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Protein interactions between surface annexin A2 and S100A10 mediate adhesion of breast cancer cells to microvascular endothelial cells

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Helene K. Myrvang, Xiaoxia Guo, Chan Li, Lodewijk V. Dekker *

School of Pharmacy, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom

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ABSTRACT

Annexin A2 (AnxA2) and S100A10 are known to form a molecular complex. Using fluorescence-based binding assays, we show that both proteins are localised on the cell surface, in a molecular form that allows mutual interaction. We hypothesized that binding between these proteins could facilitate cell–cell interactions. For cells that express surface S100A10 and surface annexin A2, cell–cell interactions can be blocked by competing with the interaction between these proteins. Thus an annexin A2-S100A10 molecular bridge participates in cell–cell interactions, revealing a hitherto unexplored function of this protein interaction.

1. Introduction

Annexin A2 (AnxA2) is a member of the larger annexin family of Ca^{2+} and phospholipid binding proteins [1–3]. AnxA2 has been implicated in cellular functions which generally involve membrane surface-associated events, such as intracellular trafficking. In common with all annexins, AnxA2 contains a conserved C-terminal core domain, which confers Ca^{2+} and phospholipid binding properties, and a less conserved, smaller N-terminal domain. The N-terminal domain of AnxA2 consists of 30 amino acids of which the first 14 residues constitute the binding site for its typical binding partner S100A10, a member of the S100 protein family [4].

S100A10 requires dimerisation to accommodate the AnxA2 N-terminus such that an S100A10 dimer can bridge two AnxA2 molecules forming a heterotetramer structure [2,5–7]. Complex formation affects several properties of AnxA2. It reduces the Ca^{2+} requirement of AnxA2 for membrane association and alters the intracellular distribution of AnxA2 compared with monomeric AnxA2 [8,9]. The tetramer localises to the cytosolic surface of the plasma membrane in association with the submembranous cytoskeleton [10]. In addition, the tetramer displays binding and bundling of F-actin at physiological Ca^{2+} concentrations [11,12].

Apart from being localised inside the cell, AnxA2 has also been detected on the cell surface of various cells. Whilst the mechanisms by which surface expression occurs are still actively investigated, from a functional point of view some patterns are emerging. Thus cell surface AnxA2 participates in cell–cell interactions. Localised on macrophages or epithelial cells, it provides a signal for interaction with and phagocytosis of apoptotic cells, most likely via interactions with phosphatidyl serine on the juxtaposed apoptotic cell surface [13–15]. AnxA2 expressed on apoptotic cells themselves binds complement factors as signal for cell–cell interaction and phagocytosis [16,17]. Furthermore, the AnxA2-S100A10 heterotetramer has been implicated in tight junction maintenance in epithelial MDCK cell monolayers in a model in which AnxA2 is associated with the lipid membrane with the S100A10 dimer bridging two AnxA2 molecules [18,19].

A relatively early study showed that an AnxA2 antibody inhibited adhesion of liver-metastatic RAW117-H10 cells to human umbilical vein endothelial cells (HUVECs) [20] suggesting an involvement of surface AnxA2 in tumour–host cell interactions during metastasis. Interactions between osteoblast AnxA2 and its receptor have also been implicated in prostate cancer metastasis to the bone [21] and in the support of multiple myeloma cell growth and adhesion in the bone marrow [22].

Amongst breast cancer cells, surface AnxA2 levels were apparently higher in metastatic than in non-metastatic cells [23–25].

However, little is known about the implications of this for breast cancer cell–host cell interactions. Interestingly, S100A10 has been detected on the cell surface of HUVECs [26]. Thus direct interactions between surface AnxA2 and S100A10 could mediate cell–cell interactions between breast cancer cells and endothelial cells. Using probes recently developed in our lab, we have investigated this further. We show that surface AnxA2 and S100A10 can act as mutual receptors on the cell surface and that breast cancer cells that express surface AnxA2 can form cell–cell contacts with human microvascular endothelial cells (HMECs) through an AnxA2-S100A10 molecular bridge.

