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N-acetylgalactosamine glycans function in cancer cell adhesion to endothelial cells: a role for truncated O-glycans in metastatic mechanisms.

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ABSTRACT

Failure in O-glycan chain extension exposing Tn antigen (GalNAc-O-Ser/Thr) is clinically associated with cancer metastasis. This study provides evidence of a functional role for aberrant GalNAc-glycans in cancer cell capture from blood flow and / or adhesion to endothelium. Adhesion of breast cancer cells to human umbilical vein endothelial cell monolayers was modelled under sweeping flow. Adhesion of metastatic, GalNAc glycan-rich, MCF7 and ZR 75 1 cells to endothelium increased over timepoints up to 1.5 hour, after which it plateaued. Adhesion was significantly inhibited ($p < 0.001$) when cell surface GalNAc-glycans were masked, an effect not seen in GalNAc glycan-poor, non-metastatic BT 474 cells. Masking irrelevant galactose- and mannose-glycans had no inhibitory effect. Imaging of cells post-adhesion over a 24 hour time course using confocal and scanning electron microscopy revealed that up to 6 hours post-adhesion, motile, rounded cancer cells featuring lamellipodia-like processes crawled on an intact endothelial monolayer. From 6-12 hours post-adhesion, cancer cells became stationary, adopted a smooth, circular flattened morphology, and endothelial cells retracted from around them leaving cleared zones in which the cancer cells proceeded to form colonies through cell division.

Keywords *Helix pomatia* agglutinin , Tn antigen, glycosylation, metastasis, adhesion

Abbreviations: GalNAc - N-acetylgalactosamine; HPA - *Helix pomatia* agglutinin; HUVEC - human umbilical vein endothelial cells; PBS - phosphate buffered saline; EDTA - ethylenediaminetetraacetic acid; TNF- α - tumour necrosis factor- α ; HPTS - 8-hydroxypyrenetrisulphonic acid; PNA -peanut agglutinin; Con A - concanavalin A; SEM - scanning electron microscopy; IL-1 - interleukin-1; IL-6 - interleukin 6; ICAM-1 -intercellular adhesion molecule-1; GM1 - monosialotetrahexosylganglioside; MMP9 - matrix metalloproteinase 9

1. INTRODUCTION

Altered glycosylation has frequently been reported in cancer cells, and in some instances is associated with increased potential for invasion and metastasis (reviewed by [1]). An increase in exposure of N-acetylgalactosamine (GalNAc) glycans, which can be detected by binding of the lectin from *Helix pomatia* (HPA, *Helix pomatia* agglutinin), has been reported to be associated in clinical studies with poor patient prognosis and metastatic competence in a range of cancers, including those of breast [2-9], oesophagus [10], stomach [11,12], colorectum [13], lung [14], prostate [15], thyroid [16] and malignant melanoma [17]. HPA recognises heterogeneous O-linked glycans on cancer cells, including, but not exclusively, the cancer-specific Tn-antigen, GalNAc-O-Ser/Thr [18], which itself has been consistently reported as being associated with metastasis and poor prognosis in several cancer types, and has received interest as a target for vaccine development (reviewed by [19,20]).

Although metastasis is the commonest cause of death in cancer patients, owing to its complexity, it remains incompletely understood. The metastatically successful cancer cell is rare because it needs to successfully accomplish a number of steps, each requiring different behavioural characteristics supported by complex molecular signatures. These steps include (1) induction of angiogenesis to support the needs of the growing tumour (2) motility and invasion of stroma (3) intravasation into lymphatic system or blood circulatory system (4) adhesive interaction with the endothelial lining of blood vessels or lymphatics (5) and extravasation and establishment of a new tumour at a distant site (reviewed by [21,22]). It is clearly not possible to study the metastatic cascade in human subjects *in vivo* and animal models have provided some insight into the process, although they have well-recognised limitations. The altered GalNAc-glycotype recognised by HPA is associated with increased metastatic potential in animal models of several cancer types (eg [23-25]). *In vitro* models of defined aspects of metastatic mechanisms can be helpful in elucidating parts of the process in detail.

Previously, we have reported that GalNAc-glycans are not functionally involved in cancer cell adhesion to, or invasion through, basement membrane [26]. In the study reported here we investigate the potential functional role of GalNAc-glycans in cancer cell adhesive interactions with endothelium during hematogenous dissemination. While mechanical entrapment of tumour cells undoubtedly accounts for their arrest in small

vessels, there is evidence of specific adhesive interactions between cancer cells and endothelium that contributes to the distinctive organ-specific pattern of metastatic spread in many types of cancer [27-30]. Thus, we focus on cancer cell interactions with endothelial cells under conditions of sweeping flow mimicking that encountered in blood circulation. Assays were performed using the breast cancer epithelial cell lines BT 474, ZR 75 1 and MCF7. We have previously thoroughly characterised these cells for their synthesis of HPA-binding glycans of interest [26,31,32]. As summarised in Table 1, BT 474 was derived from a primary breast cancer and stably synthesises negligible levels of HPA-binding GalNAc glycans. MCF7 and ZR 75 1 were derived from breast cancer metastases, are tumorigenic in animal models, and HPA-binding GalNAc glycan-rich. Furthermore, they stably synthesise a profile of HPA-binding glycoproteins that is shared by clinical breast tumours [26,31] thus validating their use in studies investigating this feature of aberrant glycosylation in cancer.

2. MATERIALS & METHODS

2.1 Cells and cell culture

BT 474 and ZR 75 1 were obtained from the European Collection of Cell Cultures and MCF7 were a gift from Dr. Jostein Dahle (Oslo University, Norway). Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza, UK. Details of culture media for each cell type is given in Table 2.

