW-HA to bring about changes in epicardial cell behavior (Fig. 7).

4. Discussion

HA is a linear glycosaminoglycan composed of disaccharide units of glucuronic acid and N-acetyl glucosamine. This polymer is produced by hyaluronan synthases (Has) 1, 2 and 3, with Has2 being the predominant source of HA during embryonic development [30]. Once synthesized as a high molecular weight molecule, HA can be incorporated into the extracellular matrix, where it provides structural support, or it can bind cell surface receptors and induce biological signals [31]. HA can also be degraded into smaller oligosaccharides by enzymes known as hyaluronidases [32]. However, the role of these different HA sizes remains controversial as wide range of HA molecular weights have been shown to induce cellular responses [33-35].

In the present study we demonstrate that HMW-HA, but not LMW-HA, induces cellular invasion and differentiation in epicardial cells (Fig. 4). These results add to the growing evidence suggesting a specific role for HMW-HA during embryogenesis. While stimulation with HA oligosaccharides has been associated with negligible or even inhibitory effects on developmental EMT and cellular invasion [13,36], treatments with HMW-HA have yielded opposing effects, with embryonic cells exhibiting increased biological activity [6,13,37]. Additionally, these findings highlight a novel HA-mediated response in the epicardium and indicate a contribution for this molecule in the formation of the coronary vasculature.

In trying to determine how HA exerts its biological effects in the epicardium, we first focused on the role of the HA receptor CD44, as this protein is highly expressed in the heart during embryonic development [38]. Binding of HA to CD44 has been associated with the activation of the ERK and NF κ B pathways [9,13], both of which can also be under the control of the MAPK cascade component MEKK1 following several stimuli [39,40]. MAPKs are cytoplasmic serine-threonine kinases that transduce extracellular signals to regulate a variety of physiological responses in a context-specific manner. In the classic arrangement, induction of a receptor leads to activation of a MAPK kinase kinase (MAP3K), which phosphorylates and activates a MAPK kinase (MAP2K), which in turn phosphorylates and activates a MAPK. Because the MAP3K MEKK1 is also important for epithelial cell motility, we hypothesized that this kinase may participate in the transduction of HA signals to induce epicardial cell invasion. Here we have shown that stimulation with HMW-HA, but not LMW-HA, induces association of CD44 with MEKK1 and promotes phosphorylation of MEKK1 in epicardial cells (Fig. 1). These findings not only demonstrate that MEKK1 is indeed responsive to HA but also highlight a previously unknown interaction between CD44 and MEKK1. Also, these

proteins play an important role in HA-mediated EMT as blockade of CD44 or the presence of a kinase-inactive form of MEKK1 significantly impairs the ability of HMW-HA to promote epicardial cell invasion and differentiation (Fig. 5). Together, these observations further imply that MEKK1 functions to convey HA signals and thus may lead to the activation of MAPK cascades.

Through our study of possible downstream effectors, we have demonstrated that HMW-HA is able to activate ERK1/2 in epicardial cells. Furthermore, blockade of CD44, the lack of MEKK1 kinase activity or inhibition of MEK1/2 significantly decreases ERK1/2 phosphorylation by HMW-HA (Fig. 2). These results are consistent with previous findings in other systems suggesting that HA induces ERK1/2 activation through CD44 and also provides additional evidence for the involvement of MEKK1 in HA-mediated signaling. To further investigate whether HA is able to regulate gene transcription through this MAPK pathway, we analyzed the effect of HMW-HA on serum response element (SRE) activation. The SRE is a 29 bp oligonucleotide responsible for the formation of a complex that regulates the transcriptional machinery of target genes following induction by intracellular kinases such as ERK1/2 [41]. Similarly to what was observed with ERK1/2, HMW-HA induces SRE activity through a mechanism requiring CD44 and MEKK1 (Fig. 2D). Additionally, pretreatment with a MEK or ERK inhibitor completely abrogates the ability of HMW-HA to activate SRE, indicating that SRE is specifically regulated by ERK1/2 in response to HA. It is worth noting that, although inhibition of CD44 and MEKK1 signals significantly decreases HA-mediated induction of the ERK1/2/SRE cascade, it does not completely abrogate it. Thus, it is possible that HMW-HA may regulate ERK1/2/SRE through additional pathways that are independent of CD44 and MEKK1.