2. Materials and methods

2.1. Antibodies, peptides and recombinant human S100A10 protein

AnxA2 mouse monoclonal antibodies were from Santa Cruz Biotechnology, Heidelberg, Germany (#sc-47696) and from Becton, Dickinson and Company (BD), Oxford, UK (#610068). The S100A10 monoclonal mouse antibody (#610070 and total mouse IgG (#556648) were from BD, Oxford, UK. Alexa Fluor 488-conjugated goat anti mouse antibody was from Invitrogen, Dorchester, UK (#A11017) and horseradish peroxidase-conjugated goat anti mouse antibody was from Upstate, Watford, UK (#12-349). The Cy3-labelled AnxA2(Ac1-14) peptide, non-labelled peptide, Cy5-labelled S100A10 and non-labelled S100A10 were obtained as described [27]. A second batch of non-labelled wildtype (STVHEILSKLSLEC) and scrambled peptide (KIETLSEHVSLSLC) were purchased from Genscript, Piscataway, NJ, USA.

2.2. Cell maintenance

HUVECs [28] were maintained in (endothelial growth medium-2) EGM-2 (Lonza, Slough, Berkshire, UK) supplemented with their BulletKit containing: heparin, VEGF, rhFGF-B, R³-IGF-1, hydrocortisone, gentamicin sulphate amphotericin-B, rhEGF, ascorbic acid, FBS. HMEC-1 cells [29], a kind gift from Dr Francisco Candal at the Center for Disease Control and Prevention (Atlanta, GA, USA), were maintained in EGM-2 supplemented with EGF (10 ng/ml) (BD), hydrocortisone (1 μg/ml) and 10% foetal bovine serum. Human bone marrow endothelial cells [30], a kind gift from Dr Kenneth Pienta at the University of Michigan (MI, USA), were maintained in DMEM (Dulbecco's modified eagle medium) 10% foetal bovine serum, 2% penicillin/streptomycin. Human breast adenocarcinoma MDA-MB-231 and MCF7 cells [31] were maintained in RPMI-1640 supplemented with 10% foetal bovine serum.

2.3. Flow cytometry

Cells were harvested using trypsin and washed twice with phosphate–buffered saline (Sigma). For antibody experiments, 5×10^5 cells were gently resuspended in 100 μl phosphate–buffered saline containing 2.5 μg primary antibody and incubated for 45 minutes at room temperature. Cells were then washed three times with phosphate–buffered saline and incubated for 30 min at 4 °C in the dark with secondary antibody diluted 1:50 in 1% foetal bovine serum in phosphate–buffered saline. Cells were washed three times with phosphate–buffered saline and resuspended in 1 ml phosphate–buffered saline. For experiments with Cy3-labelled AnxA2(Ac1-14) peptide and S100A10 protein 5×10^5 cells were incubated with the tracers for 15 minutes at 4 °C in the dark. The cells were then washed three times with phosphate–buffered saline at 250 g for 5 min and resuspended in 1 ml phosphate–buffered saline.

Analysis was done on a Beckman Coulter Epics XL-MCL flow cytometer using Expo32 flow cytometry software. For every experiment, a minimum of 2×10^4 cells were analysed per sample and gated during analysis to exclude doublets and debris.

2.4. Biotinylation of cell surface proteins

The Pierce Cell Surface Protein Isolation Kit (#89881, Perbio Science, Cramlington, UK) was used to biotinylate and identify cell surface proteins. Briefly, confluent cells was washed in Phosphate-buffered Saline and incubated with 10 ml sulfo-NHS-SS-biotin solution for 30 minutes at 4 °C. 500 μl of a quenching solution was added after which cells were harvested by centrifugation, washed and lysed in lysis buffer. The cell extract was centrifuged at 10 000 g for 2 min at 4 °C and the supernatant was collected. Biotinylated proteins were recovered by immobilization on NeutrAvidin Gel and eluted in SDS sample buffer containing 50 mM DTT. The eluates were then analysed by Western blot as described [32].

2.5. Cell adhesion assay

Endothelial cells (2×10^5 per well) were seeded in black 96-well plates with a clear bottom (Corning Costar, Amsterdam, The Netherlands) and grown overnight to a confluent monolayer. Breast cancer cells in 75 cm² flasks were incubated with 1 μM 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester (BCECF-AM; Invitrogen) for 15 min at 37 °C in cell culture medium. Breast cancer cells were then trypsinised, resuspended in media and counted. 3×10^4 cancer cells were added to the endothelial monolayers and incubated at 37 °C for 1 h or 15 min. Plates were washed three times with phosphate–buffered saline to remove unbound tumour cells and the fluorescence was read on a fluorescence plate reader (PerkinElmer Envision 2104 Multilabel Reader) with excitation at 490 nm and emission intensity detected at 535 nm. The volume was adjusted so that for every experiment, the total volume in the well was always 60 μl after the addition of the breast cancer cells. AnxA2(Ac1-14) peptide, S100A10, S100A4 and antibodies, when added to the adhesion assay, were incubated with the endothelial monolayers for 30 min before the addition of the breast cancer cells diluted in media to the correct concentration.