All assays were performed using breast cancer cells from within five consecutive passages and HUVEC from within three passages to minimise phenotypic variation. All plastics were pre-coated in 0.2% w/v bovine gelatin (Sigma Aldrich Co. Ltd., UK) in phosphate buffered saline (PBS), pH 7.4. Adherent cell monolayers were harvested for passaging and for assays using, for HUVEC, 0.025%-trypsin-0.02% EDTA (Gibco, UK) and for breast cancer cell lines 0.05%-trypsin-0.02% EDTA (Gibco, UK). Cultures were maintained under 5% CO₂ / air at 37°C.

2.2 Cancer cell-endothelial cell adhesive interactions under conditions of sweeping flow:

2.2.1 Quantifying MCF7 and ZR 75 1 cancer cell adhesion to endothelial cell monolayers over time

HUVEC were grown to confluence in 35mm confocal microscope imaging-compatible ibidi dishes (Thistle Scientific, UK) pre-coated in 0.2% v/v bovine gelatin. They were stimulated with 10ng/ml TNF- α (Sigma Aldrich Co. Ltd., UK) in EBM-2 media for 2 hours prior to the assay. At the same time, the cells from the cell lines originally derived from breast cancer metastases, MCF7 and ZR 75 1, were labelled overnight prior to the assay in 10mg/ml solution of fluorescent 8-hydroxypyrenetrisulphonic acid (HPTS; Sigma Aldrich Co. Ltd., UK). Unincorporated HPTS was aspirated and the cells washed in 5 changes of PBS before trypsinising, as described previously, to obtain a cell suspension. 1ml of cell suspension containing 2×10^5 HPTS-labelled breast cancer cells was introduced to each HUVEC monolayer and the dishes rocked gently on a platform with a north-south / east-west rocking motion for time periods of 5 mins; 15 mins; 30 mins; 45 mins; 1 hr; 1.5 hr and 2 hr. At the end of the rocking period, the HUVEC monolayer was gently washed in 3 changes of PBS to remove any non-adherent breast cancer cells and imaged using a Zeiss confocal microscope using a 10x plan Neofluar objective and 458nm laser and 475 LP filter. Two technical replicates and three biological replicates were performed for each assay. For each assay dish, 15 randomly chosen images were captured, giving a total of 90 images per time point for each cell line. Adherent HPTS-labelled cancer cells were enumerated.

2.2.2 Exploring the effect of masking cancer cell surface glycans on MCF7, ZR 75 1 and BT 474 cancer cell adhesion to endothelium

To determine the effect of masking cell surface GalNAc-glycans on cancer cell adhesion to endothelial cell monolayers, BT 474, ZR 75 1 and MCF7 cancer cell suspensions were prepared and HPTS-labelled as described previously then incubated with 10 μ g/ml solution of the GalNAc-binding lectin HPA. In order to control for the potential effect of steric hindrance from the relatively large HPA molecule, as well as for the effects of blocking GalNAc-glycans specifically, cells were also incubated with peanut agglutinin (PNA) or concanavalin A (Con A) (all from Sigma Aldrich Co. Ltd., UK) in culture medium, or incubated with culture medium alone (positive control), for 2 hours to achieve lectin binding to the cells. The carbohydrate-binding preferences of the lectins are summarised in Table 3. Cells were washed thoroughly to remove unbound lectin before proceeding. 1ml of cell suspension containing 2×10^5 HPTS-labelled cells were then applied to a HUVEC monolayer and the dishes rocked gently, as described previously, for 1.5 hr. Each test consisted of 4 dishes, one each for positive control (no lectin), plus HPA, PNA and Con A groups, and three technical replicates of each were performed. Images of 20 randomly chosen fields were captured per dish, totalling 60 images per group for each cell line tested.

Adherent HPTS-labelled cancer cells were enumerated and the number of adherent cancer cells in the positive control (no lectin masking) in comparison to HPA, PNA and Con A glycan-masking groups were analysed.

Data were analysed using a Shapiro-Wilks test for normal distribution. A T-test was applied to normally distributed data and a non-parametric Wilcoxon signed ranks test was applied to data that was not normally distributed. p values of <0.05 were considered to be statistically significant.

2.3 Imaging post-adhesion MCF7 cancer cell behaviour

2.3.1 24 hr time course, live cell imaging

Confluent HUVEC monolayers were established in a 6-well confocal microscope-compatible plate (Wafergen Biosystems UK 'smartslide system'). HPTS-labelled MCF7 cells, were introduced on the endothelial cell monolayer and allowed to interact under conditions of sweeping flow, for 1.5 hr, as described previously. Non-adherent cells were removed by gentle washing in 3 changes of PBS and then 5mls of fresh EBM 2 media was added. The plate was introduced into a micro-incubator system (Wafergen Biosystems UK 'smartslide system') mounted on the stage of Zeiss confocal microscope and cells maintained at 37⁰c in 5% CO₂ / air. A single, representative field of view was selected and cells were imaged using a 10x plan Neofluar objective and 458nm laser and 475 LP filter every 5 mins for 24 hr.

2.3.2 24 hr time course imaged using scanning electron microscopy

Confluent HUVEC monolayers were established on plastic coverslips placed within 24 well plates. Unlabelled MCF7 cells were introduced on the endothelial cell monolayer and allowed to interact under conditions of sweeping flow, for 1.5 hr, as described previously. Non-adherent cells were removed by gentle washing in 3 changes of PBS and then 5mls of fresh EBM 2 media was added. The plates were then returned to a 37⁰c incubator and maintained under 5% CO₂ / air. Coverslips were removed at time points: 30 mins, 1 hr, 2 hr, 6 hr, 12 hr and 24 hr and processed for imaging for scanning electron microscopy (SEM) as follows. Cells were fixed with 2% v/v glutaraldehyde (TAAB, U.K) and 2% v/v paraformaldehyde (Agar Scientific) in 0.1M sodium cacodylate buffer (Agar Scientific) at pH 7.4 for 1 hr. They were then washed in 1M sodium cacodylate buffer,

then incubated in 1% v/v osmium tetroxide (Agar Scientific) in distilled water for 1 hr. Cells were then washed three times in 1M sodium cacodylate buffer to remove excess osmium tetroxide. They were then dehydrated through a series of graded ethanol solutions and dried in a critical point dryer (Tousimis, Samdri-780). They were sputter coated with gold using a conductive metallic sputter coater (Agar auto sputter coater) and imaged using a Hitachi 3400N scanning electron microscope.

