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Biochimica et Biophysica Acta 1693 (2004) 111–123

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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Annexins expressed on the cell surface serve as receptors for adhesion to immobilized fetuin-A

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Received 28 April 2004; received in revised form 9 June 2004; accepted 11 June 2004
Available online 23 July 2004

Abstract

Fetuin-A is a major constituent of the fetal bovine serum used extensively in cell culture media. We hereby present data demonstrating that breast carcinoma cells can adhere to immobilized fetuin-A in a calcium-dependent fashion. Interestingly, the cells can also divide and attain confluency under these conditions. Using a proteomic approach, we have identified annexin-II and -VI as the putative cell surface receptors for fetuin-A in the presence of Ca^{2+} ions. Biotinylation of cell surface proteins followed by immunoprecipitation revealed that annexin-VI was expressed on the extracytoplasmic surface of the cell membranes. Finally, to demonstrate that annexin-II and -VI were the adhesive receptors for fetuin-A, siRNA knockdown of expression of the annexins significantly reduced the calcium-mediated adhesion. Interestingly, we demonstrated that the tumor cells could also adhere to immobilized fetuin-A in the presence of magnesium ions, and that this adhesion was most likely mediated by integrins because neutralizing antibodies against β1 integrins substantially reduced the adhesion. Our studies suggest that the expression of annexin-II and -VI and possibly other members of the family mediate novel adhesion and signaling mechanisms in tumor cells.

Keywords: Fetuin-A; Annexin-II; Annexin-VI; Adhesion; MDA-MB-231; Calcium

1. Introduction

Fetuin-A is a serum glycoprotein initially identified in fetal and newborn calf serum and subsequently detected in sheep, pig, goat, mice, rat and humans [1]. During development, the concentration of fetuin-A in the fetus increases to approximately 3 mg/ml and rapidly decreases after birth to a level of ~0.6 mg/ml in the adult human plasma. Fetuin-A can be detected in allantoic, amniotic [2] as well as the cerebrospinal fluids [3]. It is a member of the cystatin superfamily [4] and is produced and secreted into the blood by the liver [5]. It has been linked to several physiological functions ranging from brain development [6] to bone remodeling and immune function [7,8]. Probably its most important function in normal physiology is its ability to prevent ectopic calcification [9].

Fetuin-A has long been known to support the attachment and growth of a variety of cell types in culture [2]. It is the major constituent of fetal bovine serum, the preferred growth supplement in most tissue culture media [2,10,11]. However, its role in adhesion and growth of cells in culture

Abbreviations: ECIS, electric cell-substrate impedance sensing; FDA, fluoresceine diacetate; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; FRET, fluorescence resonance energy transfer; PAGE, polyacrylamide gel electrophoresis

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doi:10.1016/j.bbamcr.2004.06.005

has not been without controversy. Many studies have suggested that its biological properties are mediated by the growth and adhesion factors which co-purify with it in the serum [2]. We and others have reported the presence of fetuin-A binding sites on tumor cells [12,13]. In the present study, we demonstrate, using a proteomic approach, the presence of annexin-II and -VI in the plasma membranes of the breast carcinoma cell line MDA-MB-231. Of all the cell surface proteins in these cells, the annexins have a preferential binding interaction with immobilized fetuin-A in a calcium-dependent manner.

Annexins belongs to a ubiquitous multigenic superfamily of water-soluble, calcium-dependent membrane aggregating proteins that share certain properties and are related by the amino acid sequence [14]. They are present in both plant and animal cells and there are about 13 members in the family identified so far [15,16]. Annexins, like fetuins, have also been implicated in various biological processes. Extracellular molecules like tenascin C, plasminogen and tissue plasminogen activator (t-PA) have been demonstrated to be ligands for annexin-II [17]. Annexin-VI is a unique protein in this family both structurally and functionally. While other annexins contain four copies of the 'annexin repeats', annexin-VI has eight, possibly as a result of gene duplication [18]. Annexin-VI has a widespread tissue distribution [19] and plays a role in cell growth regulation, intracellular homeostasis and secretory pathways [18,20–22]. It has also been demonstrated to be differentially expressed in murine melanoma cell lines [21]. Despite their initial identification as intracellular proteins because of their lack of signal peptides, annexins can be transported to the extracellular environment where they are likely to play key physiological roles. Annexin-II and -VI are expressed on murine RAW 117 large-cell lymphoma cells where they serve as surface receptors for tumor cell-endothelial cell interaction [23,24]. Annexin-VI is also expressed on the surface of several human and rodent tumor cell lines [25].

We hereby report that annexin-II and -VI are the putative receptors for fetuin-A on the surface of tumor cells and that the receptor–ligand interaction has a requirement for calcium ions and intact fetuin-A with its full complement of sialic acid residues.

2. Materials and methods

Fetuin-A (F3004, processed by gel filtration) and asialofetuin (types I and II) were purchased from Sigma. Asialofetuin types II and III were prepared by enzymatic cleavage using neuraminidase. Rabbit polyclonal antibodies to annexin-II and -VI were from Biodesign (Saco, ME). The immunogens were HPLC-purified synthetic peptides corresponding to residues 9–30 of the N-terminus of annexin-II monomer and residues 1–16 of the N-terminus of annexin-VI. Annexin-II and -VI siRNA and their corresponding scrambled siRNA were purchased from Qiagen (Valencia, CA). All the other reagents unless otherwise stated were purchased from Sigma.

The breast tumor cell lines MCF-10A, MDA-MB-231 and MDA-MB-435 were purchased from ATCC and are routinely maintained in DMEM medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin. The DMEM/F12 medium (Sigma; cat # D-9785) used in this study does not contain Ca^{2+} and Mg^{2+} ions. When supplemented with 10% fetal bovine serum, all the divalent ions are derived from the serum. Concentrations of Ca^{2+} and Mg^{2+} ions in the serum-free medium were adjusted accordingly.

2.1. Cell adhesion assay

The cells were grown in DMEM with 10% FBS. They were detached with 2 mM EDTA and washed three times with sterile physiological buffer (150 mM NaCl, 10 mM HEPES, pH 7.4) and the number of cells/ml determined using a hemocytometer. Sterile 96-well cell culture plates were either coated with fetuin-A (0–5 mg/ml) or collagen-IV (0–5 mg/ml). The nonspecific sites in these wells were blocked with 3% BSA. Cells resuspended in physiological buffer at a concentration of 4×10^4 cells/well were added to the coated plates in the presence or absence of the respective ions (Ca^{2+}, Mg^{2+}). The plates ware incubated at 37 °C for 90 min. The nonadherent cells were then washed off and to the adhered cells, fluoresceine diacetate (FDA) in DMSO was added to a final concentration of 20 μM in 100 μl of the physiological buffer. The fluorescence at 520 nm following excitation at 485 nm (slits—5 nm), emitted from the cells (arbitrary units of fluorescence) was determined by a luminescence spectrophotometer LS 55 (Perkin Elmer) with a fluorescence plate reader attachment, and was proportional to the number of cells per well.

2.2. Analysis of adhesion of breast carcinoma cells to fetuin-A and collagen IV in the absence and presence of function-blocking antibodies against β1 integrins

In an effort to determine whether the Mg^{2+}-dependent adhesion of the breast carcinoma cells to fetuin-A was mediated by integrins, we repeated the adhesion studies in the absence and presence of function-blocking anti-integrins antibodies. We made use of MDA-MB-435 cells which have been shown to use α1β1 integrins to adhere to collagen IV [26] (positive control). The wells of 96-well microtiter plates were coated with the optimal concentration of either fetuin-A or collagen IV as determined above. To these wells, 4×10^4 cells were added in the presence of either 2 mM Mg^{2+} or 2 mM Ca^{2+} and in the presence of either nonimmune IgG or mouse anti β1 integrins (MAB1965) (Chemicon, Temecula, CA) at a concentration of 5 μg/100 μl. The cells were allowed to adhere for 2 h, after which the nonadherent cells were washed off and the number of adhered cells determined as above.

