Clustering of monosialyl-Gb5 initiates downstream signalling events leading to invasion of MCF-7 breast cancer cells

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Invasion is a complex process controlled by secretion and activation of proteases, alteration of integrin levels and GSL (glycosphingolipid) patterns. Differential organization of GSLs with specific membrane proteins and signal transducers in GEMs (GSL-enriched microdomains), initiates signalling events to modify cellular phenotype. Although the GSL monosialyl-Gb5 has been linked with invasion, its functional role in invasion is poorly described and understood. To investigate this problem, we induced the invasion of human breast cancer cells and subsequently explored the underlying mechanism. In the present study, the invasion of human MCF-7 breast cancer cells is highly dependent on clustering of monosialyl-Gb5, and the subsequent activation of monosialyl-Gb5-associated focal adhesion kinase

and cSrc in GEM leading to the downstream activation of extracellular-signal-regulated kinase (ERK). As a result, we observed increased expression levels and activity of matrix metalloproteinases-2 and -9, which correlated with decreased expression of integrins $\alpha 1$ and $\beta 1$. Together these results suggest that the organization of crucial molecules in GEMs of MCF-7 cells is critical for their invasive properties.

Key words: extracellular-signal-regulated kinase (ERK), glycosphingolipid (GSL), glycosphingolipid-enriched microdomain (GEM), integrin, matrix metalloproteinase (MMP), monosialyl-Gb5 (MSGb5).

INTRODUCTION

The majority of all cancer-related death arises from the metastatic spread of primary tumours. The mechanisms for the acquisition of invasive and metastatic potential of tumour cells are, however, not well understood at either the molecular or the biochemical level. Investigating these mechanisms is therefore one of the main challenges for exploratory and applied cancer research.

A number of complex invasion-associated cellular activities have been identified and characterized, including alterations in expression levels of adhesion molecules and proteolytic degradation of the extracellular matrix, along with changes in expression or activities of a variety of cellular proteins in multiple branched signalling pathways [1]. In addition, previous studies [2] have highlighted the importance of aberrant glycosylation as a key event in the induction of invasion and metastasis. Altered glycosylation of cell-surface glycoproteins [3] and GSLs (glycosphingolipids) [4], which have been identified as tumour-associated antigens, are often found in malignant cells. Of particular importance is that GSLs can cluster and assemble with membrane proteins and signal transducers to form GEMs (GSL-enriched microdomains), where they are able to initiate signal transduction [5], and thus profoundly affect the cellular activities associated with tumour cell invasion. Roles for GSLs in invasion were demonstrated recently for GM3 (monosialoganglioside 3) and GM1 [6,7].

MSGb5 (monosialyl-Gb5), also known as SSEA-4 (stage-specific embryonic antigen-4) [8], is a GSL found in GEMs that are maximally expressed in human renal cell carcinomas and correlates with metastasis [9,10]. Furthermore, previous work [11] has shown that stimulation of MSGb5 by the mAb (monoclonal

antibody) RM1 accounts for the invasive properties of MCF-7 human breast carcinoma cells. The molecular mechanisms regulating synthesis of MSGb5 have been studied previously [12]; however, no clear functional role of MSGb5 in the invasive and metastatic behaviour of tumour cells has been demonstrated.

In the present study, we have induced the invasion of MCF-7/AZ breast cancer cells by the ether lipid ET-18-OMe (1-*O*-octadecyl-2-*O*-methyl-glycerophosphocholine) [13] and analysed changes in organization of MSGb5, integrins and signal transducers. Subsequently, we identified the signalling pathway that leads to the activation of MMPs (matrix metalloproteinases). The findings of the present study may help to explain the mechanism by which GEM organization and composition results in the invasion of MCF-7 breast cancer cells.