We have previously shown that HMW-HA promotes cellular invasion in NIH-3T3 cells through a mechanism requiring NF κ B and Snail2. Therefore, we explored the possibility of this pathway also being activated in epicardial cells. Interestingly, NF κ B activity and Snail2 expression are both induced in response to HMW-HA in a CD44 and MEKK1 dependent manner (Fig. 3). However, this pathway appears to be activated to a lesser extent in epicardial cells when compared to our previous studies in NIH-3T3 cells, suggesting a compensatory but not prevalent role for this NF κ B/Snail2 cascade in epicardial cells. Furthermore, inhibition of MEK1/2 or ERK1/2 does not affect the ability of HA to induce NF κ B and Snail2, which indicates that the ERK1/2 and NF κ B pathways are distinctly activated by HMW-HA in epicardial cells and function independently of each other. As we investigated the role of these proteins in HA-mediated EMT we noted that, while inhibition of ERK1/2 effectively decreased the cellular response to HMW-HA, blockade of NF κ B or silencing of Snail2 did not significantly affect it (Fig. 6). Thus, other mechanisms appear to sufficiently compensate for the absence of NF κ B signals. Interestingly, if both ERK1/2 and NF κ B are simultaneously blocked, the invasive response to HA is totally inhibited, indicating a synergistic effect between these two pathways.

This study is the first to our knowledge to demonstrate that HA-mediated activation of NF κ B and ERK1/2 is dependent on the CD44/MEKK1 signaling axis and to identify these cascades as crucial for the induction of epicardial cell motility and differentiation (Fig. 7). These observations define a novel integrated model for the regulation of epicardial cell biology by HA, suggesting a global role for this carbohydrate during cardiac development.

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Fig. 1.

MEKK1 phosphorylation and association with CD44 in response to HMW-HA. (A) Epicardial cells were treated with HMW-HA (300 $\mu\text{g/mL}$) for 0-15 min, followed by immunoprecipitation (IP) with anti-CD44 and Western blotting (WB) with anti-MEKK1 (top panel) or anti-CD44 antibody (bottom panel). The graph represents WB quantification by densitometry. The results are expressed as MEKK1 band intensity relative to the CD44 bands. (B) Cells were treated with HMW-HA (300 $\mu\text{g/mL}$) and then immunofluorescently stained with antibodies against MEKK1 and CD44. (C) Cells were left untreated (lane 1), treated with LMW-HA (lane 2) or HMW-HA (lane 3) for 5 min or incubated with CD44 blocking antibody followed by treatment with HMW-HA for 5 min (lane 4). Lysates were subjected to IP with anti-CD44 and WB with anti-MEKK1 (top panel) or anti-CD44 antibody (bottom panel). Densitometry analysis was performed as in 1A. (D) Cells were treated with HMW-HA or LMW-HA (300 $\mu\text{g/mL}$) for 0-15 min, followed by WB with anti-phospho MEKK1 (top panel) and reblotting with anti-MEKK1 (bottom panel). Densitometry measurements were performed as in 1A and are expressed as phospho-MEKK1 band intensity relative to the MEKK1 bands. *, $p < 0.05$ as compared to the untreated control.

Fig. 2. Detection of ERK1/2 and SRE activity in HMW-HA-treated cells. (A) Cells treated with HMW-HA (0-300 μ g/mL) for 15 min were employed for WB with anti-diphospho-ERK1/2 (top panel) and reblotted with anti-ERK1/2 (bottom panel) or were subjected to FACE assays (graph) and the absorbance values were measured. (B) Cells were left untreated (lane 1), or stimulated with LMW-HA (lane 2), HMW-HA alone (lane 3), HMW-HA in the presence of a CD44 blocking antibody (lane 4), KI-MEKK1 (lane 5) or U0126 (lane 6). Lysates were employed for WB with anti-diphospho-ERK1/2 (top panel) and reblotted with anti-ERK1/2 (bottom panel) or cells were subjected to FACE assays (graph) and the absorbance values were measured. (C) Cells were transfected with a SEAP-SRE reporter plasmid and treated with HMW-HA for 30 min. SEAP activity was determined after a 24 hr incubation period. (D) Cell were transfected with SEAP-SRE and left untreated or treated with LMW-HA, HMW-HA alone, HMW-HA in the presence of CD44 blocking antibody, KI-MEKK1, U0126 or ERK

inhibitor peptide. SEAP activity was determined after a 24 hr incubation period. (E) Cells were left untransfected or transfected with KI-MEKK1 and incubated for 30 hrs. Lysates were subjected to WB with anti-hemagglutinin and anti-actin antibodies. *, $p < 0.05$ as compared to the untreated control; #, $p < 0.05$ as compared to treatment with HMW-HA alone.

