Fetuin-A (ahsg) is a Major Serum Adhesive Protein that Mediates Growth Signaling in Breast Tumor Cells.

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Running title: Fetuin-A, a cell attachment and growth factor in serum.

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The identity of the cell adhesive factors in fetal bovine serum, commonly used to supplement growth media, remains a mystery due to the plethora of serum proteins. In the present analyses, we show that fetuin-A whose function in cellular attachment in tissue culture has been debated for many years, is indeed a major serum cell attachment factor particularly for tumor cells. We are able to report this because of a new purification strategy that has for the first time, given us a homogeneous protein band in colloidal Coomassie stained gels that retains biological activity. The tumor cells adhere to immobilized fetuin-A and not alpha-2-macroglobulin, its major contaminant. The interaction of cells with fetuin-A is driven mainly by Ca²⁺ ions and cells growing in regular medium supplemented with fetal bovine serum are just as sensitive to loss of extracellular Ca²⁺ ions as cells growing in fetuin-A. Fractionation of human serum revealed that cell attachment is confined to the fractions that have fetuin-A. Interestingly, the tumor cells also uptake fetuin-A and secrete it back to the medium using an unknown mechanism that can be observed in live cells. The attachment of tumor cells to fetuin-A is accompanied by PI3 kinase/Akt activation that is down-regulated in cells that lack annexin-A6, one of the cell surface receptors for fetuin-A. Taken together, our data show the significance of fetuin-A in tumor cell growth mechanisms in vitro and opens new research vistas for this protein.

Serum, particularly fetal bovine serum is widely used as a supplement in culture media, required for growth of most cells in culture, reviewed in (1). In the absence of serum, most cells fail to adhere properly, spread and grow on culture dishes. Serum has a plethora of adhesion (2-4) and growth factors (5). The prevailing assumption over the past two decades has been that integrins are the major cellular receptors for adhesion in cell cultures (6). Cellular adhesion to extracellular adhesion molecules such as vitronectin, fibronectin and laminin using integrins, requires the divalent ions Mg²⁺/Mn²⁺ (7). In addition, cell signaling cues for spreading and growth mechanisms in anchorage dependent cells emanate from the interaction of cells with the adhesion molecules (8). Whereas it is easy to define such signals in situations where cells are allowed to adhere to known purified extracellular matrix proteins such as fibronectin and
collagen (9), adhesion in the presence of fetal bovine serum is complex in that any one of the myriad attachment proteins and or growth factors has the potential to mediate adhesion and growth signals.

Ever since it was first purified and described, fetuin-A/ahsg was suspected of being the principal cell adhesion molecule in serum (1,10). Fetuin-A isolated and purified from serum by the Pedersen method (hereafter referred to as Pedersen fetuin-A), demonstrated cell adhesive properties in the presence of divalent ions (11). The Pedersen fetuin-A, however, is not considered pure because it is contaminated with a number of proteins including alpha 2-macroglobulin (α2M) (12). Fetuin-A purified using the Spiro method on the other hand, gave a more pure fraction but lacked attachment properties (1,13). These discrepancies raised serious doubts as to whether fetuin-A plays any role in cellular adhesion. Previous studies from our laboratory and others demonstrated that the cellular attachment to Pedersen fetuin-A was mediated by annexins, particularly annexin-A2 and -A6 (14,15). This adhesion is Ca2+ ion dependent and requires the sialic acid moieties on fetuin-A since asialofetuin-A lacks this property (16).

Fetuin-A is a serum glycoprotein synthesized and secreted by the liver and to a lesser extent the kidneys, placenta and the tongue (17). It has a high content of sugar moieties including sialic acid residues (1,18). Its molecular weight ranges from 51 to 67 kDa depending on the carbohydrate content (19). It is a member of the cystatin family of proteins even though it lacks cysteine protease inhibitory capacity (20). A number of studies suggest that fetuin-A is a multifunctional protein (21). A key physiological function attributed to the protein, determined with the aid of fetuin-A knockout mice, is its ability to inhibit ectopic calcification (22). We demonstrated that fetuin-A is capable of binding to matrix metalloproteinases particularly MMP-9 and protects this enzyme from autolytic degradation (23). This interaction is most likely mediated by the cystatin domains in fetuin A, because other members of the family such as cystatin C also interact with matrix metalloproteinases (23). Interestingly, it has been shown that fetuin-A is also able to stabilize m-calpain, a cytoplasmic cysteine proteinase (24). Other studies in our laboratory demonstrated that the Pedersen fetuin-A mediates the activation of PI3 kinase/Akt (16). Furthermore, a critical functional role of this protein may depend on its rapid uptake by cells as well as its ability to act as an opsonin in the blood (14,25).

