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Research Article

Hyaluronan synthase 3 (HAS3) overexpression downregulates MV3 melanoma cell proliferation, migration and adhesion

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ABSTRACT

Malignant skin melanoma is one of the most deadly human cancers. Extracellular matrix (ECM) influences the growth of malignant tumors by modulating tumor cells adhesion and migration. Hyaluronan is an essential component of the ECM, and its amount is altered in many tumors, suggesting an important role for hyaluronan in tumorigenesis. Nonetheless its role in melanomagenesis is not understood. In this study we produced a MV3 melanoma cell line with inducible expression of the hyaluronan synthase 3 (HAS3) and studied its effect on the behavior of the melanoma cells. HAS3 overexpression expanded the cell surface hyaluronan coat and decreased melanoma cell adhesion, migration and proliferation by cell cycle arrest at G1/G0. Melanoma cell migration was restored by removal of cell surface hyaluronan by *Streptomyces* hyaluronidase and by receptor blocking with hyaluronan oligosaccharides, while the effect on cell proliferation was receptor independent. Overexpression of HAS3 decreased ERK1/2 phosphorylation suggesting that inhibition of MAP-kinase signaling was responsible for these suppressive effects on the malignant phenotype of MV3 melanoma cells.

1. Introduction

Hyaluronan (HA) is a high molecular weight glycosaminoglycan, formed by repeating disaccharide units, composed of N-acetyl glucosamine (GlcNAc) and glucuronic acid (GlcUA) [1]. In mammalian cells it is synthesized by three different hyaluronan synthases (HAS1-3) [2–4]. These are plasma membrane-bound enzymes, which synthesize and extrude the growing hyaluronan chain into the extracellular matrix. Although there are tissue and cell type specific differences in the HAS isoform expression, in many cases isoforms are all expressed simultaneously [4–6].

Hyaluronan forms extracellular and pericellular matrices [7] when bound to its cell surface receptors like CD44 [8], RHAMM [9] and matrix proteoglycans like aggrecan [10,11]. It is present in most tissues and especially abundant in the skin [12], vitreous fluid [13] and cartilage [14]. It is associated to many processes involving active tissue remodeling such as embryonic development and cancer progression. For example in breast and ovarian cancers increased hyaluronan accumulation, especially in the tumor stroma but also around the cancer cells, indicates aggressive tumor type and results in a poor prognosis for the patient [15,16]. There is however, a discrepancy between the effects of hyaluronan in cancers originating from simple epithelia and stratified epithelia. In the former, the formation of a strong hyaluronan-rich matrix associates with aggressive tumor behavior, while in the latter it is the loss of the originally abundant hyaluronan coating around the epithelial cells, which is associated with aggressive tumor type [16].

In melanoma, as in squamous cell carcinomas, hyaluronan metabolism appears to be complex. In benign nevi the hyaluronan content is high and it is further increased at the early stages of melanoma (in situ melanoma). However, at the later stages of melanoma progression (deep melanoma) it is decreased both in the tumor cells and in the tumor stroma [17,18]. HAS1 and HAS2 show a tendency to decreased expression in cancer and stromal cells during melanoma progression, while Hyal2 expression is increased, suggesting a role for increased hyaluronan degradation in addition to decreased synthesis [18]. Recent results have shown that decreased expression of HAS1 and HAS2 associates with shorter disease time and recurrence free survival for the patients in cutaneous melanoma [Poukka et al. manuscript].

Results of the experimental work studying the influence of

Abbreviations: BSA, bovine serum albumin; bHABC, biotinylated hyaluronan binding complex; fHABC, fluorescent hyaluronan binding complex; EGFP, enhanced green fluorescent protein; HA, hyaluronan; HAS, hyaluronan synthase; PBS, phosphate buffer saline

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increased hyaluronan production on the functional properties of cells, such as proliferation and migration, have produced contradictory results. In most cell types (umbilical cord cells, skin fibroblasts, prostate cancer cells) elevated expression of HAS2 or HAS3 can be associated with increased cell motility, proliferation, migration and invasion [19–24]. HAS2 upregulation was found to be essential for epithelial–mesenchymal transition in normal breast epithelial cells [25]. In contrast in CHO cells HAS1-3 overexpression was found to reduce cell migration [26] and in glioma cells HAS2 overexpression reduced the tumorigenic potential of glioma cells [27]. In addition to controlling the behavior of the cells, HAS1-3 may indirectly influence the gene expression and cellular functions in neighboring cells thereby influencing angiogenesis and inflammation [19].

These contradictory findings could also relate to the molecular size of hyaluronan. High molecular mass hyaluronan inhibits endothelial cell migration [28] and proliferation of human vascular smooth muscle cells [29], while small hyaluronan fragments stimulate cell migration. As a consequence the ability of Hyal2 to transform an inhibitory native hyaluronan into bioactive oligosaccharides may play a crucial role in angiogenesis [30] and tumor cell invasion [31]. Mechanistically, the effects of hyaluronan on S-phase entry and cyclin D1 expression, which influence cell cycle progression, are mediated through CD44, ERK and Rac signaling pathways [32], while the proposed pro-inflammatory activity of hyaluronan oligosaccharides have also been suggested to involve NF-κB-activation by either CD44 or TLR-4 receptors [33].

