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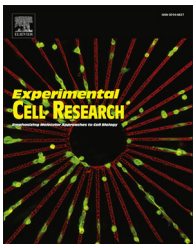

Research Article

Hyaluronan production enhances shedding of plasma membrane-derived microvesicles

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ABSTRACT

Many cell types secrete plasma membrane-bound microvesicles, suggested to play an important role in tissue morphogenesis, wound healing, and cancer spreading. However, the mechanisms of their formation have remained largely unknown. It was found that the tips of long microvilli induced in cells by overexpression of hyaluronan synthase 3 (HAS3) were detach into the culture medium as microvesicles. Moreover, several cell types with naturally active hyaluronan synthesis released high numbers of plasma membrane-derived vesicles, and inhibition of hyaluronan synthesis reduced their formation. The vesicles contained HAS, and were covered with a thick hyaluronan coat, a part of which was retained even after purification with high-speed centrifugation. HAS3 overexpressing MDCK cells cultured in a 3-D matrix as epithelial cysts released large amounts of HAS- and hyaluronan-positive vesicles from their basal surfaces into the extracellular matrix. As far as we know, hyaluronan synthesis is one of the first molecular mechanisms shown to stimulate the production of microvesicles. The microvesicles have a potential to deliver the hyaluronan synthase machinery and membrane and cytoplasmic materials to other cells, influencing tissue regeneration, inflammation and tumor progression.

Introduction

Membrane-bound vesicles are released from most cell types [1]. The vesicles have been classified into different size categories, although this classification is ambiguous [2]. Those ranging from 100 nm to 1 μm in diameter are usually called microvesicles and their secretion has been associated with normal physiological events like embryonic development [3], cell differentiation [4] and maintenance of homeostasis in general, as well as pathological conditions [5] like inflammation [6], tissue regeneration [7] and tumor progression [8]. Microvesicles are thought to be shed directly from plasma membrane and include cytosolic

Abbreviations: bHABC, Biotinylated hyaluronan binding complex; DIC, Differential interference contrast; ELSA, Enzyme-linked sorbent assay; fHABC, Fluorescent hyaluronan binding complex; FBS, Fetal bovine serum; GFP, Green fluorescent protein; HAS, Hyaluronan synthase; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy.

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components, proteins, RNA, ribosomes, and selected plasma membrane proteins [5]. Microvesicles can be incorporated into other cells, enabling the transfer of bioactive molecules [5]. Microvesicles have been suggested to participate in tumor–stroma interactions and enter into circulation to help in the establishment of a favorable tumor cell niche in distant organs [9–11].

Hyaluronan is a large extracellular glycosaminoglycan showing enhanced synthesis during embryonic development, inflammation, wound healing, and malignant tumors, i.e. whenever rapid tissue remodeling takes place. Unlike other glycosaminoglycans, hyaluronan is synthesized on the plasma membrane, and the newly synthesized chain is secreted into the extracellular space directly through plasma membrane [12]. The hyaluronan synthase isoenzymes (HAS1-3) are enriched and active in special plasma membrane regions like lipid rafts [13,14], long microvillus-like protrusions [13,15], filopodia [16] and lamellipodia [17,18]. Moreover, we have found that plasma membrane shape is actually modified by hyaluronan synthesis, resulting in the induction and maintenance of membrane protrusions [13,15].

While studying cells overexpressing Has3 we found hyaluronan-positive microvesicles in the culture medium. This prompted us to check whether hyaluronan synthesis is associated to the release of the microvesicles. It turned out that active hyaluronan synthesis, either by endogenous or transfected HAS, increased shedding of membrane-bound, hyaluronan-coated microvesicles. The vesicles contained HAS, CD44 and actin, and likely a number of other cytoplasmic and plasma membrane components that may have local or systemic targets and functions unknown at present.

Materials and methods

Cell culture

LP-9 Human mesothelial cells (LP-9) were cultured in MCDB 110 medium (Sigma-Aldrich, St. Louis, MO) and medium 199 (Sigma) in a ratio of 1:1, supplemented with 15% fetal bovine serum (FBS, HyClone, Thermo Scientific, Epsom, UK), 2 mM glutamine (EuroClone, Pavia, Italy) and 50 μg/ml streptomycin sulfate, 50 U/ml penicillin (EuroClone), 10 ng/ml epidermal growth factor (Sigma) and 0.05 μg/ml hydrocortisone (Sigma).

