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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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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].

Abbreviations: AnxA2, annexin A2; HUVEC, human umbilical vein endothelial cell; HMEC, human microvascular endothelial cell; HBME cells, human bone marrow endothelial cells; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethylester; EGM-2, endothelial growth medium-2

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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, R^3-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 10000 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 cm^2 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