3. Results

3.1. Cell surface AnxA2 is capable of interacting with S100A10

Recent studies revealed the presence of AnxA2 on the surface of breast cancer cells [23,24]. Here we investigated if surface-localised AnxA2 could bind S100A10, known as its main binding partner.

A Cy5 fluorophore-labelled S100A10 [27], was used as tracer and surface binding to the intact cell population was assessed using flow cytometry. Cy5-S100A10 can bind to intact MDA-MB-231 cells, indicating that an S100A10 receptor exists on these cells (Fig. 1A). Cy5-S100A10 binding can be competed with non-labelled S100A10 (Fig. 1B) and is blocked by a synthetic peptide based upon residues 1–14 of AnxA2 [28] (Fig. 1C), suggesting that AnxA2 expressed on the cell surface of these cells is the relevant receptor. In support of this conclusion, an antibody to AnxA2 also inhibits binding of Cy5-labelled S100A10 to the MDA-MB-231 cell surface (Fig. 1D).

The presence of AnxA2 on the surface of MDA-MB-231 cells was confirmed using surface biotinylation. Cells were labelled using Sulfo-NHS-SS-Biotin (a membrane-impermeable biotin linker) and the biotinylated (surface protein) fraction was immobilised on a streptavidin column and recovered using DTT. AnxA2 was recovered in the surface fraction of MDA-MB-231 cells (Fig. 2A). By contrast, -MCF7 breast cancer cells showed lower AnxA2
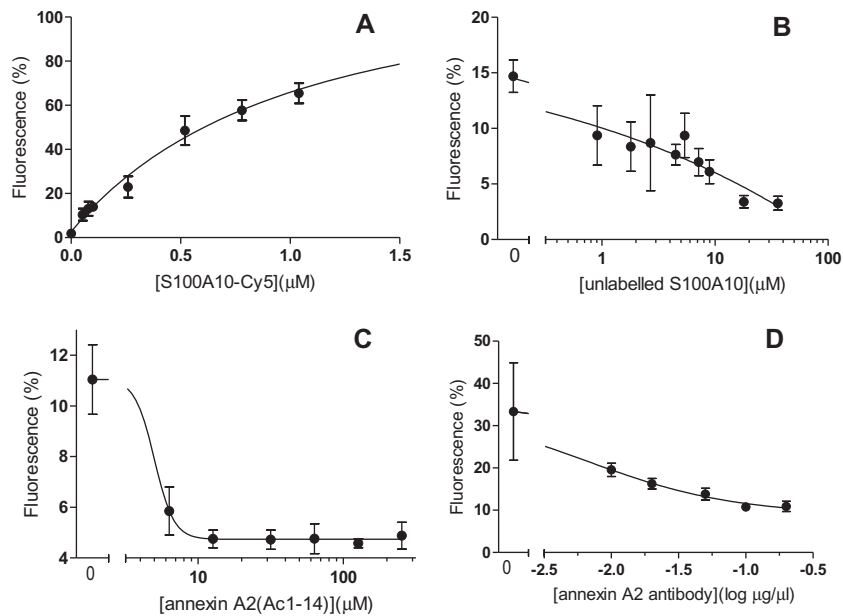
Fig. 1. AnxA2-dependent binding of Cy5-labelled S100A10 to MDA-MB-231 cells. (A) Concentration dependence of S100A10-Cy5 binding to MDA-MB-231 cells. 5×10^5 cells were incubated with increasing concentrations of Cy5-labelled S100A10 (0–10 μM) and binding was measured by flow cytometry. (B) Competition of S100A10-Cy5 binding to MDA-MB-231 cells with unlabelled S100A10. 5×10^5 cells were pre-incubated with increasing concentrations of unlabelled S100A10 (0–35 μM) for 30 min and then with 0.1 μg Cy5-labelled S100A10 for 15 min. Binding of S100A10-Cy5 was measured by flow cytometry. (C) Competition of S100A10-Cy5 binding to MDA-MB-231 cells by AnxA2(Ac1-14) peptide. 5×10^5 cells were incubated with increasing concentrations of unlabelled N-terminal peptide (0–50 μg) and then with 0.1 μg Cy5-labelled S100A10 for 15 min. Binding of S100A10-Cy5 was measured by flow cytometry. (D) Competition of S100A10-Cy5 binding to MDA-MB-231 cells by an AnxA2 antibody. 5×10^5 cells were incubated with increasing concentrations of AnxA2 antibody (0–20 μg) for 30 min and then with 0.1 μg Cy5-labelled S100A10 for 15 min. Binding of S100A10-Cy5 was measured by flow cytometry. All data points shown are the average ± S.E.M. from three independent experiments, with 20 000 events being counted each time.