Human chondrosarcoma cell line (HCS) was cultured in DMEM (Dulbecco's Modified Eagle Medium, high glucose, EuroClone) supplemented with 10% FBS (HyClone), 2 mM glutamine, 50 μg/ml streptomycin sulfate, 50 U/ml penicillin, 50 μg/ml fungizone (Gibco) and ascorbic acid (0.05 mg/ml, Sigma).

C8161 melanoma cell line, originally isolated from abdominal wall metastasis [19] was cultured in DMEM (high glucose, EuroClone) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Euroclone) and penicillin–streptomycin (50 μg/ml streptomycin, 50 U/ml penicillin; Euroclone).

The MDCK and MCF-7 cells were cultured in MEM (minimum essential medium, EuroClone, Pavia, Italy) supplemented with 10% FBS (HyClone), 2 mM glutamine (EuroClone), 50 μg/ml streptomycin sulfate and 50 U/ml penicillin (EuroClone). LP9, MCF-7, HCS, and MDCK cells were subcultured twice a week, and C8161 cells three times a week using 0.05% trypsin (w/v) and 0.02% EDTA (w/v) (Biochrom AG, Berlin, Germany).

Rat (male Wistar Rats, HsdBrlHan, Harlan Netherland 2002) mesothelial cells were isolated as in [20]. Briefly, abdominal cavity was opened and the *peritoneum parietale* was dissected and placed on a petri dish. Plastic cylinder was placed on peritoneum parietale. Cylinder was filled with 5 ml of digestion solution containing DMEM/F12 (1:1, Lonza, Verviers, Belgium) and 0.5 mg/ml collagenase, pH 7.4 (Sigma). After incubation in 37 °C for 30 min, the surface was gently scraped, medium was collected and sedimented at 1000xg for 10 min. Sedimented cells were resuspended in growth medium, DMEM/F12 (1:1) supplemented with 20% FBS (HyClone), 2 mM glutamine (EuroClone), EGF 10 ng/ml (Sigma), 0.05 μg/ml hydrocortisone (Sigma), penicillin–streptomycin (EuroClone). Cells were plated in plastic petri dishes, grown in 100% confluency and used for experiments between 2 and 3 passages.

Transfections and transductions

Stable transfections of MDCK cells were performed as described previously [21]. Production of lentivirus for doxycycline-induced expression of EGFP-HAS3 has been described previously [22]. Inducible C8161 melanoma cells were produced by transducing 200,000 cells in the wells of 6-well plates, with $4–5 \times 10^4$ EGFP-HAS3 virus particles in the presence of polybrene. The following day cells were switched to selection medium containing 100–200 mg/ml Hygromycin B (Clontech Laboratories, Mountain View, CA) for 1–3 days, then to maintenance medium containing 50 mg/ml Hygromycin B. For transient transfections, Exgen transfection reagent (Fermentas, St. Leon-Rot, Germany) was used according to manufacturer instructions.

Collagen cultures of melanoma cells

To prepare collagen gels, type I collagen (2.7 mg/ml; BD Biosciences, Two Oak Park, Bedford, MA, USA) was mixed with Earle's balanced salt solution (10x; Life Techologies), 7.5% sodium bicarbonate (Life Technologies), and 1 M sodium hydroxide solution, at a volume ratio of 8:1:0.3:0.2, respectively, in an ice bath. Collagen gel was added on the top of 70% confluent C8161 melanoma cell cultures on 8-well Ibidi chamber slides (Ibidi GmbH, Martinsried, Germany) and the gel was allowed to polymerize at 37 °C for 1.5 h before the medium was added. After 24 h, live cultures were imaged by confocal microscopy.

MDCK 3D cultures

For 3D culturing, cells were mixed with the basement membrane extract gel Cultrex® (Trevigen Inc., Gaithersburg, MD) to obtain a homogenous suspension of 2×10^5 cells/ml. Aliquots of the suspension were pipetted onto chambered coverglasses (Ibidi GmbH). The plates were kept at 37 °C to let the gel solidify for 30 min, after which culture medium (MEM) was added into wells. Cells were cultured at 37 °C and fresh medium was changed every other day. After 7 days incubation the cultures were inspected as live, or fixed for histology.

Visualization of hyaluronan in live cells with fluorescent HABC

For visualization of hyaluronan on live cells, a fluorescent group (Alexa Fluor ®594 or Alexa Fluor ®647) was directly coupled to