EXPERIMENTAL CELL RESEARCH 328 (2014) 388–400

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/yexcr

Research Article

Fetuin-A associates with histones intracellularly and shuttles them to exosomes to promote focal adhesion assembly resulting in rapid adhesion and spreading in breast carcinoma cells

Gladys Nangami[a], *Rainelli Koumangoye*[b], *J. Shawn Goodwin*[a], *Amos M. Sakwe*[a], *Dana Marshall*[c], *James Higginbotham*[d], *Josiah Ochieng*[a,*]

[a]*Departments of Biochemistry and Cancer Biology, Meharry Medical College, 1005 D.B. Todd Boulevard, Nashville, TN 37208, USA*
[b]*Division of Surgical Oncology and Endocrine Surgery, Vanderbilt University Medical Center, Nashville, TN 37232, USA*
[c]*Departments of Pathology, Anatomy and Cell Biology, Meharry Medical College, Nashville, TN 37208, USA*
[d]*Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, USA*

ARTICLE INFORMATION

Article Chronology:
Received 19 April 2014
Received in revised form
8 August 2014
Accepted 23 August 2014
Available online 4 September 2014

Keywords:
Fetuin-A
Histones
Exosomes
Adhesion
Tumor cells

ABSTRACT

The present analyses were undertaken to define the mechanisms by which fetuin-A modulates cellular adhesion. FLAG-tagged fetuin-A was expressed in breast carcinoma and HEK-293T cells. We demonstrated by confocal microscopy that fetuin-A co-localizes with histone H2A in the cell nucleus, forms stable complexes with histones such as H2A and H3 in solution, and shuttles histones to exosomes. The rate of cellular adhesion and spreading to either fibronectin or laminin coated wells was accelerated significantly in the presence of either endogenous fetuin-A or serum derived protein. More importantly, the formation of focal adhesion complexes on surfaces coated by laminin or fibronectin was accelerated in the presence of fetuin-A or histone coated exosomes. Cellular adhesion mediated by histone coated exosomes was abrogated by heparin and heparinase III. Heparinase III cleaves heparan sulfate from cell surface heparan sulfate proteoglycans. Lastly, the uptake of histone coated exosomes and subsequent cellular adhesion, was abrogated by heparin. Taken together, the data suggest a mechanism where fetuin-A, either endogenously synthesized or supplied extracellularly can extract histones from the nucleus or elsewhere in the cytosol/membrane and load them on cellular exosomes which then mediate adhesion by interacting with cell surface heparan sulfate proteoglycans via bound histones.

*Corresponding author. Fax: +1 615 327 6442.
E-mail address: jochieng@mmc.edu (J. Ochieng).

http://dx.doi.org/10.1016/j.yexcr.2014.08.037
0014-4827/

Introduction

Fetuin-A is a serum glycoprotein synthesized by the liver and secreted into the blood stream. The widely accepted physiological function of this protein is the inhibition of ectopic calcification [1]. However, it also modulates a number of signaling pathways including those involving TGF-β and insulin [2–5]. Because of its widespread involvement in so many physiological mechanisms, fetuin-A is now considered a multi-functional protein [6].

Its role in cellular adhesion has been controversial. For many years, the prevailing dogma was that adhesion proteins that co-purify with fetuin-A such as fibronectin, promote the adhesion of cells to extracellular matrices [7,8]. We, however, demonstrated that highly purified fetuin-A was just as effective as crude Pedersen fetuin-A in mediating cellular adhesion [9]. Initially we proposed that annexins particularly AnxA2 and AnxA6 were the putative cell surface receptors for fetuin-A mediated adhesion [10]. However, further mechanistic studies failed to demonstrate the receptor role of annexins. Nevertheless, annexins particularly AnxA6 were deemed essential for fetuin-A endocytic uptake by tumor cells [9]. Interestingly, this fetuin-A uptake was obligatory for strong cellular adhesion and spreading on plastic. Cells in which AnxA6 was knocked down failed to adhere and spread properly on plastic [9].