2.3. Electric cell-substrate impedance sensing (ECIS)

Having determined that the breast cancer cells could adhere to immobilized fetuin-A in a calcium-dependent manner, we next questioned whether this adhesion also involved cell spreading. We therefore analyzed the adhesion by ECIS, an instrument designed to measure the degree of cell spreading. MDA-MB-231 cells in DMEM/F12 (lacking calcium and magnesium ions) were counted and resuspended. The ECIS plates were coated with bovine fetuin-A for 2 h and blocked with 3% BSA for 30 min. Electrodes were washed with appropriate media and 200 μl of the media was added to each well and the basal resistance and impedance was measured. Cells (4×10^5 cells/well) in DMEM/F12 with or without Ca^{2+} ions were added to the wells in a total volume of 400 μl/well. The attachment and spreading was then measured using ECIS [27]. Cell motions on the scale of nanometers are manifested as fluctuations in the impedance of small gold film electrodes that serve as cell substrata by itself or in conjunction with another adhesion molecule (fetuin-A).

2.4. Identification of cell surface receptors for fetuin-A

Plasma membranes were obtained from MDA-MB-231 cells by subcellular fractionation using a sucrose gradient as described [28]. The membrane fraction was aliquoted into two equal fractions and incubated with Affigel 15(BioRad) agarose beads linked to bovine fetuin without and with 2 mM Ca^{2+} for 3 h at 37 °C. The fetuin-agarose linked proteins were pelleted down at 10,000×*g* for 3 min and the beads were washed extensively with physiological buffer in the absence and presence of 2 mM Ca^{2+}. The pellets were eluted with 2 mM EDTA and the proteins denatured with sample loading buffer and resolved on a precast 12% SDS-PAGE (Invitrogen), and stained with silver stain (Sigma).

2.5. Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF)

The protein bands were excised from the silver-stained 1D SDS-PAGE gel on a light box and control bands were excised for each band. They were then cubed and equilibrated in 100 mM NH_4HCO_3. Reduction and alkylation was carried out with DTT (3 mM in 100 mM NH_4HCO_3, 37 °C for 15 min) and iodoacetamide (6 mM in 100 mM NH_4HCO_3 for 15 min). The gel slices were then destained and dehydrated with 50% acetonitrile in 50 mM NH_4HCO_3, followed by 100% acetonitrile. Gel slices were rehydrated with 15-μl 25 mM NH_4HCO_3 containing 0.01 μg/μl modified trypsin and trypsin digestion was carried out for 2 h at 37 °C. Peptides were extracted with 60% acetonitrile, 0.1% trifluoroacetic acid, dried by vacuum centrifugation, and reconstituted in 10-μl 0.1% trifluoroacetic acid. Peptides were then desalted and concentrated into 2-μl 60% acetonitrile, 0.1% trifluoroacetic acid using ZipTipC18 pipette tips. Of the sample, 0.4 μl was applied to a target plate and overlaid with 0.4-μl alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 60% acetonitrile, 0.1% trifluoroacetic acid). MALDI-TOF mass spectrometry was carried out using a Voyager 4700 mass spectrometer (Applied Biosystems) operated in reflectron mode. The mass spectrum was calibrated to within 20 ppm using trypsin autolytic peptides present in the sample (m/z=842.50, 1045.56 and 2211.09 Da). Ions ([M+H]) corresponding to peptide masses were used to interrogate the SWISS PROT and NCBInr databases using the MASCOT (http://www.matrixscience.com) and ProFound (http://prowl.rockefeller.edu) algorithms, respectively.

2.6. Fluorescence resonance energy transfer (FRET) analysis of the binding of fetuin-A with annexin-VI

Having determined by MALDI-TOF that annexin-II and -VI are the prominent cell surface proteins which interacted with fetuin-A, purified annexin-VI was purchased from Chemicon and its interaction with fetuin-A in solution analyzed by FRET. FRET allows the quantitative determination of noncovalent molecular associations at Angstrom levels. It is a process by which radiationless transfer of energy occurs from a fluorophore of donor to an acceptor molecule in close proximity [29]. The tryptophan residue of annexin-VI was used as the donor and the Dans moiety in the Dans-labeled fetuin-A as the acceptor. Fetuin-A was labeled using Dansyl chloride as previously described [30]. The stoichiometry of labeling was calculated by determining absorbance at 280 and 350 nm. Fluorescence excitation at 350 nm and emission at 450 nm were determined for Dans-fetuin-A.

Fetuin A was added to 5 nM solution of annexin-VI in 40 mM HEPES, 0.15 mM NaCl, pH 7.5 and 1 mM DTT without and with 2 mM $CaCl_2$, or 2 mM $MgCl_2$, or 10 mM EDTA at a final concentration of 0–30 nM. FRET was determined by Luminescent Spectrometer LS 55(Perkin Elmer) by the measurement of decreasing fluorescence at 344 nm (slit 10 nm) following excitation at 296 nm (slit 4 nm). The difference between the experimental (fetuin-A+annexin-VI) and control (without annexin-VI) was determined and the binding constant (K_b) calculated using the Chipman method [31], using the equation $dF/C=a-K_b*dF$, where dF is the decreasing fluorescence at 344 nm and C is the concentration of fetuin-A added.

2.7. Expression of annexin-VI on the surfaces of MDA-MB-231 cells

To further analyze the interaction between annexins on MDA-MB-231 cells and fetuin-A, the plasma membrane fractions were incubated with fetuin-A-affigel beads in the absence and presence of 2 mM Ca^{2+} ions. The bound proteins were resolved in a precast 12% SDS-PAGE, transferred to immobilon membranes and incubated with

the rabbit polyclonal antibody against annexin-VI. The MDA-MB-231 cells were also surface biotinylated using NHS-Biotin according to manufacturer's protocol (BioRad). The cells were subsequently lysed and the lysate pre-cleared with protein-A agarose. The lysate was then divided into two equal parts. One part was incubated with nonimmune rabbit IgG, and the other part with the rabbit polyclonal antibody against annexin-VI overnight at 4 °C followed by immunoprecipitation using protein-A agarose. The pulled down proteins were resolved in 12% SDS-PAGE, transferred to immobilon membranes and the blot probed with avidin-HRP and developed as described above.