In the present work we aim to investigate, how increased hyaluronan production influences the behavior of metastatic MV3 melanoma cells. To this end we generated a stable cell line expressing doxycycline-inducible EGFP-HAS3. Induction of HAS3 expression and hyaluronan synthesis was associated with rapid formation of an enlarged coat around the MV3 cells and down-modulation of the activity of several signaling molecules, especially ERK1/2, which led to decreased melanoma cell proliferation, migration and adhesion.

2. Materials and methods

2.1. Cell culture

Parental MV3 melanoma cell line, originally isolated from a lymph node metastasis [34] and C8161 melanoma cell line, originally isolated from abdominal wall metastasis [35] were cultured in Dulbecco's modified Eagle Medium (DMEM, containing 4.5 mg/ml glucose; Gibco, Life Tecnologies, NY, USA) supplemented with inactivated 10% FBS (HyClone, Thermo Scientific, Epsom, UK), 1 mM L-glutamine (EuroClone, Pavia, Italy) and penicillin–streptomycin (50 μg/ml streptomycin, 50 U/ml penicillin; EuroClone, Pavia, Italy). Both cell lines were subcultured three times a week at 1:6 ratio. During the experiments serum (FBS) concentration was reduced to 1% because it contains several growth stimulatory factors, which may influence cell behavior and thus mask the effects of doxycycline-induced HAS3 expression and hyaluronan accumulation on cells.

2.2. Lentivirus production

The entry vector penTTGmirc2 was cut with NcoI and EcoRI, gel purified and ligated with EGFP-HAS3 NcoI–EcoRI. EGFP-HAS3 was recombined into pSLICK-Hygromycin [36]. Lentivirus production was previously described in [37].

2.3. Inducible cell line production

MV3/C8161 cells were plated on 6-well plates, 2×10^5 cells/well, and after 24 h cells were transduced with 6–9 $\times 10^4$ of the EGFP-HAS3 virus in the presence of polybrene. In the following day the medium was changed to medium containing 100 μg/ml Hygromycin B (Clontech Laboratories, CA, USA) for several days and thereafter with medium containing 50 μg/ml of Hygromycin B (Clontech Laboratories, CA, USA). Stable inducible EGFP-HAS3-MV3 and EGFP-HAS3-C8161 cell lines were cultivated in a maintenance medium, as mentioned above, supplemented with 50 μg/ml Hygromycin B and subcultured three times at a week 1:6 ratio. During the experiments cells were grown without Hygromycin B.

2.4. Hyaluronan assay

Cells were seeded on 12-well plates, 6×10^4 cells per well. On the following day fresh medium (DMEM with 1% FBS) was changed containing several doses (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, 0.5 and 1.0 μg/ml) of doxycycline (Doxycycline hydrochloride, Sigma, St. Louis, USA). After 48 h incubation the media were collected to measure the amount of hyaluronan using an enzyme-linked sorbent assay (ELSA) as described in detail previously [38] utilizing biotinylated hyaluronan binding complex (bHABC) prepared in the laboratory. Cells were detached using trypsin and cell numbers were counted using a hemocytometer to normalize the hyaluronan contents to the number of cells (ng/10^4 cells).

2.5. Hyaluronan molecular mass assay

To analyze the molecular mass distribution of the secreted hyaluronan, 2.5×10^5 cells were seeded on 6-well plates. On the next day, fresh culture medium (DMEM, 1% FBS) containing either 0 or 0.5 μg/ml doxycycline was changed. After 24 h incubation the media were harvested and cell numbers were counted. The media were chromatographed on an 1×30 cm^2 column of Sephacryl S-1000 (Amersham Biosciences), equilibrated and eluted at 4 ml/h with 150 mM sodium acetate, 0.1% CHAPS (Sigma), pH 6.8. The amount of hyaluronan was analyzed in each fraction (3–27). The void volume of the Sephacryl S-1000 column (V_0) was considered to be in the first hyaluronan positive fraction for a 2500-kDa standard hyaluronan (Hyalose, L.C.C., Oklahoma City, OK). The size distribution of hyaluronan in the samples was estimated from the peak fractions of known hyaluronan standards (150, 500, and 2500 kDa), provided by Hyalose.

2.6. qRT-PCR

To measure mRNA expression levels, 2×10^5 cells were seeded on 6-well plates. Doxycycline (0, 0.05, 0.5 μg/ml) treatment was started on the following day. After 24 h incubation with doxycycline the media were collected to hyaluronan-ELSA and cells were lysed with TRI Reagent® (Molecular Research Center Inc., Cincinnati, USA) for mRNA analyses. Total RNA was extracted with chloroform–isopropanol according to the standard procedure, washed once with 75% ethanol, diluted to RNAse-free water (Baxter, Zurich, Switzerland) and heated for 10 min at 57 °C. RNA concentration was measured with NanoDrop spectrophotometer (ND-1000 spectrophotometer, Thermo Scientific, DE, USA) and 1 μg of RNA was used for cDNA synthesis. cDNA was made using Verso™ cDNA Kit according to the manufacturer's protocol (Thermo Ficher Scientific, Surrey, UK) and reverse transcription was performed with MJ Research PTC-200 Peltier Thermo Cycler (MJ Research Inc., Watertown, USA). Quantitative real-time PCR (qRT-PCR) of different genes (Table 1) were performed with a MX3000P thermal cycler (Stratagene, La Jolla, CA) using a FastStart