3. RESULTS

3.1 Cancer cell-endothelial cell adhesive interactions under conditions of sweeping flow:

3.1.1 Quantifying MCF7 and ZR 75 1 cancer cell adhesion to endothelial cell monolayers over time

Adhesion of MCF7 and ZR 75 1 cells, originally derived from cancer metastases, to endothelial monolayers under conditions of gentle sweeping flow was quantified over time. Results are summarised in Table 4 and illustrated in Figure 1. There was minimal adhesion of cancer cells to endothelial monolayers at early time points, 5-15 min, but numbers of adherent cancer cells gradually increased from 30 min until the 1 hr 30min time point after which no further increase in numbers of adherent cells was seen. The endothelial monolayer remained intact over this time frame with no evidence of cell loss, damage or detachment.

3.1.2 Exploring the effect of masking cancer cell surface glycans on MCF7, ZR 75 1 and BT 474 cancer cell adhesion to endothelium

Physically masking the GalNAc glycans by pre-incubation of the cells with HPA resulted in a significant decrease in the adhesion of GalNAc glycan-rich MCF7 and ZR 75 1 cancer cells to the endothelial cell monolayer ($p < 0.0001$). Pre-incubating the GalNAc glycan-poor BT 474 cells with HPA had, predictably, no significant effect on their adhesion. Moreover, pre-incubation of the three cell lines with PNA to mask galactose-glycans or Con A to mask mannose-glycans, and the steric effect of their presence, had no significant effect on cell adhesion.

These results are summarised in Table 5 and illustrated in Figure 2. Pre-incubation of the cells with lectins had no detectable adverse effects on their viability or morphology (data not shown).

3.2 Imaging post-adhesion MCF7 cancer cell behaviour

3.2.1 24 hr time course, live cell imaging

Live cell imaging of cancer cell interactions with endothelial monolayers for up to 24 hr post-adhesion revealed that at early time points, up to 12 hr post adhesion, the adherent cancer cells exhibited a rounded morphology and were motile, moving on the endothelial monolayer. Towards the end of this 12 hr time frame, cancer cells settled, ceased to move, and their morphology changed to a markedly flattened appearance. During the time frame 12-24 hr post-adhesion, endothelial cells retracted away from the adherent, now stationary cancer cells, forming a cleared zone. Cancer cells then proceeded to undergo cell division within the cleared zone forming small colonies by the end of the imaging period. These observations are illustrated in Figure 3 and in Movie 1.

(for print version the last sentence of the above paragraph should read 'These observations are illustrated in Figure 3')

3.2.2 24 hr time course imaged using scanning electron microscopy

Observations recorded using live cell imaging were supported by SEM. At early time points (1-6 hr), adherent cancer cells were roughly spherical but many exhibited processes resembling lamellipodia consistent with their crawling along the endothelial monolayer. The endothelial monolayer resembled an unbroken and continuous pavement (Figure 4A and B). By 6-12 hr post-adhesion, endothelial cells had begun to retract from the cancer cells forming a cleared zone from around them (Figure 4 C and D). Cancer cells adopted a circular, flattened morphology with minimal evidence of cell processes (Figure 4D). By 12 hr post-adhesion, cancer cells had settled into the cleared areas and cell division was imaged (Figure 4E). Transcellular migration was also imaged (Figure 4F).

4. DISCUSSION

In the study reported here, we employed an assay involving sweeping flow to model interactions where cancer cells are moving in the blood stream. This type of assay is commonly used to investigate the interactions between leukocytes and endothelial monolayers during the inflammatory response, which has frequently been proposed as a paradigm of cancer cell adhesion and extravasation during metastasis. A rocking platform with a north-south/east-west direction of motion avoided vortices that are induced by circular rocking motion. Prior to the assays, the endothelial cells were stimulated with TNF- α as we have previously demonstrated that this increases the rates of cancer cells adhesion to endothelial cells markedly, indicating that molecules that are up-regulated by TNF- α may be involved in adhesive interactions [25].

Initial time series experiments indicated that there was little cancer cell adhesion to the endothelial monolayer at early time points, up to 30 mins. From 30 mins to 1 hr 30mins, adhesion increased steadily, then plateaued. The endothelial monolayer remained intact and apparently undamaged during the course of the assays. Mechano-stimulation of the endothelial cells by the sweeping flow may contribute to the gradual increase in cell adhesion as shear forces of flow have been demonstrated to up-regulate molecules potentially involved in cancer cell-endothelial cell interaction and adhesion, including IL-1 and IL-6, and mediate adhesive behaviour [30]. It has also been reported that ICAM-1 expression is induced by shear stress in HUVEC monolayers [42]. There was no significant further adhesion after 1hr 30 mins and prolonged assays under flow resulted in the endothelial monolayer becoming damaged and detached (data not shown).