To revisit the question of whether or not fetuin-A plays any role in cellular adhesion and signaling, we have developed a purification protocol in which the less pure Pedersen preparation was further purified by glycerol gradient centrifugation. With this purification strategy, we have separated fetuin-A from α2M (its major co-purifying protein) and other contaminating proteins including inter alpha globulin inhibitor H2 (identified by MALDI-TOF mass spectrometry), starting with the less pure Pedersen preparation. We also resolved human serum on glycerol gradient and determined that cell attachment and growth is associated with the fractions that contain ahsg/fetuin-A.
EXPERIMENTAL PROCEDURES

Materials. Partially purified fetuin-A (Pedersen fetuin-A) was purchased either from Sigma (Fetuin-A (S) or Calbiochem (Fetuin-A (C)). Polyclonal antibodies to alpha-2-macroglobulin (α2M) were purchased from Sigma. Antibodies to fetuin-A, AnxA2, AnxA6 and ERK2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to phospho-ERK1/2 and phospho-Akt (S473) were from Cell Signaling Technology (Danvers, MA, USA). GFP-AnxA2 expression plasmid was kindly donated to us by Dr Carl E. Creutz (University of Virginia).

Cells: The breast carcinoma cell line (BT-549) was purchased from ATCC (Manassas, VA, USA). A derivative of BT-549 stably over-expressing Galectin-3 herein denoted as BT-Gal3 was kindly donated by Dr. Avraham Raz, Karmanos Cancer Research Institute (Detroit, MI, USA). These cell lines were propagated in Dulbecco's modified Eagle's nutrient F12 (DMEM/F12) medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (50 units/mL) in a 95% air and 5% CO2 incubator at 37°C. DMEM/F12 used lacked (Mg2+ and Ca2+) and serum was the sole source for these ions. Where indicated, serum-free medium consisted of DMEM/F12 in which FBS was replaced with 0.1% bovine serum albumin (0.1% BSA).

Knockdown of annexins. For down regulation of AnxA2 and AnxA6, BT-549 cells were transfected with small hairpin RNAs (shRNA) in plasmid pSM2c (Open Biosystems, Huntsville, AL) targeting the coding sequences of these proteins. The sequences of the targeted sense region are for AnxA6, 5’-CACTCGGACCAATGCTGA-3’ and for AnxA2 a mixture of 5’-CCTGCTTTCAACTGAAATTTGTT-3’, 5’-GCAGGAAATTAACAGAGTCTA-3’, 5’-CTGTACTATTATATCCAGGAA-3’ and 5’-CGGGATGCCTTTGAAACATTTGAA-3’. The parental controls were transfected with the empty vector. Transfected cells were selected in complete medium containing 2.5µg/ml puromycin, cloned and expanded. Clones in which the down regulation of these proteins was most efficient as tested by immuno-blotting were selected for further studies.

Ultrafiltration: Pedersen fetuin-A was dissolved in HBS (Hepes buffered saline: 11 mM Hepes, 137 mM NaCl, 4 , mM KCl, 1 mM glucose) and transferred to 100,000 molecular cut-off filter units (Millipore, Billerica, MA, USA), centrifuged at 10,000 x g until totally filtered. The flow through was transferred to new tubes while the retentate was reconstituted in an equal volume. The protein concentration in the fractions was determined using the Bradford assay and equal amounts of protein analyzed in 4-12% SDS-gels and visualized by colloidal Coomassie staining (Sigma).

Affinity purification of Pedersen fetuin-A Wheat germ agglutinin-agarose (WGA) beads (Sigma) were washed twice with HBS followed by incubation with Pedersen fetuin-A for 4 h at 4°C. The beads were abundantly washed with the starting buffer and then eluted with 0.1M N-Acetyl glucosamine in HBS.
Equal volumes of the eluates were resolved by SDS-PAGE as above.

**Glycerol gradient centrifugation.** Glycerol step gradients (10-15-30-45-60%) were made in 13 ml ultracentrifugation tubes (Beckman) from 60% glycerol buffered with 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.5 mM MgCl₂. About 10 mg of Pedersen fetuin-A, fractionated fetal bovine serum as described recently (26) or human serum (Sigma) in a total volume of 1.0 ml were layered on the step gradient. This was then centrifuged at 35,000 rpm (Beckman, SW40Ti rotor) for 18 h at 4°C. Gradients were fractionated from the top. The fractions analyzed by SDS-PAGE in 4-12% gels followed by staining with colloidal Coomassie or western blotting with the indicated antibodies. Before use in other experiments, glycerol was removed by centrifugation through a 3000 molecular cut-off ultrafiltration unit and the retentate washed with HBS and reconstituted in the initial volume of HBS. Fractions from six identical gradients containing identical protein profiles were pooled and dialyzed against HBS. These were designated from the top of the gradient, as fractions S1, S2 and S3 and stored in aliquots at -80 ºC until required for use.

**Cell attachment assays** Wells of 96-well plates were coated in quadruplicates with glycerol gradient fractions (diluted 1:2 in HBS, or the indicated concentrations of the pooled and dialyzed fractions overnight at 4°C. Prior to use in cell attachment/spreading assays, the coating solutions were discarded and 1 × 10⁶ cells in serum-free DMEM/F12 were added to each well and incubated overnight (for 16-24 h) at 37ºC. As controls, cells were seeded in DMEM/F12 containing Pedersen fetuin-A or serum-free DMEM/F12. Cells were photographed using DCM200 digital camera equipped with Scopephoto software and cell numbers and viability estimated by Alamar blue colorimetric assay (absorbance read at 570 nm or fluorescence read at 595 nm after excitation at 530 nm). In other experiments, the cells were fixed in either cold methanol or 2.2% w/v paraformaldehyde, stained with crystal violet and photographed.

