Hyaluronan Stabilizes Focal Adhesions, Filopodia, and the Proliferative Phenotype in Esophageal Squamous Carcinoma Cells*

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Hyaluronan (HA) can be produced by three isoforms of the hyaluronan synthase family (HAS1–3), which are located at the plasma membrane and extrude the growing HA polymer into the extracellular space. HAS isoenzymes produce HA of different chain lengths, and HA is subsequently degraded by hyaluronidases. HA is an unbranched high molecular weight polysaccharide that is composed of D-glucuronic acid β(1–3)-β-d-N-acetyl-glucosamine-β(1–4) without further modifications. A variety of different types of cancer is characterized by high amounts of tumor-cell-associated HA (e.g. colon and gastric cancer), and in some of these malignancies, such as colon cancer, tumor-associated HA is an independent prognostic factor for poor outcome (1–2). The activity of all three isoforms of the hyaluronan synthase family (HAS1–3) can be inhibited by 4-methylumbelliferone, which interferes with HAS activity by depleting the activated uridine diphosphate-glucuronic acid precursor pool (3). Consequently, 4-MU inhibits tumor progression in animal models (4–5). The biological effects of HA have largely been attributed to activation of the HA receptors RHAMM (receptor of HA-mediated motility, CD168) and CD44 (6). CD44 is an adhesion receptor and an HA receptor that serves, together with the expression of CD24 or CD133, as a surface marker for the tumorigenic potential of breast cancer and colon cancer cells (7–9) and is implicated in the HA-mediated chemoresistance of cancer cells (10). RHAMM is particularly interesting because it is a cytoplasmic protein that not only is exported into the extracellular compartment but also exerts oncogenic effects through both extracellular and intracellular functions (11–12). Importantly, RHAMM is believed to be transforming under control of RAS signaling in tumor cells (13) and to be required in part for the signaling of CD44 (11, 14). Furthermore, both CD44 and RHAMM can associate with the cytoskeleton (15–16) and control tumor cell invasiveness (17–18). In synthesis, the HA-rich matrix is important for a variety of aspects of tumor pathobiology, including anchorage-independent growth, migration, angiogenesis, suppression of apoptosis (2, 19), and metastasis (20–21).

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2 The abbreviations used are: HA, hyaluronan; ESCC, esophageal squamous cell carcinoma; 4-MU, 4-methylumbelliferone; FAK, focal adhesion kinase; HAS3, HA synthase 3; shRNA, short hairpin RNA; HABP, hyaluronan-binding protein; ERK, extracellular signal-regulated kinase; tFAK, total FAK; pFAK, phosphorylated FAK; FCS, fetal calf serum.

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With respect to esophageal cancer, it is known that HA accumulates in the parenchyma and stroma (22). However, the role of HA synthesis and the potential mechanistic links between HA synthesis, individual HAS enzymes and ESCC cell phenotype have not yet been explored.

The understanding of the role of the HA matrix in the pathophysiology of esophageal cancer may contribute to the definition of targets for novel HA-based therapeutic approaches. Therefore, the aim of the present study was to analyze the role of HA synthesis and individual HA receptors in human ESCC cells in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were obtained from the indicated sources: 4-MU and latrunculin A from Sigma-Aldrich (Munich, Germany); AG82 from Calbiochem, Merck (Darmstadt, Germany); *Streptomyces hyaluronidase* from MP Biomedicals Germany (Eschwege, Germany); and a lentiviral gene silencing system MISSION™ from Sigma-Aldrich. Sequences are indicated in Table 1. Blocking anti-CD44 monoclonal antibody Hermes-1 was from Thermo Fisher Scientific (Bonn, Germany), and blocking anti-RHAMM IgG (R36) has been described previously (23). Pep-1 (GAHWQFNALTVR) and scrambled control peptide (SATPASAPYPLA) (24) were synthesized by Biosyntan (Berlin, Germany).

**Cell Culture**—OSC1 cells were a gift from M. Sarbia (25) and were used for experiments addressing molecular mechanisms throughout the present study. Key experiments were also performed in other ESCC lines (Kyse 30, -270, -410, -520) (26) obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). OSC1 and Kyse cells were maintained as monolayer cultures in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and streptomycin at 37 °C, 5% CO2 and 95% humidified air. DNA synthesis was determined by [3H]thymidine incorporation, and migration was assessed using a microchemotaxis assay as described previously (27).