Masking exposed GalNAc-glycans by pre-incubating cancer cells with the GalNAc-binding lectin HPA had a marked significant inhibitory effect on the MCF7 and ZR 75 1 cells' ability to adhere to the endothelial monolayers under conditions of flow. Unsurprisingly, no inhibitory effect was seen after pre-incubating the GalNAc-glycan poor BT 474 cells. Masking of, in this context, irrelevant (ie non-GalNAc-) galactose- and mannose-glycans by lectins PNA and Con A, respectively, also had no inhibitory effect, even though both lectins recognise binding partners on breast cancer cells which have, like HPA, been reported to have some prognostic significance (eg [43-47]). Moreover, that the observed inhibition in cancer cell adhesion to endothelium was as a result of specifically masking GalNAc-glycans, and not of steric hindrance in the presence bound HPA, is exemplified by the lack of inhibition in the presence of either bound PNA or Con A. The

hexameric (actually, a non-covalent trimer of covalent dimers) HPA molecule has a reported molecular weight of 79 kDa, although it occurs in different glycoforms with slightly variable molecular weights [36,48]. Molecular weights of PNA and Con A are comparable to this. The tetrameric PNA molecule has a molecular weight of 100.8 kDa [49]. There has been controversy in the literature as to the molecular weight of Con A, but at the pH employed in the experiments reported here, it is reported to exist as a mixture of monomers of molecular weight 53kDa and also dimers composed of these subunits [50]. In addition to the evidence reported here supporting a functional role for GalNAc-glycans in cancer cell adhesion to endothelium, inhibition of adhesion of GalNAc-rich cancer cells to endothelial monolayers was also observed in the presence of free GalNAc. However, the presence of monosaccharide had a detrimental effect on the endothelial monolayer, causing it to become partially detached from the plasticware and thus rendering analysis unreliable (data not shown) and further optimisation of the assay, resulting in the approach reported, here was therefore necessary.

These data, therefore, are consistent with HPA-binding GalNAc-glycans having a functional role in cancer cell adhesion to endothelium, although the GalNAc glycan-poor BT 474 cells were also able to adhere to endothelial monolayers, presumably through alternative mechanisms. While both HPA-binding and detection of Tn antigen have been much reported in the literature to be associated with aggressive biological behaviour and poor patient prognosis in a range of cancer types, as described previously, the functional reason for this has not previously been apparent. Intriguingly, the results presented here are consistent with a report [19] of an anti-Tn monoclonal antibody not only specifically recognising Tn antigen on cancer cells, including MCF7 cell tumours in a mouse model, but also inhibiting adhesion of MCF7 cells to lymphatic endothelial cells, thus functionally implicating GalNAc-glycans in lymphatic as well as blood-borne dissemination.

While there is ample evidence that cancer cells become physically trapped in small-bore capillaries during hematogenous dissemination (eg [51,52]), capture of cancer cells from blood flow also contributes to metastatic mechanisms [27-30], as modelled here. This process has been shown to involve similar adhesive interactions as those involved in the well-studied leukocyte capture from blood flow. In leukocyte adhesion to endothelium under conditions of flow, there is initial recognition of glycan Lewis a and Lewis x antigens by E-selectin expressed by activated endothelial cells (reviewed by [53]). There is no evidence that GalNAc-glycans can act as binding partners for selectins. Indeed, we have observed that while cancer cells are able to roll on

both HUVEC monolayers and recombinant E-selectin, but not P-selectin, coated microslides in a manner reminiscent of that of leukocytes, this behaviour is not inhibited by blocking the exposure of GalNAc glycans (unpublished observation). Moreover, there is evidence confirming this in a scid mouse colorectal cancer model [54]. Here, the labelling distribution of HPA for GalNAc-glycans and of selectins to the cancer cells, while somewhat overlapping, were distinct and, possibly, complementary. It seems likely, therefore, that the GalNAc glycans are functionally implicated in later, firm adhesion once initial selectin-mediated tethering has been achieved, or under circumstances where cancer cells are slowed or trapped in vessels and come into close contact with the endothelial wall. In the assay system employed in the current study, while the cancer cells were subject to constant sweeping flow, they also had an extended time period in which to interact with the endothelial monolayer with which they would inevitably come into repeated, close contact. While there has been greater focus on T-antigen and sialyl-T antigen than on Tn antigen in this context, it has been reported that cancer-associated truncated O-linked glycan structures like these promote metastasis through their recognition by galectin-3 expressed on other cancer cells, thereby causing homotypic aggregation of cancer cells and thus promoting metastasis, or on endothelial cells thus facilitating adhesion to endothelium [55-57], thus exemplifying the potential role of glycan recognition in metastatic mechanisms. Intriguingly, the galectin-3 is also reported to regulate accumulation of GM1 and N-cadherin at cell-cell junctions, thus destabilising them and promoting cancer cell migration [58]. Integrins appear to be key players in the development of the later, stable and firm adhesive attachments [59] and here an increase in the synthesis of trimeric Tn antigen on syndecan 1 in complex with alpha 5 beta 1 integrin and MMP9 has recently been demonstrated to enhance invasion and metastasis in a murine Lewis lung cancer model [60], consistent with GalNAc-glycans functioning here.

Imaging events over a 24 hr post-adhesion time course using both live cell imaging and SEM revealed that, initially, cancer cells exhibiting a rounded morphology crawled on the endothelial monolayer by extending cellular processes. Recently it has been demonstrated that in cells where Tn antigen density was increased by relocating the relevant glycosyltransferases to the endoplasmic reticulum rather than Golgi apparatus, Tn antigen density was especially enhanced in the lamellipodia [61]. This was associated with enhanced motility and invasiveness. Thus, in addition to a putative functional role in cancer cell capture from blood flow and / or securing cancer cell adhesion to the endothelium, GalNAc glycans may also function in cancer cell crawling on