**Immuno-blotting** Cells were grown in 15 cm dishes until 80-90% confluent in complete DMEM/F12. These were washed once in ice-cold PBS and harvested by scraping. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease inhibitor cocktail (Sigma), and phosphatase inhibitors (20 mM sodium fluoride, 50 mM β-glycerophosphate and 1mM sodium orthovanadate). Cell lysates or various fractions of glycerol gradients were separated in 4-12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were subsequently probed with the indicated antibodies and revealed by enhanced chemiluminescence as previously described (26).

**Uptake of labeled purified fetuin-A by BT-549 breast carcinoma cells.** Purified fetuin-A (fraction S1) was labeled with rhodamine isothiocyanate (Sigma) as described previously (26). For real time imaging of
rhodamine-labeled purified fetuin, BT-Gal3 cells were transfected with GFP-annexin A2. Following 24 h in culture, the cells were trypsinized and replated on microscope cover slips in 6-well culture plates. Cells were subsequently serum-starved for another 24 h, then washed twice with HBS without Ca\(^{2+}\) and Mg\(^{2+}\) and incubated with 0.8 \(\mu\)M purified fetuin-A (fraction S1) labeled with rhodamine isothiocyanate in HBS containing 2 mM Ca\(^{2+}\). The uptake of rhodamine-labeled fetuin-A (red) by annexin A2 transfected cells (green) was followed in real time for up to 30 min using a Nikon A1R Confocal microscope (Nikon, Melville, NY, USA). GFP was excited with a 488 nm laser (emission filter 505-550 band pass) and Rhodamine was excited with 561 nm laser (emission filter 575-650).

Adherent cells were also grown on microscope cover slips, serum-starved for 48 h in DMEM supplemented with 0.1% BSA and washed twice with Ca\(^{2+}\) and Mg\(^{2+}\)-free HBS. Cells were then incubated for 30 min in HBS containing 1 mM of divalent ions Ca\(^{2+}\)/Mg\(^{2+}\), and 10 \(\mu\)g/ml rhodamine-labeled fetuin-A. The cells were rinsed twice with cold PBS before and after fixation with -20°C cold methanol for 10 min. After washing, the cover slips were mounted on slides using Prolong Gold with DAPI (Invitrogen). Up-take was visualized by fluorescent microscopy using a Nikon Eclipse TE-2000-E inverted microscope equipped with NIS-Elements AR software (Nikon, Melville, NY, USA).

## RESULTS

### Ultrafiltration and Wheat-germ Agglutinin purification of Pedersen Fetuin-A.

The rationale behind using ultra-filtration as a strategy to further purify Pedersen fetuin-A was to separate uncomplexed fetuin-A from its high molecular weight aggregates formed with its contaminants. This approach was unable to separate fetuin-A from its higher molecular weight contaminants (data not shown). Similarly purification strategy for fetuin-A employing WGA-affinity chromatography also failed to offer an improvement over the Pedersen method. The fractions eluted by N-Acetyl-glucosamine had numerous protein bands by colloidal Coomassie blue staining even though only one homogeneous band was reported when stained with regular Coomassie (27). More importantly, the dominant \(\alpha_2\)M contaminant was not separated from fetuin-A (data not shown).

### Resolution of bovine serum on glycerol gradients.

In addition to the soluble proteins such as fetuin-A, serum contains nano-vesicles known as exosomes that are frequently purified by glycerol or sucrose gradient centrifugation. We recently showed that fetuin-A is equally distributed between the exosome free and exosome-enriched fractions of bovine serum (26). We therefore sought to examine whether fetuin-A in the fractionated serum could be separated from its contaminants by floating these fractions on glycerol step gradients. To do this bovine exosome-free serum (EFS) and exosome enriched serum (EES) were prepared as recently described (26). These were layered on glycerol step gradients, separated by
centrifugation and then fractionated from the top. As shown in Fig. 1A, the fractionation yielded one major protein peak. However, analysis of fetuin-A or α2M by western blotting in the EFS (Fig. 1B) surprisingly revealed that fetuin-A was eluted in the less rapidly sedimenting fractions (fractions 3-6), while α2M came off in the rapidly sedimenting fractions (fraction 8-11). It is interesting to note that this strategy effectively separated the majority of fetuin-A from its major contaminant α2M and that the separation was more efficient when EFS was used. This suggests that to purify soluble fetuin-A from serum, it is necessary to remove serum exosomes by differential centrifugation and then resolve the exosome-free serum on glycerol gradients. Fetuin-A is associated with serum exosomes which also elute in the less rapidly sedimenting fractions (fractions 3-7) (Fig. 1C). We previously reported that serum exosomes contain fetuin-A (26).