present on the cell surface (Fig. 2A), due to lower overall levels of annexin A2 in the cell. Based on these observations we predicted that S100A10 binding to MCF7 cells would be lower. Indeed, using the above flow cytometry measurement method, binding of Cy5-labelled S100A10 to MCF7 cell was substantially lower than binding to MDA-MB-231 cells (Fig. 2B).

Thus our data indicate that AnxA2 is present on the surface of MDA-MB-231 breast cancer cells, in a form that is capable of binding to S100A10. Competition experiments indicate that the AnxA2 N-terminus is the relevant binding site for S100A10 suggesting that for surface-bound AnxA2 the N-terminus is exposed. AnxA2 may exist on the surface directly accessible to Cy5-S100A10, or in complex with S100A10, requiring competitive displacement by Cy5-S100A10.

### 3.2. Surface localisation of S100A10 on microvascular endothelial cells

We hypothesized that the protein interaction between AnxA2 and S100A10 may mediate cell–cell interactions. This would involve the N-terminus of surface-localised AnxA2 on one cell binding to surface-localised S100A10 on a second cell. Evidence for surface localisation of S100A10 is limited however some reports indicate that S100A10 is present on the cell surface of large vessel endothelial cells [33]. In agreement with these reports we observed binding of a mouse monoclonal S100A10 antibody to intact HUVECs using flow cytometry (Fig. 3A). Using a mouse monoclonal S100A10 antibody, S100A10 surface expression was also detected on HMEC-1 and human bone marrow endothelial (HBME)-1 cells (Fig. 3A). Surface expression of S100A10 was verified by biotinylation. As shown in Fig. 3B, S100A10 was found in the surface protein fraction of HUVEC, HMEC-1 and HBME-1 cells. This indicates that S100A10 is expressed on the surface of different types of endothelial cells. To explore this further we focused on microvascular endothelial cells as model system.

3.3. Microvascular endothelial cell surface S100A10 is capable of binding the AnxA2 N-terminus

To establish whether S100A10 localised on the cell surface of endothelial cells can bind AnxA2, we performed binding experiments using a fluorescent N-acetylated synthetic peptide based upon residues 1–14 of AnxA2 [28]. A Cy3 fluorophore was covalently linked to the peptide [27] and this was then incubated with HMEC-1 cells. Binding of the peptide to the cell surface of intact cells was assessed using flow cytometry. Fig. 4A shows that Cy3-AnxA2(Ac1-14) binds to HMEC-1 cells and that the binding of Cy3-AnxA2(Ac1-14) could be competed using non-labelled peptide suggesting a specific interaction with a surface binding site. This site is most likely S100A10, since the annexin N-terminus binds strongly to this protein. An antibody to S100A10 prevented binding of the Cy3-AnxA2(Ac1-14) peptide, indicating that this binding site was indeed S100A10 (Fig. 4B). Thus S100A10 is present on these cells in a form capable of binding to the AnxA2 N-terminus.