the endothelial monolayer and subsequent invasion into local tissues. In the study reported here, after crawling on the endothelial monolayer, from around 6 hr, cells became stationary and the endothelial cells around them retracted to form clear zones. Evidence of transcellular migration was also seen. This behaviour is entirely consistent with that reported in leukocytes during transendothelial migration. Here, after leukocytes are captured from circulation, they migrate to endothelial cell borders and, through interaction of a bewildering array of signalling molecules, well reviewed, for example, by [62,63], cause the endothelial cells to retract facilitating diapedesis. There has also been much interest in transcellular migration of leukocytes through endothelia (eg [64,65]). Clearly, the similarities between leukocyte and cancer cell capture and transmigration are striking. However, leukocyte capture from blood flow and subsequent diapedesis is rapid, taking less than 1 min. Here, once cancer cells were adherent to endothelium, their movement over the endothelial monolayer and eventual retraction of endothelial cells took place over a longer time scale, reflecting the cancer cells limited ability to mobilise the same molecular players. These observations are in accordance with the studies reporting in detail the steps, and chronology, in arrest and extravasation of several cancer cell lines in an *in vivo* mouse model [51,52]. Tumour cells arrested in vessels and remained, within a platelet thrombus, in close contact with the vessel wall for 8-24 hr before the endothelial cells retracted to facilitate their firmer and stable attachment to subendothelial matrix. In the study reported here, from 6-12 hr, cancer cell division was observed such that small cancer cell colonies were formed. This is consistent with observations [51,52 and references therein] of cell division after 4-24 hr following arrest in the capillaries, depending on cancer cell type. There is strong evidence that metastases arise as a result of such intravascular proliferation of cancer cells adherent to endothelium even without active extravasation [66]. Cancer cell proliferation within the lumen of the capillary eventually results in its disruption and cancer invasion of local tissues. Thus, while the simple assay system employed here is limited in that it was not designed to model extravasation and invasion through extracellular matrix, its relevance in exploring cancer cell-endothelial cell molecular interactions remains.

While metastasis is a complex multi-step cascade of events, the focus of this study, that of cancer cell arrest within blood vessels and subsequent transendothelial migration to form tumour cell colonies at a new site is of special interest. The study reported here provides evidence for the first time that altered glycosylation, featuring GalNAc-glycans including the Tn antigen, repeatedly reported to be associated with metastasis and

poor prognosis in a range of cancers, function in specific adhesive interactions between cancer cells and endothelium.

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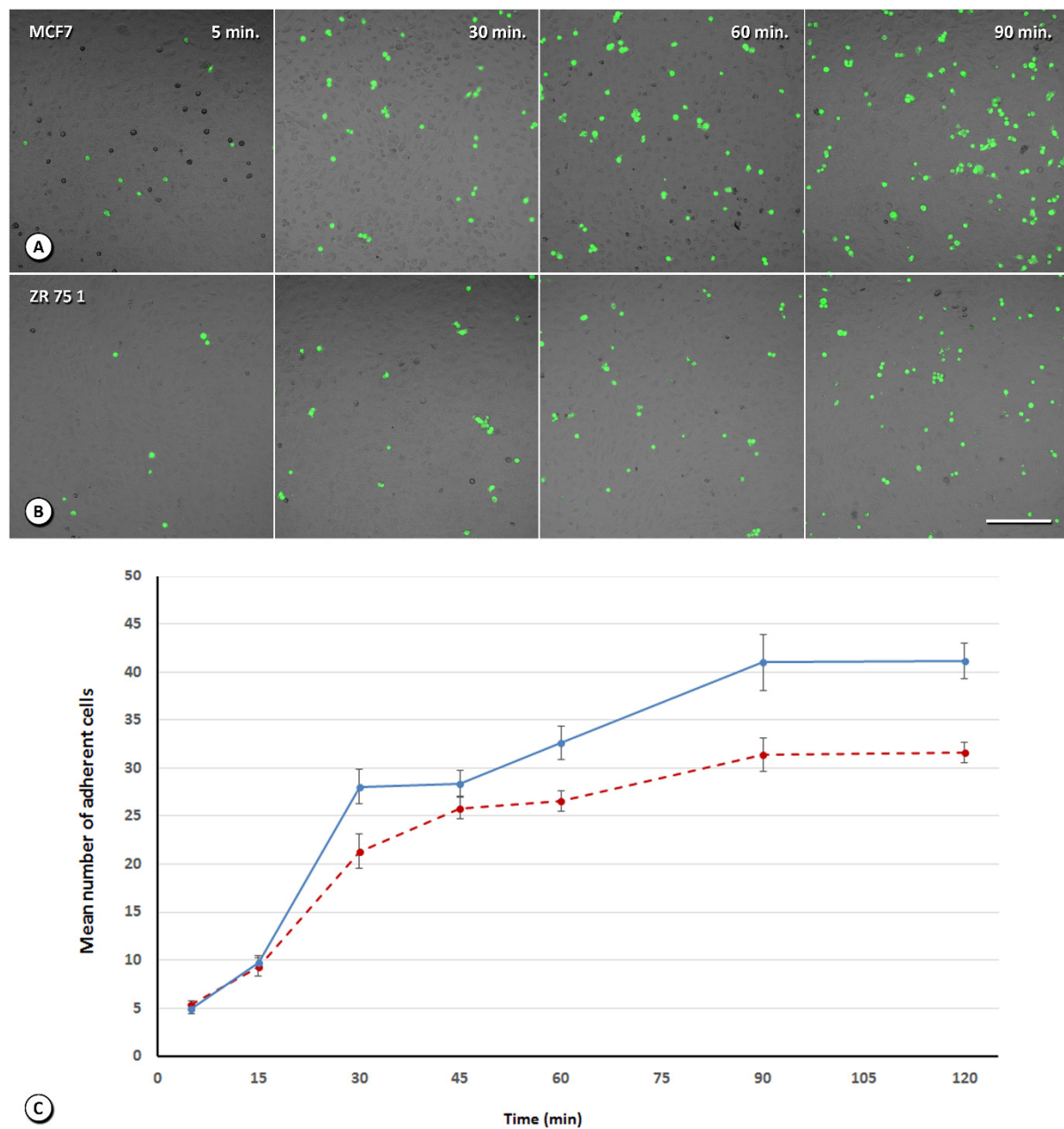


Figure 1. (A) MCF7 and (B) ZR 75 1 cancer cells (green) adhering to the endothelial cell layer at various times after seeding (5, 30, 60 and 90 mins). Scale bar = 250 μ m. (C) Number \pm SEM of adherent MCF7 (blue solid line) and ZR 75 1 (red dashed line) cells to the endothelial cell layer over time.