Purification of the Pedersen fetuin-A by glycerol gradient centrifugation. This is the first purification protocol in our hands that has been able to separate fetuin-A in Pedersen fetuin from its major contaminant α2M in solution as depicted by the colloidal Coomassie stained gel (Fig. 2A). It was critical to resolve each of the fractions in reducing gels followed by colloidal Coomassie staining, the sensitivity of which approaches that of silver staining. Thus a single homogeneous band in colloidal Coomassie stained gels is considered pure. Fetuin-A came off in the less rapidly sedimenting fractions (Fig. 2A, lanes 2-6) while α2M banded in the rapidly sedimenting fractions (Fig. 2A lanes 7-11). From the fractionation, we designated as pure fetuin-A (pooled fraction S1) eluted in tubes 1 and 2 (Fig. 2A, lanes 1 & 2); pooled fraction S2 as fetuin-A contaminated with Inter-alpha H2 globulin inhibitor (ITIH2) and other minor contaminants eluted in tubes 3-6 (Fig. 2A, lanes 3-6) and lastly, pooled fraction S3 as α2M and other contaminants eluted in tubes 8-11 (Fig 2A, lanes 8-11). The separation of fetuin-A from α2M was confirmed by western blotting (Fig. 2A, right panel). The tubes containing pure fetuin-A (pooled fraction S1), fetuin-A slightly contaminated with ITIH2 (pooled fraction S2) or those containing mainly α2M (pooled fraction S3) were once more analyzed by Coomassie staining and western blotting (Fig 2, panels B and C respectively). In terms of recovery, approximately 10% of the proteins in Pedersen fetuin were recovered in S1, 80% in S2 and 10% in S3 (Fig. 2, panel D). These fractions were then used for adhesion and cell signaling assays.

We tested the ability of proteins in each fraction of the glycerol gradient in the attachment of breast cancer cells. Even though the protein concentration of fraction #1 was quite low compared to the other fractions (Fig. 2A), it effectively supported adhesion of BT-549 cells (Fig. 3A). It however, contained the purest fetuin-A because a longer exposure of western blot revealed the fetuin-A band in this fraction. More importantly the pooled fraction S1 had the highest relative cell attachment potential (Fig. 3C). Despite having some impurities, S2 supported cell adhesion more or less to the same extent as S1 (Fig 3, panels B and C). Since S3, enriched in α2M had the lowest cell attachment potential, it can be concluded that this
serum protein is not involved in the adhesion and spreading of breast tumor cells. Similarly, the inability of fractions containing ITIH2 and other minor contaminants (S2) to affect adhesion relative to S1, show that cellular adhesion and spreading is vested in fetuin-A. Interestingly tumor cells that lacked annexin A6 (BT-A6-sh), one of the cell surface receptors for fetuin-A (15), had the poorest adhesion to the three pooled fractions and to Pedersen fetuin-A from two independent vendors (Fet-S and Fet-C) (Fig. 3D). Slight adhesion of cells to S3 is likely due to the small amount of contaminating fetuin-A. The optimum adhesion of cells to immobilized fetuin-A was realized in the presence of 1 mM Ca\(^{2+}\) ions.

**Attachment of breast tumor cells to wells coated with human serum fetuin-A.** The goal of this experiment was to fractionate human serum into fractions rich in human fetuin-A (ahsg) and fractions devoid of the protein and determine the serum fractions that have cell attachment properties. Serum exosomes and exosome free serum were resolved on glycerol gradients and each of the fractions tested for its ability to support cell attachment in the presence of Ca\(^{2+}\) ions. As expected the serum fractions that were positive for fetuin-A (Fig. 4, solid circles and inset), supported the most attachment (fractions 1-5). Serum exosomes eluting in the less rapidly sedimenting fractions 2-7(Fig. 4A, triangles) similarly supported cellular attachment as previously reported (26). The serum fractions that contained \(\alpha_2\)M (Fig. 4A, inset) on the other hand did not support cellular adhesion. Cell attachment in the presence of fetuin-A was supported by a wide range of Ca\(^{2+}\) ion concentration (0.1-1 mM) (Fig. 4B)

**Contribution of serum proteins to in vitro cell attachment.** Fetuin-A at 0.25% w/v supported the attachment of tumor cells to plastic wells to the same extent as the serum proteins at various concentrations of Ca\(^{2+}\) ions (Fig. 4C). The chelation of calcium ions in the medium drastically reduced the attachment of cells in the presence of either complete medium or 0.25% w/v fetuin-A at all concentrations of EGTA (Fig. 4C). Cells in the wells that were pre-coated with fibronectin on the other hand were less sensitive to the removal of Ca\(^{2+}\) by EGTA (Fig. 4C, open bars). The data suggest that in the presence of complete medium, integrins are not the major adhesion receptors otherwise the cells would be less sensitive to EGTA chelation. More importantly the data underscores the significance of Ca\(^{2+}\) in the adhesion of cells to plastic in the presence of medium supplemented with fetal bovine serum. Under these conditions fetuin-A appears to be a major player in the adhesive process. However, this does not rule out the contribution of serum factors such as some glycans that can also mediate adhesion in the presence of Ca\(^{2+}\) ions (28).