3.4. AnxA2–S100A10 complex in breast cancer cell adhesion to microvascular endothelial cells

Given that S100A10 and AnxA2 are localised on the cell surface of endothelial and breast cancer cells, in a molecular form that allows mutual binding, we assessed whether an interaction between these proteins can mediate cell–cell interactions between these cells. MDA-MB-231 cells were loaded with (BCECF-AM) fluorescent dye and incubated for 15 min on a monolayer of HMEC-1 cells after which non-adherent cells were washed away. Fig. 2C shows that MDA-MB-231 cells are capable of binding to HMEC-1 as well as HUVEC monolayers. MCF7 cells by contrast, showed lower levels of adhesion to either monolayer or endothelial cells (Fig. 2C). This was not due to differences in dye loading between the cells since the amount of BCECF-AM in the two cell types was identical (not
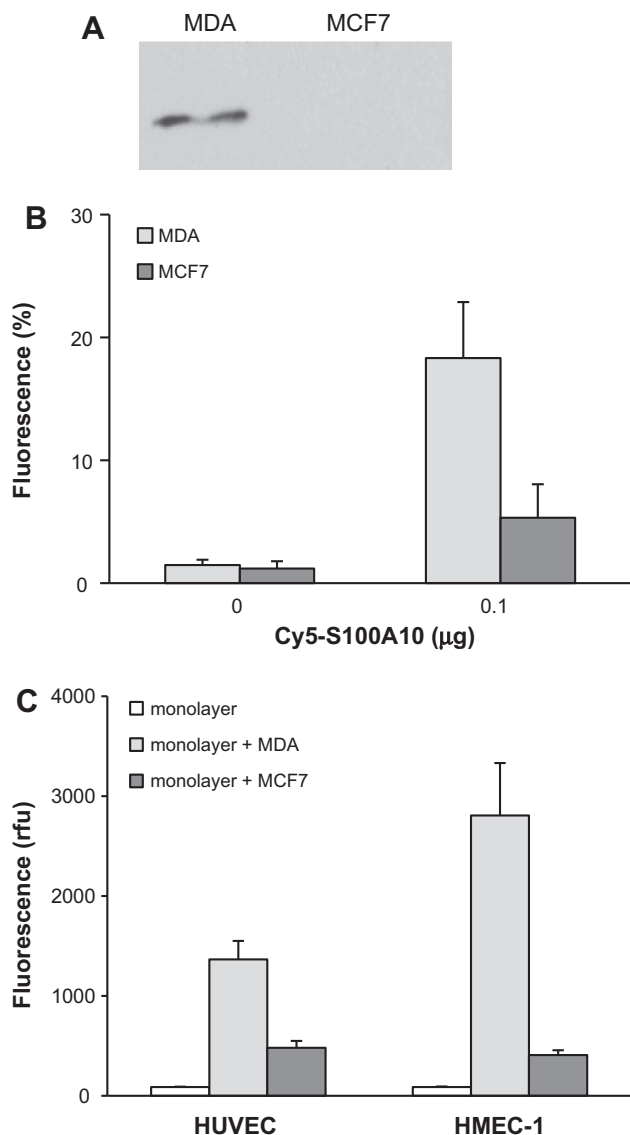
Fig. 2. AnxA2 surface expression and cell–cell interaction. (A) MDA-MB-231 or MCF7 cells were biotinylated with sulfo-NHS-SS-biotin and the isolated surface material was resolved by SDS–PAGE and analysed by Western blot. Blots were probed with an antibody to AnxA2. (B) MDA-MB-231 or MCF7 cells were incubated with 0.1 µg Cy5-labelled S100A10 and binding was measured by flow cytometry as described in methods section. Data shown are the average ± S.E.M. from 3–4 independent experiments, with 20,000 events being counted each time. (C) HUVEC or HMEC-1 were grown to monolayer after which either PBS, or 3×10^4 BCECF-AM-labelled MDA-MB-231 or MCF7 breast cancer cells were added and incubated for 15 min. Cells were then washed three times with PBS and the number of adherent cells was quantified using a fluorescence plate reader. Results are the mean ± S.E.M. from three independent experiments measured in triplicate.

shown). However, it may be due to the difference in the amount of AnxA2 present on the surface of the two breast cancer cell lines (Fig. 2A and B).