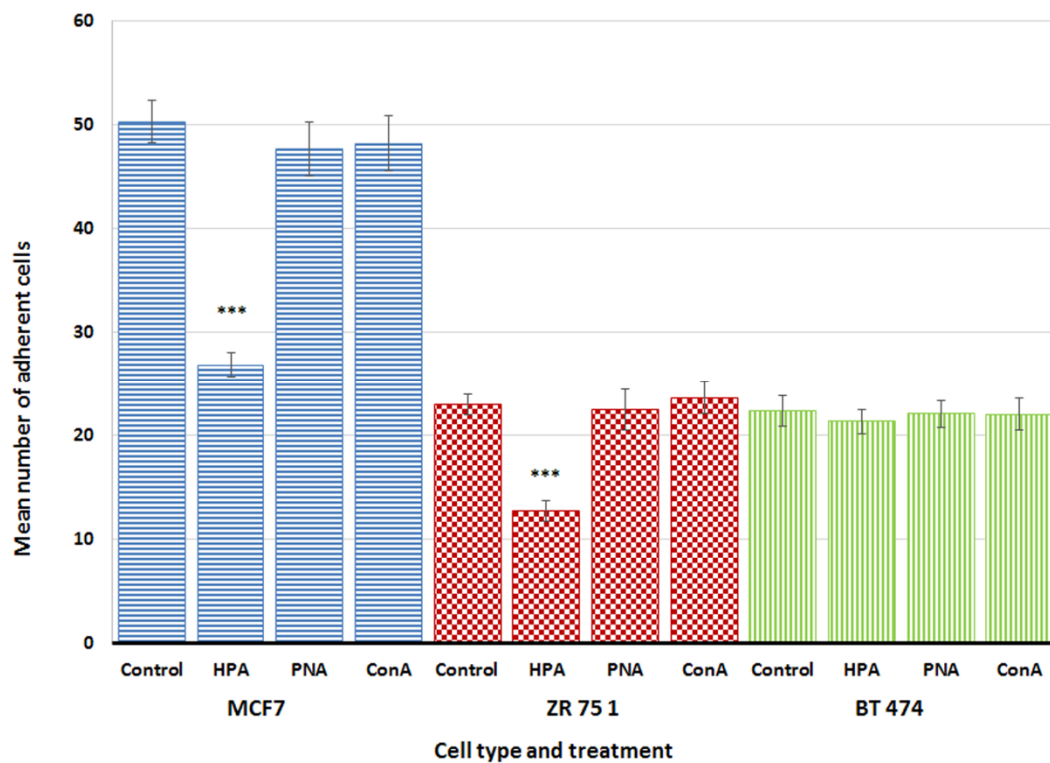


Figure 2. The effect of masking surface glycans on MCF7 (blue horizontally striped bars), ZR 75 1 (red checkerboard bars) and BT 474 (green vertically striped bars) cancer cell adhesion to endothelium. Mean \pm SEM numbers of adherent MCF7, ZR 75 1, and BT 474 cells to endothelial cell layer after GalNAc glycans are masked by HPA, galactose glycans are masked by PNA, mannose glycans are masked by ConA, or with no lectin masking (control). Masking GalNAc glycans by HPA significantly reduces MCF7 and ZR 75 1 cancer cell adhesion to endothelium relative to controls of the same cell type and relative to glycan masking using PNA or ConA (***) $p < 0.001$).