2.8. Knocking down the expression of annexin-II and -VI by siRNAs

To confirm that annexin-II and -VI were the cell surface receptors for fetuin-A, we decided to knockdown their expression in the MDA-MB-231 cells using the siRNA approach. The cells were seeded in 24-well microtiter plates at 8×10^4 cells/well and grown in the DMEM/F12 culture medium without phenol red. The siRNAs were designed to target the following sequences: annexin-II, 5′-AAGGTACAAGAGCTACAGCCC-3′ and for annexin-VI, 5′-AAGATGCCATCTCGGGCATTG-3′. At approximately 70% confluence, 1 ng of the appropriate siRNA as well as scrambled siRNA (transfection controls) were added to 100 μl of the culture medium (without phenol red). To this, 6 μl of RNAiFect (Qiagen) transfection reagent was added and mixed thoroughly by vortexing (10 s). After a brief incubation for 10–15 min at 25 °C, the transfection complex was added to the wells containing the cells and incubated for 40 h (annexin-II) and 60 h (annexin-VI). At the end of the incubations, the cells were detached, cell number determined and divided into two portions. One portion was used for adhesion assays and the other for Western blot analysis to determine the extent of knockdown. The wells of a 96-well microtiter plate were coated with 2.5 mg/ml of fetuin-A, blocked with 3% BSA, and 2×10^4 cells added to the wells (quadruplicates/treatment) in the presence of 2 mM Ca^{2+}. After 2 h of incubation, FAD was added to the adherent cells and cell number determined as above or the adhered cells were fixed in 5% glutaraldehyde in PBS for 30 min, followed by staining with 0.1% (w/v) crystal violet in 96-well microtiter plates. The stain was released from the

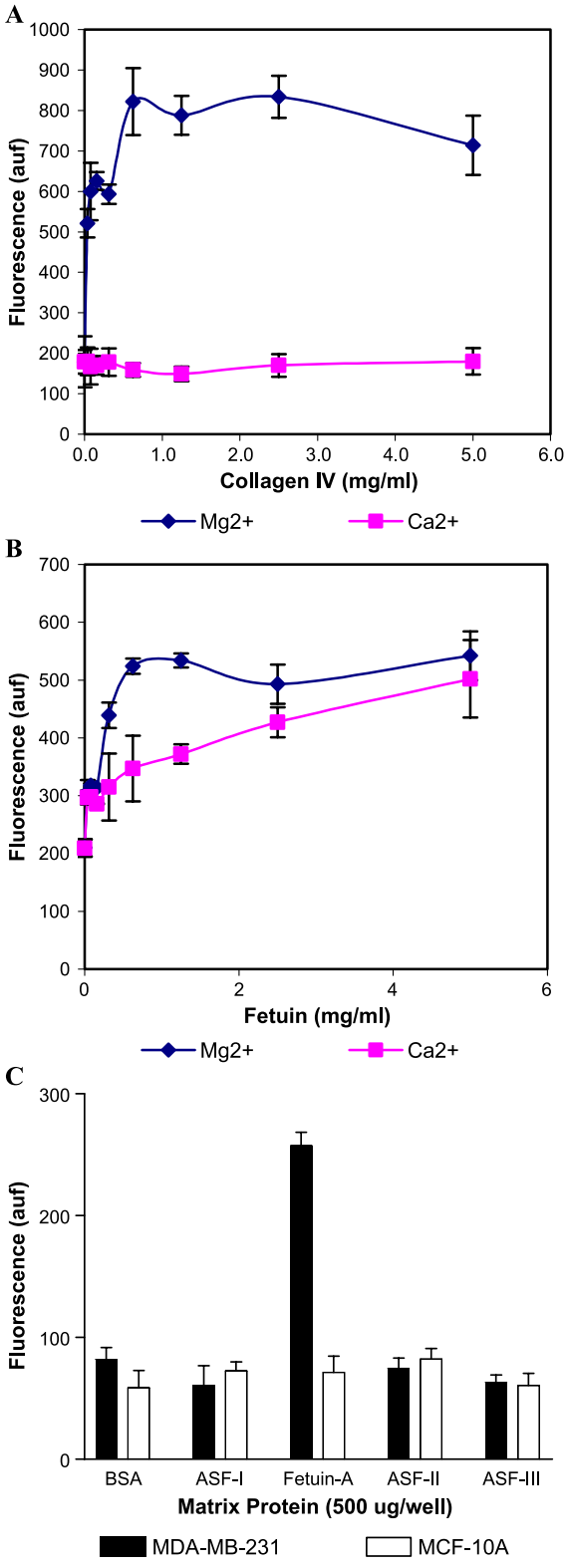

Fig. 1. Adhesion of MDA-MB-231 cells to collagen IV, fetuin-A, and asiolofetuins. The wells of a 96-well microtiter plate were coated with serially diluted doses of either collagen IV (Panel A) or fetuin-A (Panel B) in PBS overnight at 4 °C. The MDA-MB-231 cells in serumless medium were then added to the wells at 4×10^4 cells/well in the presence of either Mg^{2+} or Ca^{2+} ions. After 2 h of incubation at 37 °C, the nonadherent cells were washed off in serumless medium containing the respective divalent ions. FDA was added to the adherent cells at a final concentration of 20 μM. After 5 min of incubation at room temperature, fluorescence emission from the wells was determined by a fluorescence plate reader and reported as arbitrary units of fluorescence. In Panel C, the wells of a 96-well microtiter plate were coated with BSA, fetuin-A and three different batches of asialofetuin at ~500 μg/ml. The coating was done overnight at 4 °C, after which nonspecific sites were blocked with 2% BSA at 37 °C for 15 min. Cells (MDA-MB-231 and MCF-10A) were added to the wells (4×10^4 cells/well) in serumless medium containing 2 mM Ca^{2+} ions. After 2 h of incubation, the nonadherent cells were washed off and DAF added to the attached cells and fluorescence (auf) determined as above. (For color see online version).

cells using 10% acetic acid and the O.D. at 570 nm read by a plate reader (Dynex). The optical density was proportional to the number of cells in the wells. The other portion of the cells was lysed, protein concentration determined and 40 μg per protein sample analyzed by Western blot using a rabbit polyclonal antibody to annexin-II monomer and annexin-VI expression.

2.9. Cell proliferation assay

MDA-MB-231 cells were allowed to adhere to fetuin-A-coated wells (500 μg/well) at 2×10^4 cell/well in serum-free medium without (control) or with 2 mM Ca^{2+} in quadruplicates and incubated at 37 °C in humidified CO_2 incubator for 3 days. The cells were also allowed to grow in complete medium as a positive control. Every 24 h, the number of viable cells per well was determined by the Alamar blue assay [32]. In this assay reducing conditions, mitochondrial activity turns the Alamar blue dye from blue to pink with increased fluorescence at 590 nm after excitation at 544 nm. The dye (200 μl) was added to the wells at a dilution of 1:20 and initial fluorescence taken using a luminescence spectrophotometer LS 55 (Perkin Elmer) with a fluorescence plate reader attachment. The plate was allowed to incubate at 37 °C for 1 h, after which another reading was taken. This process was repeated three times and the average of the increase in fluorescence per hour in arbitrary units of fluorescence (auf) was determined.