Next we investigated the involvement of AnxA2 binding to S100A10 in adhesion of MDA-MB-231 cells to microvascular endothelial cells. To do so, we used the AnxA2(Ac1-14) peptide as competitor, since it can disrupt the interaction between these proteins [32]. The AnxA2(Ac1-14) peptide blocked the adhesion of MDA-MB-231 cells to HMEC-1 monolayers, indicating that binding of AnxA2 to S100A10 indeed contributes to the cell–cell interaction (Fig. 5A). The smaller inhibition of adhesion of MCF7 cells to HMEC-1 cells may reflect the observation that these cells express less AnxA2 (Fig. 2). To exclude that peptide batch effects or non
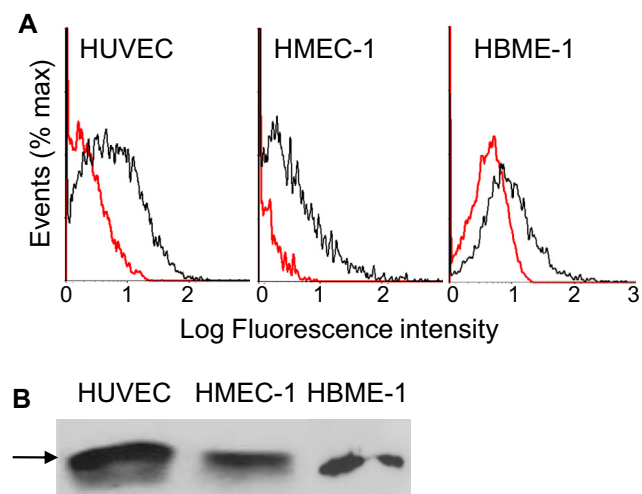
Fig. 3. Surface expression of S100A10. (A) Analysis of S100A10 antibody binding to HUVEC, HMEC-1 or HBME cells. Cells were incubated with Alexa Fluor 488 antibody either alone (red profile) or after incubation with S100A10 primary antibody (black profile). The fluorescence signal of the gated intact cell population is shown. A representative experiment is shown. (B) Three different endothelial cell types were biotinylated with sulfo-NHS-SS-biotin and the isolated surface material was resolved by SDS–PAGE and analysed by Western blot. Blots were probed with an antibody to S100A10.

specific effects caused the inhibition of adhesion, an independently synthesized, differently sourced batch of the AnxA2(Ac1-14) peptide was tested, alongside a scrambled version of the peptide. Adhesion of both MDA-MB-231 cells and MCF7 cells appeared to be inhibited by the scrambled peptide (Fig. S1). Compared to the scrambled peptide, the wildtype peptide inhibited the adhesion of MDA-MB-231 cells to HMEC-1 monolayers by 40%, whereas adhesion of MCF7 cells was not affected (Fig. S2). Thus AnxA2–S100A10 binding contributes to adhesion of MDA-MB-231 cells, but not of MCF7 cells to endothelial cells. Since the adhesion is not fully inhibited by the peptide, other mechanisms also contribute to cell adhesion. An antibody to S100A10, which blocked the binding of Cy3-AnxA2(Ac1-14) to HMEC-1 cells (Fig. 4B), blocks the binding of MDA-MB-231 cells to HMEC-1 cells (Fig. 5B), providing a further argument that surface AnxA2 on MDA-MB-231 cells can interact with S100A10 to mediate cell–cell interaction.

Since HBME-1 cells also express S100A10 on the cell surface (Fig. 3), we investigated whether surface AnxA2 interacting with S100A10 on the surface of HBME-1 cells is involved in cell–cell adhesion. As shown in Fig. S3, MDA-MB-231 cells adhere efficiently to a monolayer of HBME-1 cells whereas MCF7 cells do not. Furthermore, the AnxA2(Ac1-14) peptide inhibits the adhesion of MDA-MB-231 breast cancer cells to HBME-1 cells in a concentration dependent fashion whilst not affecting the low level of adhesion observed for MCF7 cells (Figure S3). Thus the AnxA2–S100A10 molecular complex plays a role in adhesion of breast cancer cells to multiple types of endothelium.

4. Discussion

We show that the binding of surface AnxA2 to surface S100A10 contributes to heterotypic cell–cell interactions between breast tumour cells and microvascular endothelial cells.