In order to understand the mechanism(s) by which fetuin-A mediates adhesion, we analyzed the adhesion potential and composition of exosomes secreted in the absence and in the presence of fetuin-A by detached tumor cells in suspension. We had determined in prior studies that detached cells secreted more exosomes than adhered and spread cells [11]. We showed that cellular exosomes that promoted adhesion were those secreted in the presence of fetuin-A, while those secreted in the absence of fetuin-A lacked adhesion promoting properties. Interestingly, the adhesion competent exosomes contained histones and fetuin-A in addition to the usual exosomal marker proteins such as HSP90 [12]. The adhesion incompetent exosomes on the other hand lacked histones, but contained the compendium of exosomal marker proteins [12]. A number of studies have also demonstrated the exosomal mediated adhesion and cellular growth in tumor cell lines in vitro [13,14], while others have implicated these nano-vesicles in the preparation of metastatic niches in vivo [15]. Even though studies have suggested that exosomal associated integrins drive the adhesion process [16,17], we demonstrated that both adhesion incompetent and competent cellular exosomes contain integrins [12], implying that other mechanisms are involved.

Exosomes are nano-particles (30–100 nm) that originate from the inward budding of an endosomes's limiting membrane into its lumen, giving rise to endosomes containing multiple intraluminal vesicles known as multivesicular bodies (MVBs). The outer membranes of MVBs can fuse with the plasma membrane and release their intraluminal vesicles to the extracellular milieu as exosomes [18]. Whereas interesting potential physiological roles of exosomes are being unraveled at an ever increasing pace in the literature, the mechanisms that regulate their biogenesis and function particularly in cancer cells are unclear [19].

In the present study, we questioned whether fetuin-A interacted with histones intracellularly and in solution and whether it was responsible for trafficking/shuttling histones from the nucleus to the exosomes and membranes as well as maturation of focal adhesions. A number of plasma proteins such as plasminogen have been shown to interact with histones in solution, mitigating their deleterious effects on cells [20]. Interestingly, plasminogen is capable of attenuating the exosomal mediated adhesion [12], further suggesting that histones are involved in the exosomal mediated adhesion. Even though histones have not been established as bonafide adhesion molecules, their extracellular appearance and suggested roles in this microenvironment have provoked interest in biology [21,22]. For example, a recent report indicated that extracellular histones activated a number of adhesion related signals such as PI3 kinase/Akt in platelets [23].

Materials and methods

Materials

Crude fetuin-A (Pedersen fetuin-A) and histone from calf thymus (lyophilized powder) were purchased from Sigma (St. Louis, MO). Crude fetuin-A was purified according to the procedure detailed in [9]. Antibodies to histones H2A and H3 were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal mouse anti-FLAG M2, indocarbocyanide (Cy3)-conjugated sheep anti-mouse IgG, FITC-conjugated anti-rabbit IgG and anti-vinculin antibodies were from Sigma. All other antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX) unless stated otherwise. All other reagents were from Sigma unless stated otherwise.

Cells

The breast carcinoma cell line (BT-549) and HEK293T cells were purchased from ATCC (Manassas, VA). A sub-clone of BT-549 forced to express galectin-3 and named BT-549Gal3, was kindly donated by Dr. Avraham Raz (Karmanos Cancer Research Institute, Detroit, MI). Human fetuin A (AHSG) was cloned into the pMZS-3F vector [24] to generate pMZS-3F-fetuin-A.The recombinant or empty vector was then used to transfect BT-549Gal3 cells, selected with increasing concentrations of G418 and the resulting stably transfected clones are herein designated FF94 and EV94 respectively. The parental BT-549 was also stably transfected with the fetuin-A expression vector and selected as above to yield FFBT and the empty vector transfected controls, EVBT. The generated breast carcinoma cell lines were propagated in Dulbecco's modified Eagle's medium/nutrient F-12 (DMEM/F-12) supplemented with 10% heat inactivated fetal bovine serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 50 units/ml streptomycin in a 95% air and 5% CO_2 incubator at 37 °C. Where indicated, serum free medium (SFM) consisted of DMEM/F-12 in which fetal bovine serum (FBS) was replaced with 0.1% bovine serum albumin (BSA).

Promotion of cellular adhesion and spreading by fetuin-A

The 96-well micro-titer plates were coated with either fibronectin (FN) or laminin (LN) (40 μg/ml) in PBS overnight at 4 °C, the wells blocked with 3% (w/v) BSA and an equal number of BT-549Gal3 cells (3×10^4 cells/well) added to the wells in triplicates. The cells were added in the absence (FN; LN) or presence of purified bovine fetuin-A (FetA+FN; FetA+LN). The cells were allowed to adhere for 1 h, 2 h and 8 h at 37 °C in a humidified cell incubator. At the