3. Results

3.1. Adhesion of the breast carcinoma cells to fetuin-A and collagen IV

The MDA-MB-231 and other breast carcinoma cells generally adhere well to collagen, laminin, fibronectin and vitronectin in the presence of divalent ions such as magnesium or manganese but not calcium. In such instances, the cells use integrins as the principal cell surface receptors to interact with the matrix adhesion proteins [33] (Fig. 1A). Apart from the major extracellular matrix proteins mentioned above, fetuin-A has also been shown to promote cellular adhesion. However, the cell surface receptors, which mediate cell/fetuin-A interaction, as well as the mechanism of interaction have not been elucidated to date. We hereby demonstrate that MDA-MB-231 and other breast carcinoma cells adhere rapidly to wells coated with fetuin-A in the presence of magnesium or calcium ions (Fig. 1B). Whereas the adhesion in the presence of magnesium is likely to be mediated by integrins, the adhesion in the presence of calcium ions only, we argued, was due to a novel receptor(s) on the surface of tumor cells. This adhesive interaction was rapid, taking place within the first 2 h of plating. The optimal concentration of fetuin-A at which adhesion occurred was approximately 500 μg/well. Interestingly, the calcium-mediated adhesion of cells to fetuin-A required sialic acid

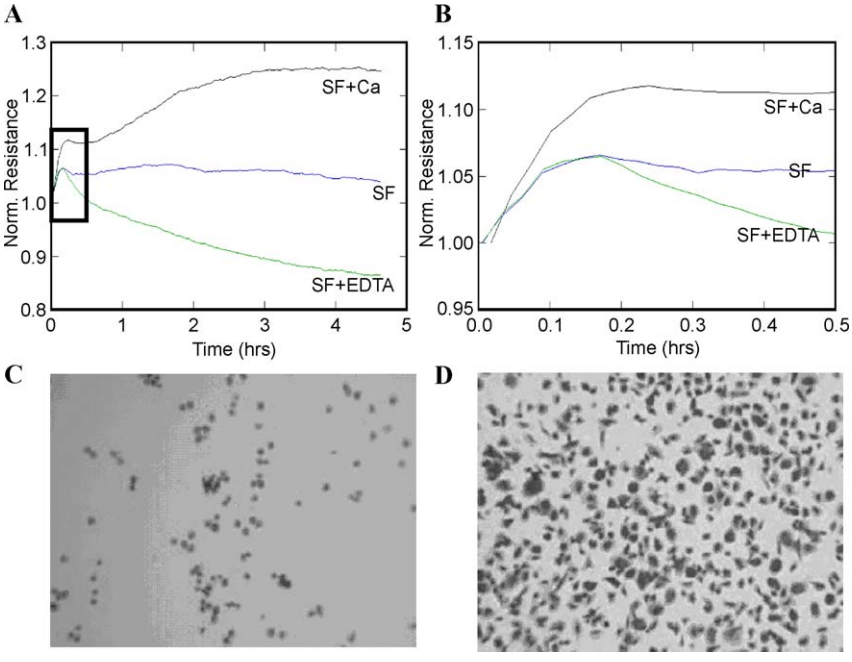

Fig. 2. Adhesion and spreading of MDA-MB-231 cells on fetuin-A. Adhesion and spreading of cells to fetuin-A was determined using ECIS. The electrodes were coated with fetuin-A at 1 mg/ml, followed by blocking of nonspecific sites by 2% BSA. Cells were resuspended in serum-free medium containing nothing (SF), 2 mM Ca^{2+} (SF+Ca), or 2 mM EDTA (SF+EDTA). Spreading was observed as increased resistance. The inset in Panel A was blown up in Panel B to represent adhesion and spreading in the first 30 min. The cells (4×10^4 cells/well) were also allowed to adhere to microtiter plates coated with fetuin-A as described in Fig. 1 in the absence (Panel C) or presence (Panel D) of 2 mM Ca^{2+} ions. The nonadherent cells were washed off after 2 h of incubation and the adherent cells fixed in 5% glutaraldehyde, stained with crystal violet and photographed. (For color see online version).

Table 1
Adhesion of MDA-MB-435 cells to fetuin-A and collagen IV in the presence of either Mg^{2+} or Ca^{2+} ions and in the absence and presence of function-blocking anti β1 integrin antibodies (% of control)

Substratum	Nonimmune mouse IgG (control)	Mouse anti β1 integrin (IgG)
Fetuin-A(Mg^{2+})	100	80.5±0.6*
Fetuin-A (Ca^{2+})	100	95.0±5
Collagen-IV (Mg^{2+})	100	63.5±12.5*

* Significant reduction in adhesion compared to control ($P<0.05$).

residues on the tri-antennary N-linked oligosaccharides of fetuin-A, because asialofetuin (ASF) did not support adhesion (Fig. 1C). Three batches of asialofetuin-A were used and cells could not adhere to any of them. Apart from MDA-MB-231, other aggressive breast carcinoma cell lines including MDA-MB-435 adhered rapidly to fetuin-A in the presence of Ca^{2+} ions (data not shown). Normal breast epithelial cells including the spontaneously immortalized MCF-10A, on the other hand, failed to adhere to fetuin-A in the presence of Ca^{2+} ions within the first 2 h (Fig. 1C). The same was true for less aggressive breast tumor cell lines such as BT-549 (data not shown).

The cells did not just adhere to fetuin-A, they also spread efficiently in this substratum, showing early signs of spreading within 20 min post-plating (Fig. 2, panels A and B). Adhesion and spreading was not observed in the absence of Ca^{2+} ions (Fig. 2, panels A, B and C). The spreading pattern (Fig. 2, panel D) was similar to that observed when the cells adhered to collagen or fibronectin in the presence of Mg^{2+}/Mn^{2+} ions (Fig. 1A).

3.2. Function-blocking antibodies against β1 integrins abrogate cellular adhesion to fetuin-A in the presence of Mg^{2+} but not in the presence of Ca^{2+} ions

In an effort to distinguish the Mg^{2+}- from Ca^{2+} ion-mediated adhesion of breast carcinoma cells to fetuin-A, we hypothesized that Mg^{2+} was involved in integrin-dependent adhesion while Ca^{2+} ions mediated a novel adhesion mechanism. We demonstrated that the adhesion of the MDA-MB-435 cells to fetuin-A in the presence of Mg^{2+} ions could be inhibited significantly by function-blocking anti-β1 integrins when compared to control cells in the presence of nonimmune mouse IgG (Table 1). As expected, the antibodies did not significantly alter the adhesion of the cells to fetuin-A in the presence of Ca^{2+} ions. The adhesion of the cells to collagen -IV was also reduced by anti-β1 integrins because these cells use mainly $\alpha1\beta1$ integrins to adhere to collagen IV. The data therefore suggest that the adhesion of breast carcinoma cells to fetuin-A in the presence of Mg^{2+}, and even Mn^{2+} (data not shown), involves β1 integrins and possibly other integrin heterodimers, such as $\alpha5\beta1$, with specificity for fibronectins which