Although neither AnxA2 nor S100A10 are classically secreted proteins, their expression on the surface of various cells has been noted. Previous studies indicated the presence of S100A10 on the surface of large vessels [33], but the presence on small vessels was less established. Our studies indicate that S100A10 is present on small dermal endothelial cells as well as bone marrow
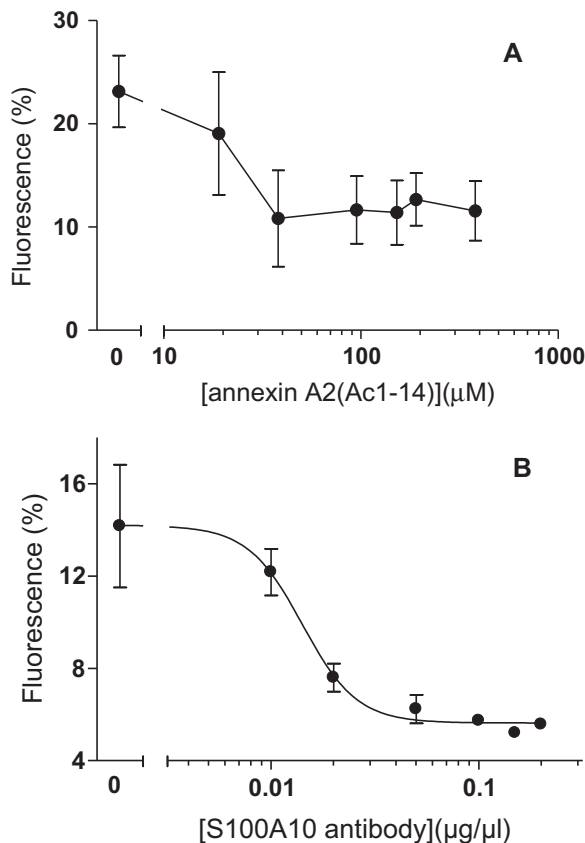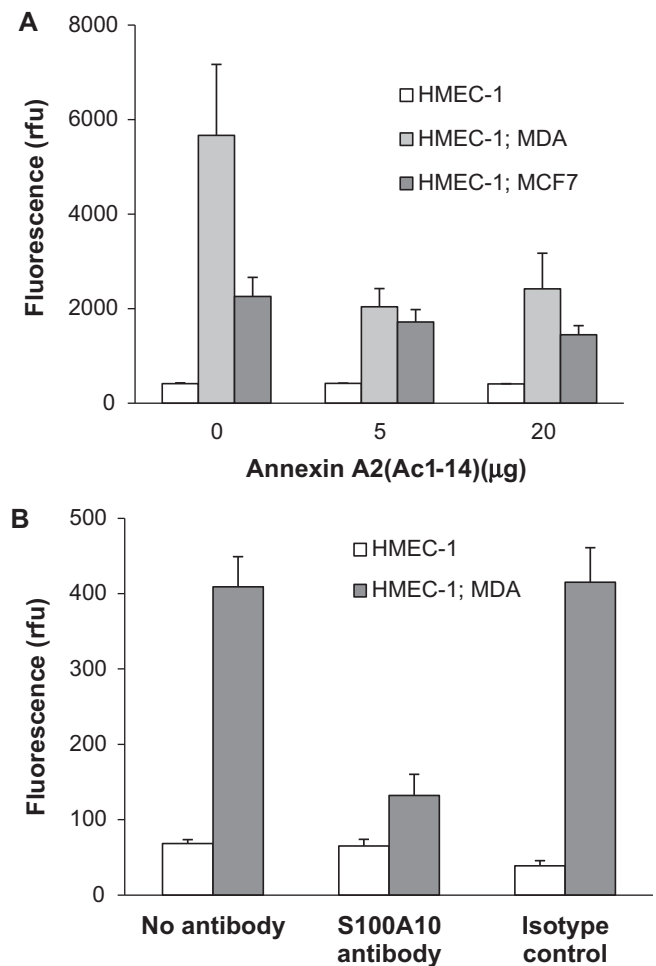
Fig. 4. S100A10-dependent binding of Cy3-labelled AnxA2(Ac1-14) to HMEC-1. (A) Competition of AnxA2(Ac1-14)-Cy3 peptide binding to HMEC-1 cells by non-labelled peptide. 5×10^5 cells were pre-incubated with increasing concentrations of unlabelled peptide (0–50 µg) for 30 min prior to a 15 min incubation with 5 µg Cy3-labelled peptide and binding was measured by flow cytometry. (B) inhibition of AnxA2(Ac1-14)-Cy3 peptide binding to HMEC-1 cells by S100A10 antibody. 5×10^5 cells were incubated with increasing concentrations of S100A10 antibody (0–20 µg) for 30 min and then with 5 µg AnxA2(Ac1-14)-Cy3 peptide for 15 min and binding was measured by flow cytometry. All data point shown are the average ± S.E.M. from three independent experiments, with 20,000 events being counted each time.