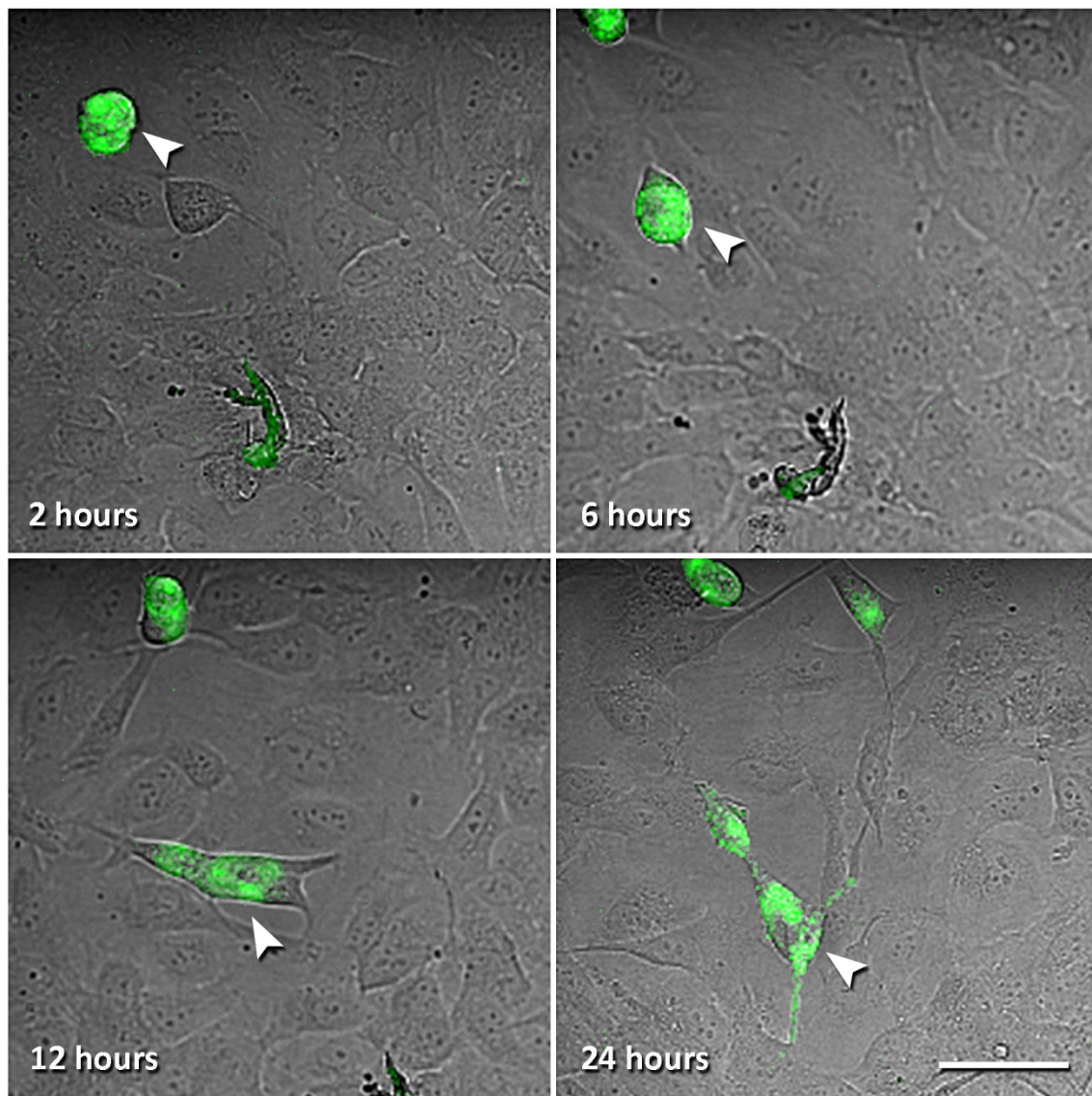


Figure 3. Confocal microscopy live cell imaging timelapse of MCF7 cancer cells (green) interacting with endothelial cell monolayer. MCF7 cancer cells were allowed to adhere to the endothelial cell monolayer under conditions of sweeping flow for 1.5 hr. Then, non-adherent cells were eluted and adherent cells imaged for up to 24hr. The arrowhead marks progress of a single cell during the time course. At early time points, up to 12 hr, cancer cells were motile. Note position of arrowed cell moving from 2 hr, to 6 hr, to 12 hr. From 12 hr, cancer cells settled, lost their motility and flattened. Note the change in the morphology of the arrowed cancer cell at 12 hr. By 24 hr, endothelial cells have retracted away from stationary cancer cells, leaving a cleared zone, and cancer cells begin to undergo cell division. Scale bar = 50 μ m.