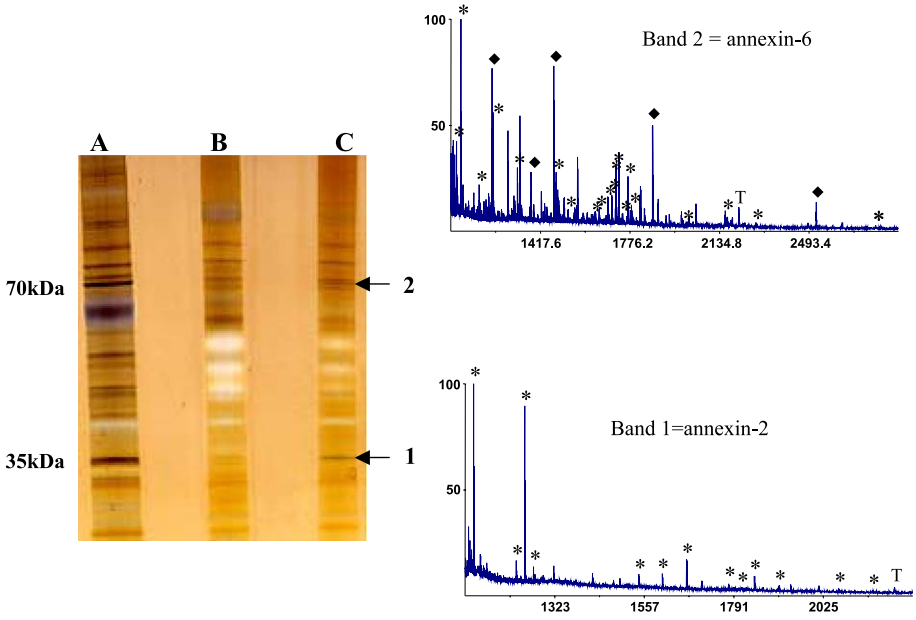

Fig. 3. Identification of cell surface proteins that bind to fetuin-A. The MDA-MB-231 cells were lysed and the plasma membrane fraction obtained by subcellular fractionation. Purified plasma membrane fractions were incubated with fetuin-A-affigel beads in the absence (lane B) or presence (lane C) of 2 mM Ca^{2+} ions. After incubation, the beads were washed extensively in the respective buffers (without and with Ca^{2+} ions). The bound proteins were eluted and resolved on a 12% SDS-PAGE. A purified membrane fraction (lane A) was also analyzed as control. Two unique protein bands, which were bound to fetuin-A-affigel beads in the presence of Ca^{2+} ions, were cut from the gel and subjected to MALDI-TOF. The ~40-kDa (Band 1) band from the silver stain was positively identified as annexin-II with a predicted molecular weight of 38.8 kDa. The MOWSE score was 247 (>73=95% confidence) and the Z score was 2.27 (>1.65=95% confidence). The total coverage was 57%. MALDI-TOF of the ~70 kDa (Band 2) band was positively identified as annexin-VI with a predicted molecular weight of 76.2 kDa. The MOWSE score was 125 (>73=95% confidence) and the Z score was 2.32 (>1.65=95% confidence). The total coverage was 47%. (For color see online version).

can co-purify with fetuin-A [2]. The calcium-mediated adhesion, on the other hand, is novel and does not involve integrins which normally require either Mg^{2+} or Mn^{2+} for proper adhesion.

3.3. Cell surface receptors for Ca^{2+}-mediated adhesion of MDA-MB-231 to fetuin-A

Using a proteomic approach, we next sought to identify the Ca^{2+}-dependent cell surface receptors for fetuin-A. The plasma membrane fraction of MDA-MB-231 was incubated with fetuin-A agarose beads in the absence and presence of calcium ions. The bound proteins were eluted after extensive washing in the respective buffers and resolved on SDS-PAGE and protein bands visualized by silver stain. In three separate experiments, two bands with approximate molecular weights of 75 and 35 kDa were observed in Fig. 3, lane C, where the membrane proteins were incubated with fetuin-A agarose in the presence of Ca^{2+} ions. The bands were not observed when the membrane proteins were allowed to interact with immobilized fetuin-A in the absence of Ca^{2+} ions (Fig. 3, lane B). All the membrane proteins are depicted in Fig. 3, lane A. These bands were excised and subjected to MALDI-TOF analysis. Protein band # 1 with the approximate molecular weight of 35 kDa was identified as annexin-II with a confidence level approaching 100%. Two independent statistical analyses were performed to determine the confidence level. The MOWSE score was 247 (>73=95% confidence) and the *Z* score was 2.27 (>1.65=95% confidence). The coverage map showed a 57% total coverage of the protein. Band #2, on the other hand, was identified as annexin-VI. The molecular weight was confirmed as being 76.2 kDa. Again two independent statistical analyses were performed to determine the confidence level. The MOWSE score was 125 (>73=95% confidence) and the *Z* score was 2.32 (>1.65=95% confidence). The coverage map showed a 47% total coverage of the protein. An interesting observation was that there were a few peptides matching long-chain fatty acid CoA ligase. The statistical analysis determined that it was not significant and the coverage was also poor. Although long-chain fatty acid CoA ligase has been demonstrated on the plasma membrane of adipocytes in mammals [34], there are no data to suggest that it is present on the cell surface of tumor cells.

3.4. Cell surface expression of annexin-VI

MALDI-TOF data strongly suggested that both annexin-II and -VI interact with fetuin-A. To further investigate this interaction, plasma membrane protein fractions were obtained from MDA-MB-231 cells as described above and incubated with fetuin-agarose beads in the absence and presence of 2 mM Ca^{2+} (Fig. 4A) for 3 h at 37 °C. After isolating the proteins that bound to fetuin-A and resolving them on SDS-PAGE gel, they were transferred onto immobilon membrane and probed with the polyclonal antibody to annexin-VI. This was then visualized using chemiluminescence. The plasma membrane fraction which was incubated with 2 mM calcium had a strong signal at 75 kDa (annexin-VI) (Fig. 4A, lane 2), as expected. A faint band at the same molecular weight (Fig. 4A, lane 1) was also evident, meaning that annexin-VI was bound to fetuin-A in the absence of Ca^{2+} albeit with a lower affinity.

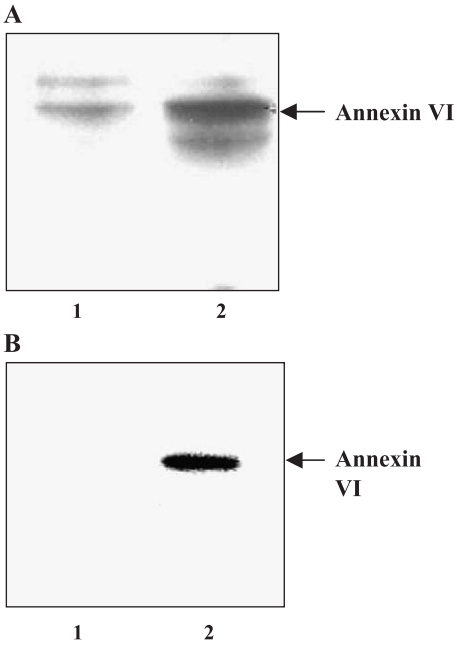

Fig. 4. Proof of the interaction between cell surface annexin-VI and fetuin-A. The MDA-MB-231 plasma membrane fraction was incubated with fetuin-A-affigel beads without (Panel A, lane 1) and with (Panel A, lane 2) 2 mM Ca^{2+} ions. After washing off the beads in the respective buffers, the bound proteins were dissolved in sample buffer, resolved on SDS-PAGE and analyzed by Western blot using antibodies to annexin-VI and visualized by enhanced chemiluminescence reagents. To demonstrate the presence of annexin-VI on the extracytoplasmic surface of the plasma membranes, biotinylated cells were washed, lysed and the lysates incubated with nonimmune rabbit IgG (Panel B, lane 1) or polyclonal rabbit antibodies against annexin-VI (Panel B, lane 2). These were then subjected to immunoprecipitation using protein-A agarose, resolved on SDS-PAGE followed by Western blot and probed with avidin-HRP.

Annexin-VI is a membrane protein which has not only been observed on the extracytoplasmic surface of the membrane, but also in intracellular vesicles engaged in endocytosis [35]. However, for the protein to be considered an ideal surface receptor for an extracellular protein such as fetuin-A, at least part of the molecule should be localized on the extracytoplasmic surface. Therefore, to determine that location of the annexin-VI motif which interacts with fetuin-A, cell surface proteins of MDA-MB-231 were biotinylated with NHS-Biotin (BioRad). The cells were lysed and the labeled proteins analyzed by immunoprecipitation using the polyclonal antibody to annexin-VI or nonimmune rabbit IgG. This was followed by a Western blot analysis with avidin-HRP to visualize the biotinylated cell surface proteins that were pulled down with anti-annexin-VI antibodies. A strong signal was observed only

in the lane where the proteins were immunoprecipitated with anti-annexin-VI antibodies (Fig. 4B, lane 2), demonstrating that at least part of the annexin-VI protein was on the extracytoplasmic surface of the plasma membrane of MDA-MB-231 cells.