Fig. 3.

CD44 and MEKK1 are required for HMW-HA to induce the NFκB pathway. (A) NFκB-SEAP reporter activity was measured in cells following 30 minute stimulation with HMW-HA and 24 hour incubation. (B) Cells left untreated or treated with LMW-HA, HMW-HA alone, HMW-HA in the presence of SN50, or SN50 alone. Nuclear extracts were employed for WB with anti-NFκB and anti-histone 3 antibodies. (C) NFκB-SEAP reporter activity was measured in cells treated with LMW-HA or HMW-HA in the absence or presence of CD44 blocking antibody, KI-MEKK1, U0126, ERK inhibitor peptide or SN50. (D) Snail2 mRNA was detected by real-time PCR following stimulation with HMW-HA for 30 minutes and a 24-hour incubation period. (E) Snail2 protein levels were analyzed by WB following stimulation with HMW-HA for 30 minutes and a 24-hour incubation period. The graph represents WB quantification by densitometry. The results are expressed as Snail2 band intensity relative to the actin bands. (E) Snail2 expression in cells treated with LMW-HA or HMW-HA in the absence or presence of CD44 blocking antibody, KI-MEKK1, U0126, ERK inhibitor peptide or SN50. *, $p < 0.05$ as compared to the untreated control; #, $p < 0.05$ as compared to treatment with HMW-HA alone.

Fig. 4.

Regulation of epicardial cell behavior by HA. (A) Fluorescently labeled cells were stimulated with LMW-HA or HMW-HA for 30 minutes and incubated for 24 hours to allow for invasion into collagen gels and through an 8 μ m pore membrane. (B) Cells were treated with LMW-HA or HMW-HA for 30 minutes and incubated for 24 hours in low serum medium (1% FBS). 10% FBS was used as a positive control. (C) Cells were treated with HMW-HA or LMW-HA for 30 minutes and incubated for 24 hours. Lysates were subjected to WB with anti-vimentin and anti-actin antibodies. The graphs represent WB quantification by densitometry. The results are expressed as vimentin band intensity relative to the actin bands. *, $p < 0.05$ as compared to the untreated control.

Fig. 5.

The role of CD44 and MEKK1 in HA-mediated cellular invasion and differentiation. (A) Fluorescently labeled cells were stimulated with HMW-HA for 30 min in the presence or absence of CD44 blocking antibody or KI-MEKK1 followed by a 24hr incubation period. (B) Cells were treated with HMW-HA for 30 minutes in the presence or absence of CD44 blocking antibody or KIMEKK1 and incubated for 24 hours. Lysates were subjected to WB with anti-vimentin and anti-actin antibodies. *, $p < 0.05$ as compared to the untreated control; #, $p < 0.05$ as compared to treatment with HMW-HA alone.

Fig. 6.

Involvement of the ERK1/2 and NFκB cascades in the regulation of cellular invasion and differentiation by HMW-HA. (A) Cells were labeled and treated with HMW-HA for 30 min in the absence or presence of control siRNA, Snail2 siRNA, SN50, ERK inhibitor peptide or SN50 together with ERK inhibitor peptide. After incubation for 24hrs, fluorescence was determined. (B) Cells were subjected to treatments as in 6A and lysates were subjected to WB with anti-vimentin and anti-actin antibodies. (C) Real-time PCR was performed to determine specificity and silencing ability of Snail2 siRNA. Both Snail1 (clear bars) and Snail2 (dark bars) expression levels were measured. Decreased Snail2 protein levels following transfection with Snail2 siRNA were also detected by WB. *, $p < 0.05$ as compared to the untreated control; #, $p < 0.05$ as compared to treatment with HMW-HA alone.

Fig. 7.

A schematic diagram of molecular interactions stimulated by HMW-HA . We propose that HMW-HA activates two distinct signaling cascades, one ERK-dependent and another NFκB-dependent, which lead to the induction of differentiation and invasion in epicardial cells. CD44 and MEKK1 are important for the activation of both cascades, although HMW-HA may also activate ERK1/2 via other mechanisms.