**Uptake of labeled purified fetuin-A by BT-549 breast carcinoma cells.** We next questioned whether fetuin-A can be up-taken by the tumor cells and whether once inside they can interact with intracellular annexin A2 (AnxA2) or annexinA6 (AnxA6). Breast carcinoma cells were transiently transfected with GFP-AnxA2 (green) and then incubated with rhodamine-labeled fetuin-A (red). The uptake of labeled fetuin-A
was then followed in live cells. The data show that fetuin-A is up-taken within seconds (Fig. 5A). By 10 min., the uptake is almost complete and the cells begin to pump out excess fetuin-A using an unknown mechanism which involved formation of membrane blebs (Fig. 5B). Interestingly the fetuin-A in the blebs does not appear to co-localize with GFP-AnxA2. The data also show that internalized fetuin-A co-localize with GFP-AnxA2 inside the cells (Fig. 5B).

In order to determine the mechanisms that mediate the uptake of fetuin-A, we questioned whether annexins play a role in this process. The annexins on the cell surface may interact with fetuin-A in solution to mediate its uptake as shown for other cell types (14). To test this, we incubated rhodamine-labeled fetuin-A (fraction S1) with BT-549 breast carcinoma cells in which AnxA2 and AnxA6 had been knocked down and renamed BT-A2-sh and BT-A6-sh respectively (Fig 6, panels A and B). As shown in Fig. 6, panels C and D, depletion of AnxA6 and AnxA2 respectively in BT-549 cells inhibited the uptake of fetuin-A compared to the parental cells.

To further investigate the adhesive interactions between carcinoma cells and fetuin-A, we analyzed the attachment of parental BT-549 transfected with empty vector and BT-549 cells in which AnxA2 (BT-A2-sh) and AnxA6 (BT-A6-sh) had been knocked down, to microtiter wells coated with either fetal bovine serum or fetuin-A in SFM. We determined in three separate experiments that whereas parental BT-549 adhered strongly to FBS and fetuin-A coated wells after 8 h of incubation, both BT-A2-sh and BT-A6-sh had reduced adhesion to these substrata (Fig. 7). The reduced adhesion of cells to both FBS and fetuin-A was more drastic in AnxA6 compared to AnxA2 depleted cells. More importantly lack of AnxA6 prevented cell spreading on either FBS or fetuin-A. Loss of AnxA2 on the other hand did not affect cell spreading (Fig. 7).

Lastly we investigated the signaling mechanisms that are turned on upon the interaction of breast carcinoma cells with purified fetuin-A. Purified fetuin-A fraction (S1) was incubated with BT-549 cells for 10 min in the absence or presence of graded doses of the protein and then monitored the activation of the MAP kinase and PI3 kinase/Akt pathways by measuring the levels of phospho-ERK1/2 and P-Akt (S473) as readouts. A higher concentration of fetuin-A, fraction-S1 was able to activate ERK to varying extents in the presence of Ca\textsuperscript{2+} (Fig. 8A). Interestingly, BT549 cells that lacked AnxA6, showed almost negligible activity of the PI3 kinase/Akt pathway compared to the parental cells (Fig. 8A). However, the MAP kinase pathway was highly active in this cell line, even in the absence of Ca\textsuperscript{2+} (Fig. 8A).

**DISCUSSION**

Serum is known to have a number of adhesion platforms which include the classical extracellular matrix proteins such as laminins, collagens, fibronectin, and vitronectin. More recent studies have revealed that chondroitin sulfate chains can also mediate cellular adhesion (28). We recently demonstrated that nano vesicles, known as exosomes, also form an important part of cellular adhesion
repertoire in serum and are more relevant in the anchorage-independent growth of tumor cells (26).

Fetuin-A is a notoriously sticky molecule that co-purifies with a number of unrelated proteins and other factors (1,18). Separating these contaminating proteins from fetuin-A has been a technical challenge ever since this bovine protein and its human homologue (ahsg) were first described (1). Our goal in this study was to re-visit an old question and ask whether or not fetuin-A plays a role in cellular adhesion in culture. From the moment it was first described until the report by Spiro (29), it was generally assumed that fetuin-A plays a role in the in vitro attachment and growth of cells. Using the standard techniques of the day, Spiro demonstrated that highly purified fetuin-A lacked the ability to support cellular attachment in vitro. The results suggested that cellular attachment and growth was mediated by serum proteins that associate with fetuin-A (29). To further support this notion, Nie et al isolated a factor in fetal bovine serum that could replace Pedersen fetuin-A as a growth factor for human skeletal muscle satellite cells (30). Subsequent studies suggested that the reagents used in the Spiro method, namely Zn$^{2+}$ ions, altered the structure of fetuin-A and consequently its ability to support cellular attachment and growth (1). An interesting report by Yu et al (31) demonstrated that when Pedersen fetuin-A was incubated with Zn$^{2+}$ followed by dialysis to remove the salts and then injected with breast tumor cells into nude mice, fetuin-A induced apoptosis in the cells which then failed to form tumors compared to untreated controls (without Zn$^{2+}$). Their data underscored the ability of Zn$^{2+}$ ions to alter the structure and consequently the biological properties of fetuin-A.