**Immunostaining**—Cultured cells were fixed for 20 min either with 3.7% paraformaldehyde in phosphate-buffered saline or with acetone:methanol (1:1) at −20 °C. HA was detected using biotinylated hyaluronan-binding protein (HABP, Seikagaku; 6 μg/ml) and fluorescein isothiocyanate- or Cy3-labeled streptavidin (Dako, Hamburg, Germany; 1:200) or Alexa 594-coupled control peptide (SATPASAPYPLA) (24) were synthesized by Biosyntan (Berlin, Germany).

**Real-time Reverse Transcription-PCR**—Total RNA from OSC1 cells was isolated by using TriReagent® (Sigma-Aldrich), and cDNA was synthesized by using the Superscript III first-strand synthesis system (Invitrogen). The PCR reactions were performed by using the 7300 real-time PCR system (Applied Biosystems, Darmstadt, Germany) with SYBR Green PCR Master Mix (Applied Biosystems). Relative expression levels were compared by using real-time PCR with the 2^−ΔΔC(T) method. The primer sequences of the genes of interest are given in Table 2.

**Lentiviral Knockdown**—HAS3, HAS2, CD44, and RHAMM knockdown were achieved by using the MISSION™ Lentiviral shRNA knockdown system (Sigma-Aldrich). The used shRNA
sequences are stated in Table 2. A scrambled shRNA was used as a control. The transfer into the packaging line HEK 293T (ATCC) was performed with the lipofection reagent FuGENE 6 (Roche Applied Science, Mannheim, Germany). After 16 h, the medium was changed to Iscove’s modified Dulbecco’s medium for better stability of the produced lentiviral particles. The next day, the lentiviruses were harvested, and target cells were transfected at a multiplicity of infection of 10 and kept for 5 days in normal growth medium before fixation.

**Statistical Analysis**—All data sets were analyzed either by analysis of variance and the Bonferroni post hoc test or by Student’s t test as appropriate. Data are presented as means ± S.E. Statistical significance was assigned at the level of p < 0.05.

**RESULTS**

**4-MU Decreases Filopodia and Focal Adhesion Complexes**—Incubating OSC1 cells with 0.3 mm 4-MU decreased the total amount of HA secreted into the medium to 50.7% ± 10.7% (n = 3, p < 0.05) of that secreted by untreated OSC1 cells. As a control, the effect of 4-MU on proteoglycan synthesis by incorporation of 35SO42− into sulfated glycosaminoglycans chains was determined. Because 0.3 mm of 4-MU specifically inhibited HA synthesis without affecting sulfated proteoglycans (94.3% ± 1.5%, n = 3 of control, p > 0.05), this concentration was used throughout the study.

Interestingly, the shape of OSC1 cells changed remarkably in response to the inhibition of HA synthesis by 4-MU. These phenotypical changes comprised cell clustering, a uniform flat appearance, and smoother cell borders than those of untreated control cells (Fig. 1A, arrows). Moreover, a dramatic decrease in actin cytoskeleton staining (Fig. 1B) occurred in the presence of 4-MU. This finding suggests that 4-MU interferes with either actin fiber formation or actin fiber anchoring, e.g. disassembly of focal adhesion complexes. In contrast, the tubulin network remained intact in response to 4-MU (Fig. 1C, green). In addition, OSC1 regularly exhibited numerous filopodia, which were detectable by the WGA Alexa Fluor® 555 conjugate as membrane marker (Fig. 1C, red). Similar filopodia have been associated previously with tumor cell transformation (30). Notably, 4-MU caused rapid resolution of these protrusions within 1 h after application (Fig. 1C, right).