MATERIALS AND METHODS

Antibodies

Abs raised against GSLs that were used in this study were: mouse IgM MBr1 against globo-H, mouse IgM 1A4 (E10) against Gb3, mouse IgG₃ DH2 against GM3, mouse IgM MK1-8 against GM2, mouse IgM 9G7 against Gb4, mouse IgM SSEA-3 against Gb5 [14], mouse IgM RM1 against MSGb5 [15] and mouse IgM 5F3 against disialyl-Gb5 [16]. The mouse IgG₃ against MSGb5 was generated by Dr D. Solter (Department of Developmental Biology, Max-Planck Institute of Immunobiology, Freiburg, Germany) and was obtained from the Development Studies Hybridoma Bank [developed under the auspices of the NICHD (National Institute of Child Health and Human Development) and maintained by

Abbreviations used: Ab, antibody; BCA, bicinchoninic acid; CMF-HBSS, calcium- and magnesium-free Hanks balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated kinase; ET-18-OMe, 1-*O*-octadecyl-2-*O*-methyl-glycerophosphocholine; FAK, focal adhesion kinase; GD3, disialoganglioside 3; GEM, glycosphingolipid-enriched microdomain; GM3, monosialoganglioside 3; GSL, glycosphingolipid; HPTLC, high-performance TLC; mAb, monoclonal Ab; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MMP, matrix metalloproteinase; MSGb5, monosialyl-globoside 5; MT, membrane type; PP1, 4-amino-1-*tert*-butyl-3-(1'-naphthyl)pyrazolo[3,4-*d*]pyrimidine; SSEA-4, stage-specific embryonic antigen-4.

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the Department of Biological Sciences, University of Iowa, Iowa City, IA, U.S.A.]. Abs directed against integrins $\alpha 1$, $\alpha 2$ and $\beta 1$, and anti-paxillin, anti-(phospho-paxillin), anti-p130^{Cas} and anti-(phospho-p130^{Cas}) were from Chemicon. Mouse anti-FAK (focal adhesion kinase) mAb and mouse anti-cSrc mAb were from Transduction Laboratories. Rabbit anti-(phospho-FAK) Abs (against phosphorylated residues Tyr³⁹⁸, Tyr⁵⁷⁷, Tyr⁸⁶¹ and Tyr⁹²⁵) and anti-(phospho-cSrc) (Tyr⁴¹⁶) were from Biosource. Rabbit anti-ERK (extracellular-signal-regulated kinase) and anti-(phospho-ERK) (Thr²⁰²/Tyr²⁰⁴) were obtained from Cell Signaling Technologies. Abs against MMP-2 and MMP-9 were from Sigma. Biotinylated anti-rabbit and anti-mouse, and FITC-labelled anti-mouse secondary Abs were from Vector Laboratories.

Cell culture

MCF-7/AZ and MCF-7/6 cells are variants of the human mammary carcinoma cell family MCF-7. The cells were maintained on tissue culture plastic substrate (Nunc) in a 50:50 (v/v) mixture of DMEM (Dulbecco's modified Eagle's medium) and Ham's F12 (Invitrogen) containing 250 units/ml penicillin, 100 μ g/ml streptomycin (Invitrogen) and 10% (v/v) fetal bovine serum (Invitrogen) at 37°C in a humidified 10% CO₂/90% air atmosphere. Both cell variants have been studied in several invasion assays *in vitro*. MCF-7/AZ and MCF-7/6 cells are not invasive into collagen type I [13], whereas MCF-7/6, but not MCF-7/AZ cells, are invasive when tested in precultured heart fragments [17].

Reagents

ET-18-OMe (clinical grade) was provided kindly by Dr P. Hilgard (ASTA MEDICA, Frankfurt am Main, Germany). Cells were cultured in the presence of 15 μ g ET-18-OMe per ml of culture medium for the indicated times. Drug toxicity was evaluated through measurement of mitochondrial dehydrogenase activities with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] reagent (Sigma) [18]. PP1 (4-amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine), a cSrc kinase inhibitor, was from Calbiochem and was used at a final concentration of 20 μ M. Micro-BCA (bicinchoninic acid) and BCA protein assay reagent kit were from Pierce. Vectastain[®] ABC-AmP[™] kit was from Vector Laboratories.

Collagen type I invasion

Collagen type I invasion was determined as described previously [19]. Briefly, six-well plates were filled with 1.25 ml neutralized collagen type I (0.09%; Upstate Biotechnology) and incubated for 1 h at 37°C to allow gelification. Single-cell suspensions were prepared with trypsin/EDTA, mixed with or without ET-18-OMe and PP1, seeded on top of collagen type I gel and cultured at 37°C for 24 h. The number of cells penetrating into the gel or remaining at the surface was counted in 12 fields of 0.157 mm², using an inverted microscope controlled by a computer program (Dr L. Vakaet Jr, McForth, Creative Solutions, Rockville, MD, U.S.A.). The invasion index expresses the percentage of invading cells over the total number of cells.