We hereby demonstrate that cellular attachment to Pedersen fetuin-A is mediated by the protein per se and not its contaminating factors as suggested by earlier reports which overwhelmingly supported the ‘contaminant theory’ (1). Clearly it is important to define the cell adhesion mechanisms that are in play in fetal bovine serum based tissue culture, since it is still the dominant model system that biologists use to study cell growth mechanisms. Our data also support earlier studies that intimated the importance of Ca$^{2+}$ for tumor cell growth (32). The interaction of cells with serum derived fetuin-A is mediated by cell surface expressed annexins (15). This is based on the observation that knockdown of annexins particularly AnxA6 on cells significantly reduce the adhesion of these cells to fetuin-A coated wells (Fig. 3) (15). Others have also shown that fetuin-A interacts with AnxA2 and AnxA6 (14,33). Kojima et al (34) demonstrated that AnxA4 binds strongly to fetuin-A coupled to sepharose columns. Our attempts to co-immunoprecipitate fetuin-A with AnxA2 or AnxA6 in cell lysates with antibodies to fetuin-A and vice-versa, however, have not given us consistently reliable data. Nevertheless the adhesive interaction of carcinoma cells with immobilized fetuin-A which depends on the levels of annexins expressed on the cell surface suggest that these proteins could interact more when associated with membranes or immobilized but only interact weakly in solution. However, the cell signals mediated by fetuin-A in solution such as
PI3 kinase/Akt, can also be transmitted by immobilized fetuin-A (16).

We have herein demonstrated the significance of annexin/fetuin-A interaction in cellular adhesion and growth of cells in media supplemented with fetal bovine serum. The data suggest that whereas annexin family members, AnxA2 and AnxA6 play significant roles in the attachment of breast carcinoma cells to fetuin-A, it is AnxA6 that mediates cell spreading. Interestingly, AnxA2 has been shown to promote cell spreading in intestinal epithelial cells (35), suggesting that the abilities of AnxA2 and AnxA6 to mediate cell spreading is cell type dependent. It has been reported that down-regulation of AnxA2 expression in human cells markedly reduced their proliferation (36). The mechanism driving this AnxA2 based cell proliferation was not defined by this report.

The data reported herein, intimate that lack of AnxA2 or AnxA6 leads to a reduced interaction of the cells with fetuin-A and consequently altered growth potential in culture. Interestingly, cells that lack AnxA2 and AnxA6 such as LNCaP prostate cancer cells, grow poorly in culture with a doubling time of ~31 hours compared to another prostate cancer line (PC3) which express AnxA2 and has a doubling time of ~24 hours (37). Whether cell-fetuin-A interaction is also as important for in vivo cell attachment and growth as it is for in vitro adhesion is yet to be defined. We previously determined that Lewis lung carcinoma cells proliferate much more vigorously after transplantation into fetuin-A wild-type syngeneic C57/BL-6 mice relative to the fetuin-A null mice. The cells also attached and proliferated well in vitro in the presence of fetuin-A (16). The in vivo extracellular matrices are populated with ligands for integrins such as fibronectin, laminin and collagen and therefore one would expect that integrin mediated adhesion is more relevant in this microenvironment (8). However, there are other extracellular matrices such as liver ECM that have a higher than normal concentration of fetuin-A. We have also demonstrated that the lung extracellular matrix has a high concentration of fetuin-A (Ochieng, unpublished observations). Fetuin-A binds to elastin fibers with micromolar affinity and tumor cells have a tendency to attach to the fibers in the presence of culture medium supplemented with fetal bovine serum (38). Taken together, the elevated levels of fetuin-A in the lung and liver ECM could be one of the reasons why these organs are considered to be 'good soil' for tumor growth (39). The metastatic tumor cells may have a preference for fetuin-A as adhesion and growth platform. Bone is another micro-environment, in which a number of metastatic tumor cells attach to and grow (40). Interestingly, fetuin-A has been shown to be concentrated in the bone extracellular matrix (41).

Our data showing the efficient uptake of labeled fetuin-A by tumor cells was interesting. Whereas we cannot rule out the possibility that some of the uptake fetuin-A are degraded by the tumor cells, the data suggest a sophisticated mechanism of trafficking fetuin-A by tumor cells and possibly other cell types (14). The uptake could be part of de-adhesion that is necessary in cellular processes such as motility and cell division (42). Another possibility is that the up-
taken fetuin-A stabilizes and protects intracellular caspases and calpains from autolytic degradation (21,24). Lastly, fetuin-A could be taken in, undergo some form of covalent modification and then secreted back as suggested by the data. We are currently doing experiments to address these possibilities. More importantly, our data suggest that both AnxA2 and AnxA6 are involved in fetuin-A uptake mechanisms as shown in other cell types (14).

We previously showed that the interaction of cells with Pedersen fetuin-A leads to the activation of PI3 kinase/Akt (16). One could argue that since Pedersen fetuin-A is not pure, the PI3 kinase/Akt activation is mediated by α2M, a major contaminant of fetuin-A. This line of argument has merit because purified α2M has been shown to mediate PI3 kinase/Akt activation (43,44). The present data, using purified fetuin-A, now confirm our earlier studies that indeed the fetuin-A mediates the activation of PI3 kinase/Akt, using phospho-Akt (S473) as a readout. More interestingly, the data confirm that PI3 kinase/Akt is activated upon the interaction of fetuin-A with members of the annexin family of membrane receptors particularly AnxA6. PI3 kinase/Akt is one of the principal growth signaling pathways utilized by tumor cells (45). Together our data demonstrate that the interaction of tumor cells with fetuin-A should be given a serious consideration since this protein is not just a mere bystander in the realm of cell growth mechanisms.