Confocal imaging of live cells that had been stained with Alexa

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**FIGURE 1. 4-MU causes changes in morphology, disruption of the actin cytoskeleton, and inhibition of HA-associated filopodia.** A, treatment with 0.3 mm 4-MU for 24 h resulted in a dramatic change in the shape of OSC1 cells, including a uniform flat appearance, cluster formation, and smoother cell borders (arrows). Scale bars, 500 μm. B, phalloidin staining revealed a retraction of actin cytoskeleton 24 h after the addition of 4-MU to OSC1 cells. Scale bars, 50 μm. C, in controls, extensive filopodial protrusions were detected by staining of plasma membranes (membr.) with the WGA Alexa Fluor® 555 conjugate (red). The filopodia did not contain tubulin (green). Filopodia were no longer detectable 24 h after the addition of 4-MU (right panel). Scale bars, 20 μm. D, the use of Alexa Fluor® 594-coupled HABP (red) during confocal microscopy of live cells showed a continuous pericellular HA coat covering also the filopodia (arrows). Hyaluronidase (20 units per ml) completely removed the pericellular HA signal (not shown). Importantly, after treatment with 4-MU for 24 h, the HA coat was no longer detectable. This finding suggests rapid turnover of the pericellular HA coat. Top, the xz-view; bottom, the xy-view. The orientation of the xz-analysis is indicated. Scale bars, 20 μm. E, the association of the HA coat with the filopodia was verified in fixed OSC1 cells by immunostaining of CD44 (green) and affinity histochemistry of HA (red). Fluorescence microscopy showed that HA aggregates are still associated with the filopodia even after fixation. Scale bars, 5 μm. F, staining actin (phallolidin, green) and the membrane (red) showed that the filopodia contained actin and that the filopodia were sensitive to the inhibitor of actin polymerization latrunculin A (2 μM, 5 min). Scale bars, 5 μm. G, filopodia showed a rapid response to inhibition of FAK by AG82 (10 μM, 1 h), as shown by WGA Alexa Fluor® 555 conjugate (red). In this figure, the images compare untreated OSC1 cells in 10% FCS (control) with OSC1 treated with the indicated agents. Representative images from more than three experiments are shown. Scale bars, 20 μm.
still intact at this time. After 5 min, the inhibition of filopodia was almost complete (Fig. 1F, right).

Subsequently, the contribution of focal adhesion (FA) function to the maintenance of filopodia was analyzed by treatment with the FAK inhibitor AG82 (10 μM, 1 h). AG82 completely inhibited the cell protrusions (Fig. 1G). Taken together, these results indicate that OSC1 cells extend actin-based filopodia that are dependent on HA synthesis and FAK activity.

Inhibition of HA Synthesis Causes Cleavage of FAK—To address the mechanism by which the inhibition of HA synthesis interferes with FAK and FAs, we used immunostaining of phosphorylated FAK (Fig. 2A, yellow) and paxillin (Fig. 2B, red) to analyze the distribution and activity of focal adhesion complexes after treatment with 4-MU. The levels of both pFAK and paxillin were dramatically lower in focal adhesions in response to 4-MU (24 h). In addition, we performed immunoblot analysis to measure the amount of total and phosphorylated FAK (Fig. 2C). In line with the results of immunofluorescence staining, these results showed a strong decrease in the levels of both total FAK (tFAK) and pFAK.

Using an antibody that detects C-terminal FAK cleavage products, we identified rapid cleavage of FAK starting between 15 and 30 min after the addition of 4-MU as the reason for the reduction in tFAK (Fig. 2D). The most prominent bands run at 125, 100, 70, and 48 kDa; these findings are in agreement with those reported previously (31). To ensure that the observed phenomena were specific responses to the inhibition of HA synthesis by 4-MU, we investigated the effects of other agents known to either degrade or displace HA. Indeed, we found that FAK degradation was also induced 5 min after the application of Streptomyces hyaluronidase (Fig. 2E, upper panel) and 24 h after the application of Pep-1 (500 μg/ml, 24 h, lower panel). To analyze the distribution and activity of focal adhesion complexes after treatment with 4-MU, we performed immunoblot analysis to measure the amount of total and phosphorylated FAK (Fig. 2C). In line with the results of immunofluorescence staining, these results showed a strong decrease in the levels of both total FAK (tFAK) and pFAK.

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Kyse 520. Furthermore, FAK degradation in response to 4-MU occurred in four out of five tested cell lines such as Kyse 520, 410, 270 (Fig. 3B). These results indicate that the pericellular HA matrix is required for filopodial plasma membrane extensions and suggest that the absence of HA results in rapid degradation of FAK and the breakdown of filopodia.