3.5. Analysis of the binding interaction between fetuin-A and purified annexin-VI by FRET

We next analyzed the binding interaction between purified fetuin-A and annexin-VI. FRET allows the quantitative determination of noncovalent molecular associations at Angstrom levels. It is a process by which radiationless transfer of energy occurs from a fluorophore of donor to an acceptor molecule in close proximity [29]. The two tryptophan residues of annexin-VI were excited at 296 nm and emitted fluorescence at 344 nm. This emission (donor) at 344 nm was used to excite the Dans group (acceptor) of labeled fetuin-A (Fig. 5A). The binding data were then used to calculate the binding constants between fetuin-A and annexin-VI under different conditions. The binding constant of 68.8 μM^{-1} (K_d=14.5 nM) in the presence of 2 mM Ca^{2+} was higher than in the absence of divalent ions, where K_b was 41.2 μM^{-1} (K_d=24.3 nM) (Fig. 5B). There was, however, substantial binding interaction between the two proteins in solution in the absence and presence of divalent ions (Fig. 5B). This is in contrast to the interactions between cell surface annexin-VI and immobilized fetuin-A where calcium ions were required and cellular adhesion and spreading inhibited in the presence of EDTA (Fig. 2). This particular finding is not surprising because the cell microenvironment surrounding the tumor cells is likely to influence the binding interaction [36,37]. Moreover, cellular adhesive interactions with matrix proteins are usually not particularly strong because rapid adhesion and detachment are necessary for proper cell growth [38].

3.6. Knockdown of annexin-II and -VI expression and calcium-mediated adhesion of MDA-MB-231 cells to fetuin-A

To demonstrate that annexin-II and -VI were the bona fide receptors which mediate the adhesion of the tumor cells to fetuin-A, the cells were transiently transfected with either

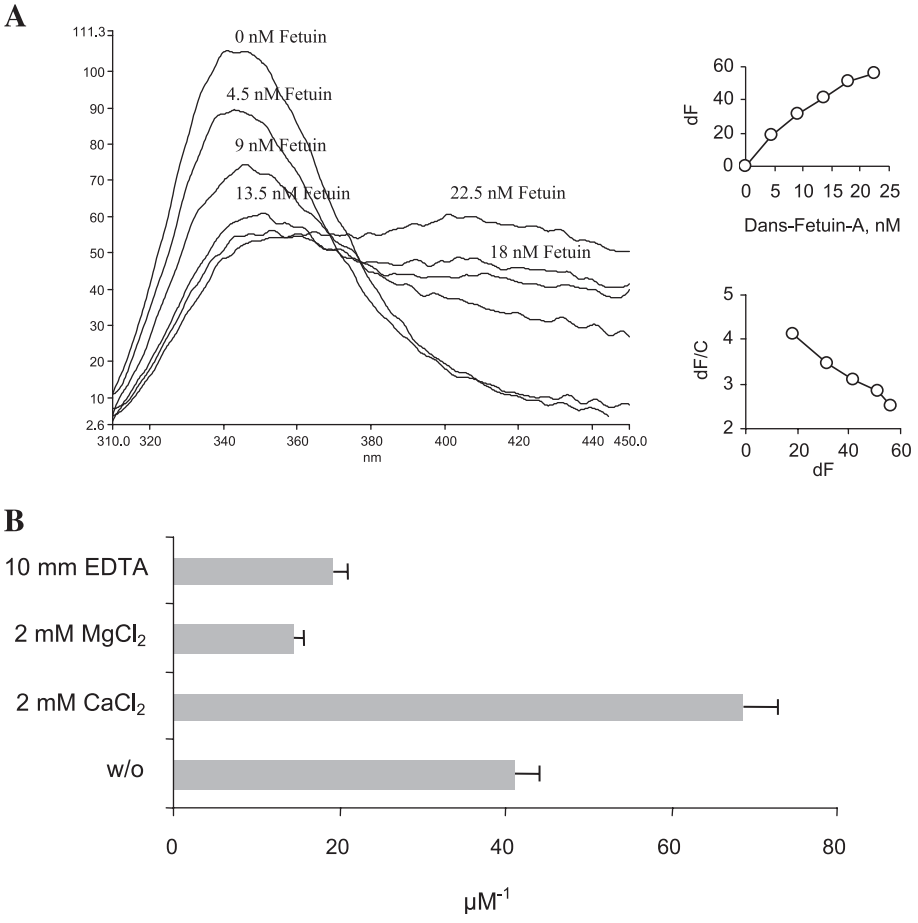

Fig. 5. Direct interaction between purified annexin-VI and Dans-labeled fetuin-A. The binding interaction between the two proteins was investigated by FRET. The energy transfer was from the Trp residues in annexin-VI to the Dans-group of the labeled fetuin-A, and was determined as decreases in fluorescence emission at 344 nm. The interaction was monitored without (w/o), with 2 mM Ca^{2+}, 2 mM Mg^{2+}, and with 10 mM EDTA. The binding constants were determined by the Chipman approach [31] from the plots of dF/*C* versus dF.

annexin-II or VI siRNA as well as scrambled double-stranded oligos as controls. The phenotypic manifestation of knocking down the expression of either annexin-II or VI was evident as early as 35 h after transfection. During the knockdown of both annexin-II and -VI, there was gradual detachment of cells from the culture flasks, underscoring the importance of the annexins in cellular adhesion. The reduced adhesion of siRNA transfected cells to fetuin-A was apparent. In three separate experiments, knocking down the expression of annexin-II resulted in approximately 30% reduction in cellular adhesion to fetuin-A in the presence of calcium (Fig. 6A, bar B), relative to untreated or cells transfected with scrambled oligos (Fig. 6A, bars A and C, respectively). The annexin-II protein, on the other hand, was reduced in the cells by almost 98% (Fig. 6B, lane 2) relative to untreated and cells transfected with scrambled oligos (Fig. 6B, lanes 1 and 3, respectively). As for annexin-VI, the siRNA knockdown resulted in almost 50% reduction in cellular adhesion to fetuin-A in the presence of Ca^{2+} ions

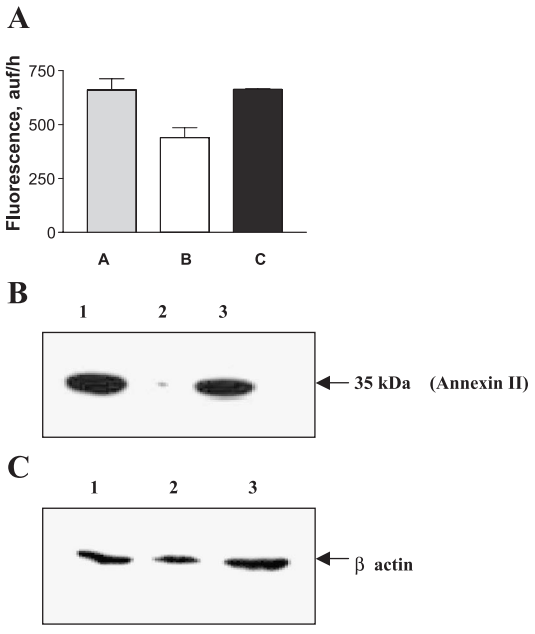

Fig. 6. Knockdown of annexin-II expression and calcium-mediated adhesion of MDA-MB-231 cells to fetuin-A. The cells were seeded in 24-well microtiter plates at 8×10^4 cells/well and grown in DMEM/F12 culture medium without phenol red. At approximately 70% confluence, 1 ng of siRNA targeted to annexin-II sequence as well as scrambled siRNA (transfection controls) were added to 100 μl of culture medium. To this, 6 μl of the transfection reagent was added and mixed by vortexing. After a brief incubation at 25 °C, the transfection complex was added to the wells and incubated for 40 h. The cells were detached, cell number determined and divided into two parts. One part was used for adhesion assays and the other for Western blot assays to determine the extent of the knockdown. Adhesion assays in the presence of Ca^{2+} ions were done as described in Fig. 1 (quadruplicates/treatment). Adhesion of control untreated cells (bar A); cells transfected with annexin-II siRNA (bar B) and cells transfected with scrambled siRNA (bar C) are represented in Panel A. The annexin-II expression in control untreated cells (lane 1); cells transfected with annexin-II siRNA (lane 2); and cells transfected with scrambled siRNA (lane 3) are depicted in Panels B. The blot in Panel B was stripped and re-probed for β-actin to show equal loading (Panel C).