In summary, we herein show that breast carcinoma cells in culture adhere in the presence of fetuin-A via annexins and that the signaling that emanates from this interaction is relevant for cell growth. More importantly the data suggest that this interaction (annexin/fetuin-A) is just as relevant (if not more) as the interaction between cell surface integrins and the extracellular adhesion proteins such as fibronectin in tissue culture. We also show that tumor cells efficiently uptake fetuin-A (via annexins) and that this could be part of an important hitherto unknown growth related mechanism. Lastly, we show that Pedersen fetuin-A can be purified to homogeneity without sacrificing its cell attachment properties.

REFERENCES

Abbreviations:  SFM-serum free medium; HBS-Hepes buffered saline; α2M-alpha-2-macroglobulin; GFP-green fluorescence protein; ECM-extracellular matrix; AnxA2-annexin-A2; AnxA6-annexin-A6; Inter-alpha H2 globulin inhibitor-ITIH2

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FIGURE LEGENDS

Figure 1. Glycerol gradient separation of fetuin-A from α2M in fetal bovine serum. A) Exosome-free and exosome-enriched fractions of bovine serum were prepared by ultracentrifugation of fetal bovine serum. After reconstitution of the exosome-enriched serum in serum-free DMEM/F12, 10 mg/ml total protein was layered on a glycerol step gradient, centrifuged and fractionated from the top (1.0 ml fractions). The protein content in each fraction was measured by the Bradford assay. B and C) Equal volumes of each fraction containing separated exosome-free (panel B) or exosome-enriched (panel C) proteins were analyzed by SDS-PAGE in 4-12% gradient gels, blotted unto nitrocellulose membranes and probed with antibodies to fetuin-A or α2M.

Figure 2. Separation of fetuin-A in Pedersen fetuin from its major contaminants by glycerol gradient centrifugation. A) Pedersen fetuin-A was layered on a glycerol step gradient, centrifuged for 18 h at 100,000 x g and the gradients fractionated from the top. The distribution of fetuin-A and its contaminants was analyzed by SDS-PAGE in 4-12% gradient gels and either stained with colloidal Coomassie (left panel) or blotted unto nitrocellulose membranes and probed with antibodies to fetuin-A or α2M (right panel). Notice the α2M peak in fraction 9 and the fetuin-A peak at fraction 4. The α2M bands are indicated by arrows and ITIH2 by asterisks. B) Fractionated fetuin-A fractions were pooled as indicated in panel A above and exhaustively dialyzed against HBS at 4°C. Equal amounts of the pooled fractions were analyzed by SDS-PAGE in 4-12% gradient gels and either stained with colloidal Coomassie (B) or blotted unto nitrocellulose membranes and probed with antibodies to fetuin-A or α2M (C). D) Densitometric analysis of protein recovery after glycerol gradient centrifugation. Bars represent recovered protein relative to the input from at least three determinations.

Figure 3. Cell attachment assays on glycerol gradient purified fetuin-A. Partially purified Fetuin-A from bovine serum (Pedersen fetuin-A) at 10 mg/ml in TBS was layered on a 10-60% glycerol step gradient, centrifuged and equal fractions collected from the top. Triplicate wells of a microtiter plate were coated overnight
at 4°C with 100 µl of a 1:2 dilution of each fraction (Panel A); the indicated concentration of the pooled and dialyzed fractions S1, S2 and S3 (Panel B); 0.5 mg/ml each of the pooled and dialyzed fractions S1, S2 and S3 as well as the crude preparation of commercially available fetuin-A (Panel C and D). BT549 cells stably expressing Galectin-3 (BT-Gal3) (panels A-D) or the indicated breast cancer cell lines (panel D) were harvested, washed and re-suspended in serum-free medium. The cells (1 x 10^4 cells/well) were then plated on the pre-coated wells and these were allowed to attach overnight at 37°C. Unattached cells were removed along with the medium by aspiration and the cell counts and viability were estimated using the Alamar blue assay. Bars represent attachment/viability of cells relative to attachment/viability in serum-free medium.