**TREATMENT WITH 4-MU IMPAIRS PROLIFERATION AND MIGRATION OF OSC1 CELLS**—In addition, the phosphorylation status of ERK1/2, an important downstream target of FAK, was analyzed between 5 min and 20 min after the addition of 4-MU. It was found that reduction of phosphorylation of ERK1/2 was strongly decreased starting at 20 min. Shown are representative immunoblots and quantitative analysis at 20 min of digitized blots.

45.4% ± 3.8% that of untreated controls (n = 3–5, mean ± S.E., p < 0.05, Fig. 4C).

**KNOCKDOWN OF HAS3 AND HAS2 CAUSES FAK DEGRADATION AND INHIBITION OF FILOPODIA**—Because HAS3 and HAS2 are the main HAS isoforms in OSC1 cells, we investigated whether the molecular and cellular events mediating the inhibitory effects of 4-MU could be recapitulated by the knockdown of HAS3 or HAS2 in OSC1 cells in vitro. Knockdown of HAS3 and to a lesser extent of HAS2 reduced HA secretion into the medium (Fig. 5A) as expected from the relative expression levels of HAS2 and HAS3. In response to knockdown of HAS3, the phenotypical changes and the resolution of filopodia (Fig. 5B) closely resembled the changes produced by 4-MU. In addition, shHAS2 partially reduced filopodia as well and induced a smoother outline of cell clusters. Western blot analysis using the C-terminal FAK anti-
body showed pronounced FAK cleavage after HAS3 and HAS2 knockdown (Fig. 6A). Concomitantly Akt/PKB and ERK phosphorylation were reduced by shHAS3 and shHAS2 (Fig. 6B). In turn, shHAS3 and shHAS2 reduced the proliferative and migratory response to FCS (Fig. 6C). Altogether, the cellular responses to HAS3 and HAS2 knockdown support the conclusion that HA synthesis plays a key role in the maintenance of filopodia and FAK protein levels as well as ERK and Akt/PKB signaling in OSC1 cells.

Blockade of RHAMM but Not CD44 Induces FAK Degradation and Inhibits Filopodia—OSC1 cells express both CD44 and RHAMM as identified by immunohistochemistry (Fig. 7, A and B). In response to 4-MU, CD44 was more pronounced in the circumference of the OSC1 cells, which might be due to a redistribution or due to the change in cell shape. In contrast, the expression pattern of RHAMM was not affected by 4-MU. To identify the HA receptors involved in the regulation of tFAK protein levels, and the maintenance of filopodia CD44 and RHAMM were down-regulated by lentiviral shRNA. Interestingly, shCD44 did not affect filopodial integrity (Fig. 7C) or tFAK levels (Fig. 8A). In contrast, shRHAMM led to a complete inhibition of filopodia (Fig. 7D) and a strong decrease in intact tFAK levels (Fig. 8A). However, both shRHAMM and shCD44 decreased Akt/PKB and ERK1/2 phosphorylation (Fig. 8B) in OSC1 cells. Furthermore, both shCD44 and shRHAMM reduced proliferation and migration in response to FCS (Fig. 8C).

In addition to shRNA, blocking antibodies against CD44 (Hermes-1) and RHAMM (R36) were used. In line with the results obtained with shRNA, only blocking RHAMM caused loss of filopodia and FAK cleavage (supplemental figure). Blocking antibodies against CD44 inhibited Akt/PKB and ERK phosphorylation and blocking RHAMM by R36 resulted only in reduced Akt/PKB phosphorylation (supplemental figure). Thus, inhibiting RHAMM closely mimics all effects of 4-MU, shHAS3, and shHAS2, whereas inhibition of CD44 lacks the effects on filopodia and FAK. Therefore, it may be concluded that RHAMM plays a crucial role in transducing the effects of pericellular HA on the maintenance of FA and filopodial integrity in OSC1 cells, whereas both HA receptors are involved in ERK and Akt/PKB signaling.

**DISCUSSION**

HA synthesis is not sufficient for malignant transformation (32), but HA, HA-binding proteins, and HA receptors provide a matrix environment that supports the malignant phenotype of cancer cells, stromal cell recruitment, and, thus, the progression of cancer (33). In human ESCC, HA accumulates in the parenchyma and stroma, and HA is produced by both tumor cells and stroma (22, 34). Here, an analysis of the molecular and cellular effects of HA synthesis on ESCC phenotype is provided.