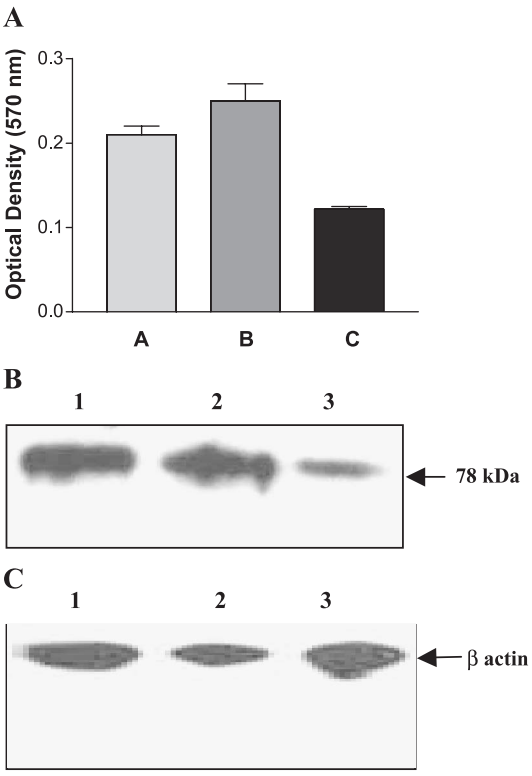

Fig. 7. Knockdown of annexin-VI expression and calcium-mediated adhesion of MDA-MB-231 cells to fetuin-A. The impact of knocking down the expression of annexin-VI on the adhesion of the cells to fetuin-A in a calcium-dependent manner was done as described in Fig. 6 with only a few modifications. The adhesion assay was done by fixing the cells in 5% glutaraldehyde and staining them with crystal violet. The stain was released into the wells by adding 100 μl of 10% acetic acid and measuring optical density at 570 nm by plate reader. Adhesion of control untreated cells (bar A), cells transfected with scrambled siRNA (bar B), and cells transfected with annexin-VI siRNA (bar C) are represented in Panel A. The annexin-II expression in control untreated cells (lane 1), cells transfected with scrambled siRNA (lane 2), and cells transfected with annexin-VI siRNA (lane 3) are depicted in Panels B. The blot in Panel B was stripped and re-probed for β-actin to show equal loading (Panel C).

(Fig. 7A, bar C) compared to control untreated cells and those transfected with scrambled oligos (Fig. 7A, bars A and B, respectively). Protein expression was reduced by almost 70% by the siRNA (Fig. 7B, lane 3) relative to the control cells (Fig. 7B, lanes 1 and 2).

3.7. Growth of MDA-MB-231 cells is mediated by fetuin-A and Ca^{2+} ions in vitro

Having demonstrated that annexin-II and -VI can mediate the adhesion of the breast carcinoma cells to fetuin-A, we finally questioned whether this interaction was sufficient to transmit growth signals to these cells. The cells were therefore allowed to adhere to culture plates in serumless medium containing Ca^{2+} ions only (Ca); Ca^{2+} ions and 0.25% w/v of fetuin-A (CF); and complete medium containing 10% fetal bovine serum (CM) and allowed to grow at 37 °C for 3 days. The increase in cell number was

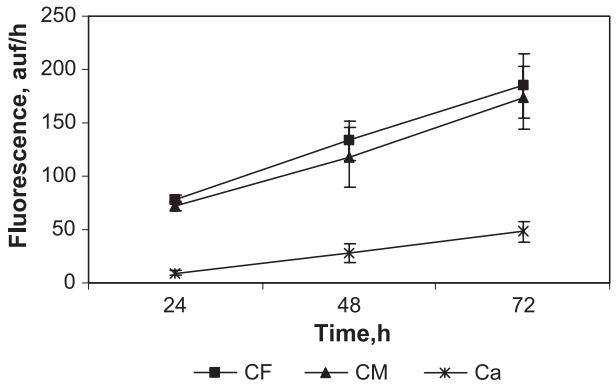

Fig. 8. Proliferation of MDA-MB-231 cells in the presence of fetuin-A and Ca^{2+} ions. The cells (2×10^4 cells/well) were plated in 96-well culture plates (quadruplicates/time point) in complete medium (CM), serumless medium containing Ca^{2+} (Ca) or serumless medium containing 2 mM Ca^{2+} ions, and added to wells coated with fetuin-A (500 μg/well) (CF). At 24-, 48-, and 72-h time points, Alamar blue was added to the wells corresponding to each time point and the rate of change of Alamar fluorescence (auf/h) used as a determinant of cell density and was measured by the luminescence spectrophotometer LS 55 (Perkin Elmer) with a plate reader attachment.

monitored by Alamar blue dye as described above. It was surprisingly evident that within 72 h, when these cells are normally in their log phase of growth, the rate of growth in serumless medium containing fetuin-A and calcium ions was the same as the rate for the cells growing in complete medium (Fig. 8). The rate of growth in the presence of calcium ions only was low as expected and there was no exponential increase in cell number. Interestingly, the cells were also able to grow to the same extent in suspension in the presence of either fetuin-A/Ca^{2+} or complete medium (data not shown). Interestingly, cells in suspension in eppendorf tubes also responded to fetuin-A/Ca^{2+} by growing exponentially (data not shown), meaning that fetuin-A can rescue these cells from anoikis [39].

4. Discussion

It has been our experience that breast carcinoma and other aggressive tumor cells such as Lewis lung carcinoma (data not shown) have the ability to adhere and spread on immobilized fetuin-A in any appropriate physiological buffer or serumless medium, with a requirement for Ca^{2+} ions. This observation suggested to us the existence of a cell surface receptor(s) for the glycoprotein other than integrins, which normally require manganese or magnesium ions [33]. In the present work, we present evidence that annexin-II and -VI are the putative tumor cell surface receptors for fetuin-A adhesion in the presence of Ca^{2+} ions. This is an important discovery because despite the fact that fetuin-A has been known to be a cell attachment factor in fetal bovine serum since the late 1950s [40], its cell surface receptor(s) has been elusive. More importantly, since that initial demonstration of its cell adhesive properties, there has been a serious debate among cell biologists concerning the mechanism of this adhesion [2]. One school of thought maintains that the proteins which co-purify with fetuin-A in vivo are responsible for this adhesion, while the other is a proponent of fetuin-A per se being the principal adhesion factor [2].