**Figure 4. Attachment of tumor cells to human serum proteins and fetuin-A.** A) Human serum exosomes and exosome free serum were fractionated on glycerol gradients as described in “experimental procedures”. The wells of a 96-well microtiter plate were coated with the aliquots of the gradient fractions (100 µl/well) overnight at 4°C. The wells were washed once with SFM and BT-549 cells added to the wells (2 x 10^4 cells/well) in SFM containing 1 mM Ca^{2+}. The cells were allowed to attach for 3 h at 37°C and the non-adherent cells washed off with SFM and the cells once more incubated in SFM containing Alamar Blue (1:10). After 6-8 hours of incubation the plates were read at 570 nm to determine the number of attached and viable cells. Wells coated with exosomal fractions (triangles) and with exosome free serum (circles). Inset, the western blots of the glycerol gradient fraction of exosome free serum probed with anti-ahsg and anti-α2M. B) BT-549 cells were seeded in the wells of a 96-well microtiter plate (2 x 10^4 cells/well) in fetuin-A dissolved in SFM (0.25% w/v) and containing different concentrations of Ca^{2+} (0.1-1 mM). C) BT-549 cells were allowed to attach to the wells of microtiter plate (2 x 10^4 cells/well) in either complete medium (right hatched bars) or 0.25% w/v fetuin-A in SFM (solid bars) for 6 h. Some of the wells of a microtiter plate were pre-coated with fibronectin (2µg/well) and then BT-549 cells added to them (2 x 10^4 cells/well) in complete medium (open bars) and incubated for 6 h. At the end of the incubation the wells were washed once with HBS containing 1 mM Ca^{2+} and then incubated with HBS containing graded doses of EGTA (0-1 mM) for 30 min. The detached cells were washed twice with HBS containing 1 mM Ca^{2+} and finally cells incubated in the respective incubation medium containing Alamar Blue (1:10) for 4-6 h or until color change to monitor the attached cells.

**Figure 5. Uptake of purified fetuin-A by BT549 breast cancer cells.** A-B) Real time imaging of fetuin-A uptake. BT-549 cells over expressing galectin-3 (clone 11-9-1-4) were transfected with GFP- AnxA2. At 24 h post-transfection, the cells were trypsinized and re-plated on microscope cover slips in 6-well culture plates. Cells were subsequently serum starved for 24 h, then washed twice with HBS without Ca^{2+} and Mg^{2+} and incubated with 0.8 µM rhodamine-labeled fetuin-A (fraction S1) in HBS containing 2 mM Ca^{2+}. The uptake of rhodamine fetuin-A (red) by GFP-AnxA2expressing cells (green) was followed in real time for up to 30 min using a Nikon A1R Confocal microscope (Panel A). The inset boxes in panel A are expanded in panel B which shows the co-localization of rhodamine-labeled fetuin-A and GFP- AnxA2.
Figure 6. Influence of Annexin-A2 and Annexin-A6 on the up-take of fetuin-A by tumor cells. BT-549, AnxA6-depleted BT-549 (BT-A6-sh; panel A) and AnxA2-depleted BT-549 (BT-A2-sh; panel B) cells were grown on glass cover slips in 6-well culture plates for 24 h. Cells were subsequently serum-starved for 48 h, washed twice with HBS and treated with 10 µg/ml rhodamine labeled fetuin-A (fraction S1) in HBS for 30 min at 37°C. The cells were once again washed in ice-cold HBS and then fixed with paraformaldehyde. The cover-slips were mounted in Prolong Gold with DAPI and visualized by fluorescence microscopy as described in Materials and Methods (panels C and D).

Figure 7. Roles of AnxA2 and AnxA6 in the adhesion and cell spreading on wells coated with either fetal bovine serum (FBS) or fetuin-A. The wells of 96-well culture plate were coated overnight with either 10% FBS or 100 µg/ml of purified fetuin-A in SFM. The wells were then washed once with HBS and then cells (BT549, BT-A2-sh, and BT-A6-sh) added (2 x10⁴ cells/well) in SFM containing 1 mM of Ca²⁺ and Mg²⁺ and incubated overnight at 37°C. The detached cells were washed once with SFM and then fresh SFM added including Alamar Blue (1:10) and cells incubated for another 4 h. The plate was read at 595 nm in a fluorescence plate reader after excitation at 530 nm. Each bar represents the average reading of six wells. The SFM was subsequently removed and cells fixed in cold methanol and stained with crystal violet. Excess dye was washed off with water, plate dried and cells photographed.

Figure 8. Fetuin-A-dependent activation of MAPK in breast carcinoma cells requires annexins and extracellular Ca²⁺. A) Parental BT-549 or AnxA6-depleted BT-549 (BT-A6-sh) cells were serum-starved for 24 h, then washed 2x with HBS and incubated in HBS with or without 2 mM Ca²⁺ and the indicated concentrations of purified fetuin-A (fraction S1). Cells were subsequently harvested using a cell scraper, washed in ice-cold HBS and lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors as described in “experimental procedures”. Equal amounts of proteins were analyzed by western blotting using antibodies to phospho-ERK1/2, phospho-Akt (S473) and total ERK2 or β-actin as the loading controls. B-C) Densitometric analysis of ERK and Akt phosphorylation in the total cell lysates. Bars represent ERK1/2 phosphorylation normalized to total ERK2 (panel B) or Akt phosphorylation normalized to β-actin (panel C) from a representative experiment.
Figure 1

A

B

Exosome-Free Serum

Fraction No  1  2  3  4  5  6  7  8  9  10  11

Blot: α₂M

Blot: Fet-A

C

Exosome-enriched Fraction

Fraction No  1  2  3  4  5  6  7  8  9  10  11

Blot: α₂M

Blot: Fet-A
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Fetuin-A (ahsg) is a major serum adhesive protein that mediates growth signaling in breast tumor cells
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