In addition to the inhibition of proliferation and migration, 4-MU also repressed the formation of cell protrusions. These cell protrusions were reminiscent of the filopodia that have in previous studies been associated with the malignant phenotype of cancer cells (30). Furthermore, it was demonstrated recently that overexpression of HAS3 in several cell lines causes pronounced microvilli that were sensitive to 4-MU and hyaluronidase (28). Therefore, microvilli are thought to provide a scaffold to support the pericellular HA-matrix (28). The present results suggest that filopodia also can be dependent on HA-synthesis and pericellular HA. Furthermore, our findings suggest that the loss of filopodia and the change in cell shape are a consequence of FAK cleavage in response to the inhibition of HA synthesis. A likely candidate protease responsible for FAK degradation is calpain, which is involved in the physiological turnover of FAK and is
PKB, whereas ERK phosphorylation was responsive only to shRHAMM. This difference between the use of the blocking antibody R36 and shRNA might point toward a role of intracellular RHAMM for ERK phosphorylation in OSC1. Inhibition of CD44 by both shRNA and Hermes1 antibody led to decreased phosphorylation of Akt/PKB and ERK1/2. These findings suggest that both HA receptors are involved in the observed inhibition of the signaling response after interference with HA synthesis. The inhibition of Akt/PKB and ERK 1/2 signaling likely also explains the inhibition of growth and migration in response to 4-MU, shHAS3, shHAS2, shCD44, and shRHAMM because the activation of the Ras-MAPK and PI3-kinase pathways by HA have been shown to mediate promigratory and proproliferative phenotypes (32, 39–41) in cultured cancer cells. Interestingly, RHAMM is also a novel susceptibility gene for breast cancer, and its overexpression is positively correlated with the phosphorylation of ERK, metastasis, and poor survival for patients with breast cancer (42–43). RHAMM peptide vaccination is currently being successfully explored in phase 1 clinical trials of acute myeloid leukemia and multiple myeloma (44). The relevance of RHAMM for esophageal cancer is further emphasized by a microarray analysis showing that RHAMM is highly induced in human ESCC cell lines and correlating RHAMM expression with the TNM Classification of Malignant Tumors (TNM) stage of human esophageal carcinoma (45).

All considered, in OSC1 cells interference with HA production, digestion of HA, displacement of HA, and inhibition of RHAMM signaling all cause FAK cleavage, suggesting a strong cross-talk between FA and hyaluronan/RHAMM. This might be important for these tumor cells to regulate adhesion, migration, and proliferation. In contrast, CD44 participates in hyaluronan-mediated signaling through Akt/PKB and ERK and through these pathways may contribute to...
versus scrambled controls. CD44 and RHAMM. Data were normalized to tubulin as loading control and to B, phosphorylation of AKT and ERK1/2 were reduced by shRNA targeting both FCS expressed as cpm per total cellular protein (n).

![Diagram](image)

**FIGURE 8. FAK cleavage is induced specifically by down-regulation of RHAMM.** A lentiviral shRNA targeting CD44 and RHAMM were used as in Fig. 7. Immunoblotting of tFAK and quantitative analysis of 125-kDa tFAK and degraded tFAK (<125 kDa) revealed that shCD44 had no effect on FAK, whereas shRHAMM induced pronounced FAK cleavage compared with the scrambled lentiviral vector. Data are normalized to tubulin and to scrambled control (n = 3, mean ± S.E.). *p < 0.05 versus scrambled control vector. B, phosphorylation of AKT and ERK1/2 were reduced by shRNA targeting both CD44 and RHAMM. Data were normalized to tubulin as loading control and to scrambled controls. C, both shCD44 and shRHAMM inhibited migration toward FCS as determined in a 24-well microchemotaxis assay and reduced DNA synthesis as determined by [3H]thymidine incorporation in response to FCS expressed as cpm per total cellular protein (n = 3). **p < 0.01, ***p < 0.001 versus scrambled control vector. scr, scrambled.

the control of migration and proliferation but has no effect on FAK and cell shape. FAK mediates many crucial events in cancer cell biology and signaling, including spreading, proliferation, migration, invasion, and metastasis (46–47); moreover, the promise of targeting FAK activity by antitumor therapy is supported by numerous studies. The novel interrelationship between HA/RHAMM and FAK turnover described here could therefore be important for a better understanding of these processes and for the development of new anticancer strategies.

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