Fig. 5. Inhibition of adhesion by AnxA2(Ac1-14) peptide and S100A10 antibody. (A) HMEC-1 were grown to monolayer and treated with the AA2(Ac1-14) peptide (5 µg or 20 µg) for 30 min. Either PBS, or 3×10^4 BCECF-AM-labelled MDA-MB-231 or MCF7 cells were then added to the monolayers and incubated for 15 min and then washed three times with PBS to remove unattached cells. The number of adherent cells was quantified using a fluorescence plate reader. (B) As under A, except that monolayer cells were pretreated with 5 µg IgG1κ isotype control or S100A10 antibody. Data are presented as the mean ± S.E.M. from three independent experiments.

endothelial cells. The conformation of surface S100A10 has not been assessed previously. Using fluorescent tracers developed in our laboratory, we probed the conformation of the protein and we show here that S100A10 is capable of binding AnxA2, suggesting that it is present on the cell surface as a dimer. We provide evidence that an AnxA2 molecule present on an opposing cell, such as a breast cancer cell, can bridge to the endothelial cell by interacting with surface localised S100A10 located on the latter.

Exactly how S100A10 associates with the endothelial cell surface is not known. It may be that S100A10 is in fact tethered to the endothelial membrane via AnxA2, which has known capability of binding to cholesterol [34] and heparin [35]. One molecule of AnxA2 on the cell surface could tether an S100A10 dimer, leaving one free annexin binding site to coordinate annexin on the surface of an opposite cell [7]. Alternatively, S100A10 could form a homo-tetramer, consisting of two dimers with one dimer binding to two surface-localised AnxA2 molecules and the other dimer free to bind two AnxA2 molecules [36]. Such a model is in principle testable by performing competition experiments and measuring release of the proteins into the cell supernatant. Attempts at this using Western blotting of cell supernatants were unsuccessful. Thus at this stage the model is mainly based upon high sensitivity reagents including fluorescence tracers.

It may be argued that the surface expression detected here is non-physiological, for instance being generated during cell

preparation. However, it should be noted that the binding assays employed here relied exclusively on flow cytometry methods, which only measure the intact cell population with any cell debris or small particular material gated out. Furthermore, we showed that the proteins localised on the cell surface are physiological in the sense that they can bind their protein partners, and not merely deposited on cells in disordered fashion. This is the first such analysis of cell surface annexin A2 and S100A10 (previous studies relied mostly on antibody detection). Hence the data support the notion that surface expression of these proteins is physiological. Nevertheless, the alternative scenario mentioned cannot be entirely excluded.

Several known adhesion molecules contribute to breast tumour cell–endothelium interactions, including selectins and cadherin [37]. Indeed the fact that we observed that cell–cell interactions were not 100% inhibited by the annexin A2 peptide indicates that other components also contribute. Changing the relative expression patterns of adhesion molecules by tumour cells forms a step in the acquisition of a metastatic phenotype. The difference in AnxA2 surface expression between MDA-MB-231 and MCF7 cells is of interest and correlates with their metastatic behaviour. Both

cell lines can establish tumours in nude mice. However, tumours derived from MCF7 cells are poorly invasive and rarely metastatic whilst tumours derived from MDA-MB-231 produce metastases in nude and SCID mice [38]. The fact that surface AnxA2 is elevated in metastatic breast cancer cells compared to normal breast epithelium and non-metastatic breast cancer cells [23] (and this study) suggests that it may contribute to this process, possibly via mediating cell–cell interactions. Further research, using a wider range of breast cancer cell lines is needed to firmly establish this notion. The direct interactions with the microvasculature observed here may aid tumour cell intravasation or extravasation during haematogenous dissemination. In vivo assessment is required to provide further confirmation of this idea.

As mentioned in the introduction, surface AnxA2 has been implicated in a number of cell–cell interaction scenarios. The current data are mostly consistent with earlier models of kidney epithelial cell monolayers which were shown to make cell–cell contacts via an AnxA2–S100A10 molecular bridge. Thus an AnxA2–S100A10 molecular bridge may facilitate cell–cell interactions in a range of cellular contexts and changes in the AnxA2-S100 axis may affect the dynamics of tumour–host cell interactions.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.012.

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