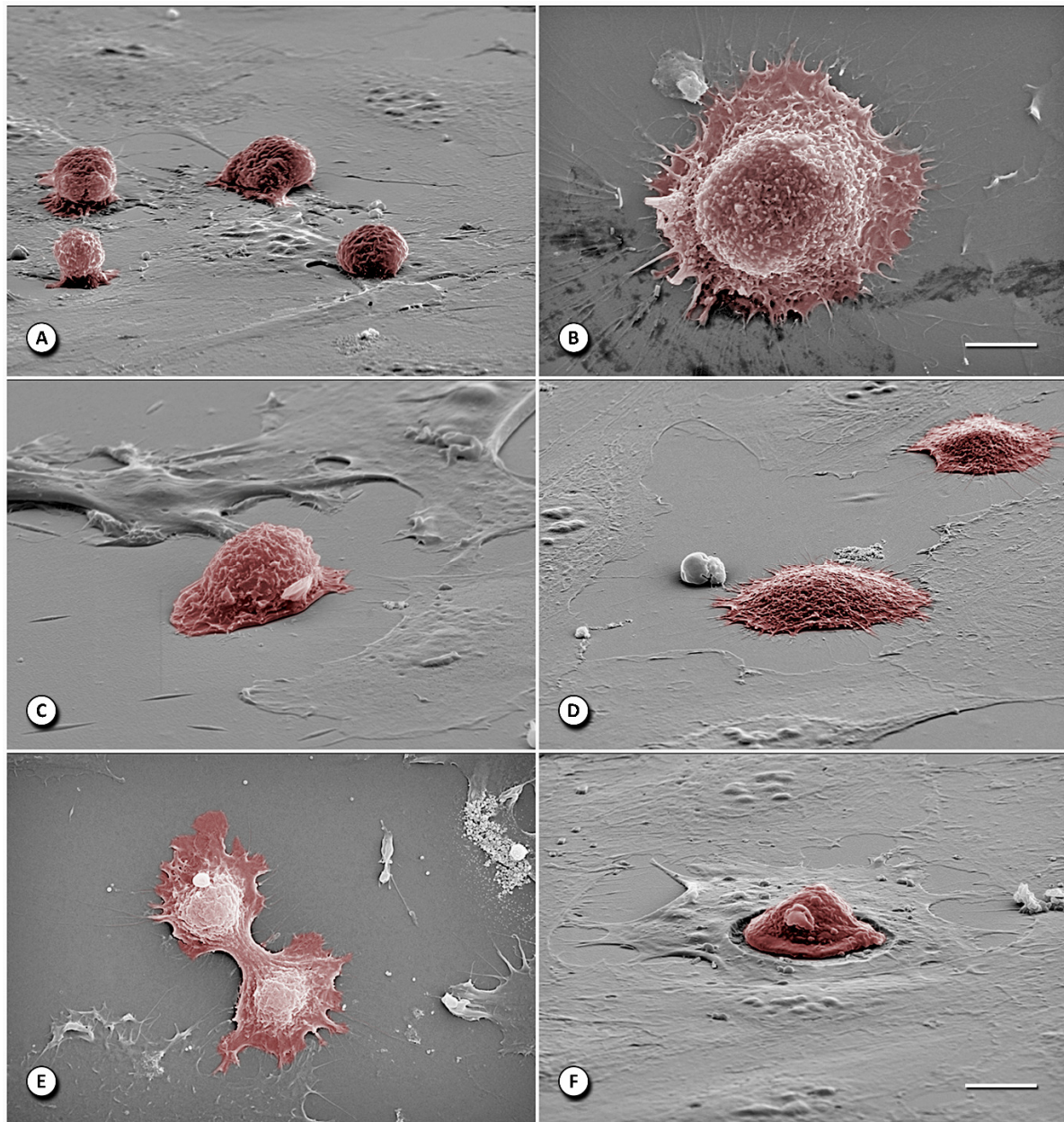


Figure 4. Scanning electron micrograph timelapse of MCF7 cancer cells (red) interacting with endothelial cell monolayer. MCF7 cancer cells were allowed to adhere to the endothelial cell monolayer under conditions of sweeping flow for 1.5 hr. Then, non-adherent cells were eluted and adherent cells imaged at time points up to 24 hr later. A) 1 hr. Cancer cells are adherent to a continuous endothelial monolayer and exhibit a rounded morphology a rounded appearance. B) 2 hr. Cancer cells extend processes consistent with their crawling on the endothelial monolayer. C) 6 hr. Endothelial cells have retracted from around cancer cells leaving a cleared zone. D) 12 hr. Cancer cells are flattened and adherent in the gap between retracted endothelial cells. E) 12 hr Cancer cell division within cleared areas in the endothelial monolayer. F) Transcellular migration. Scale bar panels A, and C-f = 25 μ m. Scale bar B = 10 μ m.

Table 1 Derivation and level of synthesis of aberrant GalNAc-glycans of cell lines employed in this study

Cell line	Derivation & description	Synthesis of aberrant GalNAc-glycans*
BT 474	Derived from a primary, invasive, ductal carcinoma of the breast [33]	Negligible [26,31]
ZR 751	Derived from malignant ascites arising from a primary invasive ductal carcinoma. Tumorigenic in nude mice in the presence of oestrogen [34].	Moderately strong. Expresses a heterogeneous profile of HPA-binding glycoproteins shared by human clinical tumour samples**[26,31]
MCF7	Derived from malignant pleural effusion arising from a primary invasive ductal carcinoma. Oestrogen receptor positive. Tumorigenic in nude mice; tumorigenic and metastatic in scid mice models [35]	Very strong. Expresses a heterogeneous profile of HPA-binding glycoproteins shared by human clinical tumour samples**[25,26,31]

*as assessed by HPA binding profile based on lectin histochemistry & flow cytometry. ** as assessed by Western blot

Table 2 Cell culture media

Cell type	Culture media	Supplements
BT 474	IMDM media (PAA, UK)	10% v/v heat inactivated fetal calf serum (Sigma Aldrich Co. Ltd., UK), 2mM L-glutamine (Gibco, UK), 1% v/v streptomycin and penicillin (Sigma Aldrich Co. Ltd., UK).
ZR 75 1	RPMI-1640 media (Gibco, UK)	as BT 474
MCF7	DMEM:F-12 media (Gibco, UK)	as BT 474
HUVEC	EBM-2 media (Clonetics, Lonza, UK)	Supplement pack (Clonetics, Lonza, UK) contains: hydrocortisone, human fibroblast derived growth factor, vascular endothelial growth factor, R3-insulin derived growth factor, ascorbic acid, heparin, human epidermal growth factor, fetal calf serum and gentamycin and amphotericin

Table 3 Lectin carbohydrate-binding partners

Lectin	Carbohydrate binding partners
HPA (Helix pomatia agglutinin)	GalNAc- α -1,3GalNAc; α -GalNAc; α -GlcNAc [36,37]
PNA (Peanut agglutinin)	Gal(B1→3)GalNAc; D-Gal; Gal(B1→3)GalNAc-Ser/Thr (T antigen)[38,39]
Con A (concanavalin A)	α -mannose; glucose; trimannosyl core of N-glycans [40,41]

Table 4 Quantification of (a) MCF7 and (b) ZR 75 1 cells adherent to HUVEC over time

Time points	5 min	15 min	30 min	45 min	1 hr	1.5 hr	2 hr
(a) MCF7 cells							
Total no. adherent cells	440	878	2524	2556	2937	3689	3706
Mean no adherent cells	5	10	28	28	33	41	41
/ field of view (n=90) ¹							
Standard deviation	4.60	6.47	17.22	12.62	16.14	27.95	17.72
Standard error of the mean	0.48	0.68	1.82	1.33	1.70	2.95	1.87
(b) ZR 75 1 cells							
Total no. adherent cells	479	838	1921	2325	2392	2827	2846
Mean no adherent cells	5	9	21	26	27	31	32
/ field of view (n=90)*							

Standard deviation	4.24	8.85	17.19	10.85	10.19	16.33	9.94
Standard error of the mean	0.45	0.93	1.81	1.14	1.07	1.72	1.05

¹Two technical replicates and three biological replicates were performed for each assay. For each assay dish, 15 randomly chosen images were captured, giving a total of 90 images per time point for each cell line

Table 5. Adhesion of MCF 7, ZR 75 1 and BT 747 cells to HUVEC monolayer after masking glycans using HPA, PNA and Con A

Cell line	MCF7				ZR 75 1				BT 474			
Group	Cont	HPA	PNA	Con A	Cont	HPA	PNA	Con A	Cont	HPA	PNA	Con A
Total number of adherent cancer cells	3019	1610	2858	2893	1380	763	1348	1418	1343	1281	1325	1323
Mean no. adherent cells / field of view (n=60 ¹)	50	27	48	48	23	13	22	24	22	21	22	22
Standard deviation	15.72	9.01	20.04	20.81	8.04	7.44	15.56	12.12	11.28	9.23	10.12	12.14
Standard error of the mean	2.03	1.16	2.59	2.69	1.04	0.96	2.01	1.56	1.46	1.19	1.31	1.57
p value ²	N/A	<0.0001	0.17	0.38	N/A	<0.0001	0.11	0.69	N/A	0.75	0.91	0.73

Cont: Control group with no lectin masking.

¹three technical replicates of each group were performed. Images of 20 randomly chosen fields were captured per dish, totalling 60 images per group for each cell line tested

²control in comparison to HPA, PNA and Con A glycan-masking groups