In the present work, we demonstrate a cell/fetuin-A interaction which requires intact sialic acid residues of the glycoprotein and Ca^{2+} ions. Therefore, wherever intact fetuin-A is immobilized, such as in the various extracellular matrices in vivo including the bones, one can expect responsive cells to adhere to or associate with such sites. Commercial tissue culture dishes can easily be coated with fetuin-A in the media, and the responsive cells in turn specifically adhere to the fetuin-A in a calcium ion-dependent manner. It should, however, be noted that the bulk of fetuin-A in vivo is soluble and only those fetuin-A molecules that are immobilized on the substrata participate directly in cellular adhesion. For example, we have observed that fetuin-A can easily be immobilized on elastin fibers, an interaction which may aid the adhesion of tumor cells to elastin-rich tissues such as the lungs (unpublished information). The expression of both annexin-II and -VI was not only deemed necessary for adhesion of tumor cells to fetuin-A; they also stimulated cell spreading as shown by siRNA studies, pointing to their potential roles as classical cell surface receptors for extracellular matrix interaction [38]. Such interactions normally do not require tight binding interaction because cellular adhesion, particularly of tumor cells, is a dynamic process involving rapid adhesion and detachment [41,42]. However, when the binding interaction between fetuin-A and annexin-VI was analyzed in solution, a much tighter binding affinity (in the nanomolar range) was demonstrated. Binding was not only evident in the presence of 2 mM Ca^{2+} as expected; there was some binding in the absence of Ca^{2+} ions and even in the presence of 10 mM EDTA. The requirement of Ca^{2+} ions for cell adhesion to fetuin-A suggests that the disposition of annexins on the cell surface depends on calcium ions [17].

Fetuin-A has been shown to interact with a number of cellular proteins [2,12,30,43], and we initially hypothesized that it could bind to several cell surface proteins. The present data suggest that annexin-II and -VI, and most likely other members of the annexin family which are expressed on the cell surface, assume lectin properties with preference for the sialic acid residues on tri-antennary sugar moieties found in fetuin-A [44]. The approximate 50% reduction in adhesion of MDA-MB-231 cells to fetuin-A, as a result of annexin-VI knockdown, suggests that the elimination of all other relevant annexins on the surface would totally abrogate the Ca^{2+}-mediated adhesion of the cells to fetuin-A.

Both annexin-II and -VI are known to be expressed on the extracellular membrane surfaces of a number of cells, despite their lack of signal peptides and obvious membrane spanning domains [17]. Moreover, the mechanisms by which they are secreted are likely to be tightly regulated because only a small proportion, ~4% of the total annexin-

II, is expressed extracellularly [45]. The regulatory mechanisms which control the secretion and cell surface disposition of the annexins are obviously important determinants in their roles as receptors for extracellular fetuin-A. Tumor cells which secrete significant quantities of annexins to the extracellular milieu are more likely, based on the current data, to adhere rapidly to fetuin-A-coated surfaces. The scenario may be more complex in vivo because stromal cells can also secrete annexins which can then bind to the surfaces of tumor cells resulting in increased adhesion to fetuin-A.

In the present work, we have demonstrated that the rapid cell surface annexin/fetuin-A interaction is predominantly a feature of breast carcinoma cells, particularly the aggressive ones such as MDA-MB-231. The interaction is weak in less aggressive breast carcinoma cells such as BT-549 [46] and normal breast epithelial cells, including MCF-10, which failed to adhere to fetuin-A even after 3 h of incubation (data not shown). Increased expression of annexin-II has been observed in large cell lymphoma and colon adenocarcinoma. Annexin-II and -VI have been demonstrated to be involved in the adhesive interactions between RAW 117 large cell lymphoma and endothelial cells [24,23]. Of note is the number of studies that have suggested that annexins are multifunctional cell surface receptors that interact with a variety of extracellular ligands such as plasminogen and tissue plasminogen activator [45], tenascin C [47], heparin [48], and chondroitin sulfate chains [49]. A number of annexins including annexin-II contain the Cardin–Weintraub consensus sequence for glycosaminoglycan recognition [48]. However, it is unlikely that this is the motif in annexin-II or -VI which interacts with fetuin-A because heparin or chondroitin sulfate was unable to abrogate the adhesion of cells to immobilized fetuin-A (data not shown).

Lastly, the present work suggests that the interaction between the breast carcinoma cells and fetuin-A via annexin-II and -VI sends 'outside in' signals into the cells, resulting in cell spreading and growth. The breast carcinoma cells attained confluence in the presence of fetuin-A and calcium ions alone (ruling out the involvement of integrins in this anchorage-dependent growth signaling). Thus, other than the stimulation of cellular attachment, the calcium-mediated cell to fetuin-A interaction could be a key mechanism adapted for growth stimulation or for rescuing the tumor cells from apoptosis due to anoikis [39]. Apart from the in vitro cell culture conditions, we recently demonstrated that fetuin-A can significantly affect adhesion and growth of tumor cells in vivo. In a two-stage skin initiation/promotion protocol of carcinogenesis, skin papillomas were developed in fetuin-A wild-type and null mice. After 6 weeks of promotion with phorbol esters, tumor volume in the wild type was significantly larger than in the fetuin-A null C57/BL-6 mice (unpublished information), indicating that fetuin-A was required for growth and expansion of the transformed cells.

The signaling mechanisms by which annexin-II and -VI promote the growth of tumor cells are currently not known. Annexins, particularly annexin-VI, can form membrane channels for calcium ion transport from the extracellular milieu to the cytoplasm [50]. Interestingly, we have demonstrated that fetuin-A is capable of transiently increasing [Ca^{2+}] in 11-9-1-4 but not in MDA-MB-435 breast carcinoma cells [46]. Therefore, the interaction of some tumor cells with fetuin-A in the presence of calcium may result in increases in intracellular calcium ions, triggering a variety of signaling mechanisms culminating in cell growth. Studies reported by Kohn and Liotta [51] have recently pointed to the importance of extracellular calcium ions as one of the modulators of tumor progression/metastasis pathways in vivo.

In as much as we have presented evidence here that cells, particularly tumor cells, are likely to use the novel fetuin-A/annexin signaling for adhesion and growth, we have also demonstrated that other protein/protein interactions are involved in this cell adhesion/growth-promoting phenomenon of fetuin-A. For example, we postulate that the Mg^{2+} ion-dependent adhesion of breast carcinoma cells to immobilized fetuin-A is mediated by activated integrins such as the VLA1 family members, which interact with their ligands such as fibronectin that associates and co-purifies with fetuin-A in serum [2]. Another possibility is that fetuin-A in vivo acts as a "sponge" or reservoir, attracting and concentrating a number of unrelated adhesion and growth factors in the vicinity of tumor cells. We have demonstrated that fetuin-A interacts with matrix metalloproteinases and, together with other members of the cystatin family, protects these enzymes from premature autolytic degradation or inactivation [30]. It is likely that association of matrix metalloproteinases with fetuin-A serves an unknown physiological function which is essential for growth.

In summary, we have demonstrated that breast carcinoma cells can use annexin-II and -VI to interact with fetuin-A. The cells adhere and spread on immobilized fetuin-A in a calcium-dependent fashion, making the annexins behave like C-type lectins or siglecs with a preference for the sialic acid residues on fetuin-A. We propose that annexin-II and -VI/fetuin-A signaling is a key tumor cell adhesion/growth pathway, and the elucidation of the mechanisms involved is our primary goal.

Acknowledgements

This work was supported by NIH grants 1U54 CA091408 (J.O. and L.M.M.), P30 CA68485 (Vanderbilt-Ingram Cancer Center), P50 CA90949 (project 1 to L.M.M) and GM 08037 (J.O.).

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