GSL composition in GEMs and soluble fractions by TLC immunostaining

The composition of GSL in GEMs and in the soluble fraction of both MCF-7 cell lines after treatment with ET-18-OMe for 1 h was compared with untreated cells. The procedure was performed as described previously [20]. Briefly, total GSLs were separated from phospholipids and other lipids by the acetylation procedure

Figure 1 Invasiveness of MCF-7/AZ and MCF-7/6 cells

Effect of ET-18-OMe (closed bars) compared with controls (open bars) on invasion into collagen type I of MCF-7/AZ, MCF-7/6 cells or MCF-7/AZ and MCF-7/6 cells treated with PP1. Single-cell suspensions were seeded on top of a collagen type I gel-layer and cultured at 37°C for 24 h. The invasion index expressed the percentage of cells invading into collagen type I over the total number of cells. Results are means $\pm$ S.D. * $P < 0.05$.

described previously [21]. The GSLs were purified further by HPTLC (high-performance-TLC) in isopropanol/hexane/water (55:25:20 by vol.) system and identified with TLC immunostaining by specific Abs. The intensity of immunoblotted bands was quantified by densitometry using the Scion Image program (Scion).

Western blotting

Cell lysates were made from cells grown to 70% confluence. The cells were treated for the indicated times with ET-18-OMe and PP1, washed three times with PBS and subsequently lysed in 0.5 ml lysis buffer [PBS containing 1% (v/v) Triton X-100, 1% (v/v) Nonidet P40 and the following protease inhibitors: 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1.72 mM PMSF, 100 mM NaF, 500 μ M sodium metavanadate and 500 μ g/ml sodium pyrophosphate]. Aliquots of lysates containing equal amounts of protein were boiled for 5 min in SDS/PAGE sample buffer [0.5 M Tris/HCl, pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 0.01% Bromophenol Blue and 5% (v/v) 2-mercaptoethanol], resolved by SDS/PAGE (7.5% gels) and transferred on to PVDF membranes (Bio-Rad Laboratories). After transfer, membranes were incubated with relevant Abs against FAK, phospho-FAK, cSrc, phospho-cSrc, ERK, phospho-ERK, MMP-2, MMP-9, integrins $\alpha 1$, $\beta 1$, $\alpha 2$, p130^{Cas}, phospho-p130^{Cas}, paxillin and phospho-paxillin, followed by incubation with a secondary biotinylated Ab (1:1000) and developed by enhanced chemiluminescence, using the Vectastain[®] ABC-AmP[™] detection kit. Images of the membranes were created and analysed using the BioChem System and analysis software (UVP).

Integrins and signal transducers in GEMs and soluble fractions

Cells were treated for the indicated times with ET-18-OMe and postnuclear fractions were prepared in TNE (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with 10 μ g/ml aprotinin and 1.72 mM PMSF and 1% (v/v) Triton X-100, followed by sucrose-gradient density centrifugation to isolate 12 fractions [0.5 ml sample mixed with 0.5 ml 85% (w/v) sucrose solution, followed by 5.5 ml 35% (w/v) sucrose solution and 4.2 ml 5% (w/v) sucrose solution. Ultracentrifuge (Beckman) with swinging buckets (SW40 Ti) at 39 000 rev./min, 4°C for 17 h], which included GEMs found in fractions 4–6, as described previously [22,23]. Integrins ($\alpha 1$, $\beta 1$ and $\alpha 2$)

Figure 2 Expression levels of activated cSrc (phospho-Tyr⁴¹⁶), FAK (phospho-Tyr³⁹⁷/Tyr⁹²⁵) and ERK (phospho-Thr²⁰²/Tyr²⁰⁴) in whole-cell lysates from MCF-7/AZ and MCF-7/6 cells

Cells at 70% confluence were treated for 0, 5, 10, 15 or 30 min with ET-18-OMe or ET-18-OMe and PP1. (A) Whole-cell lysates of MCF-7/AZ and MCF-7/6 cells (30 µg of protein) were analysed by SDS/PAGE (7.5% gels) and immunoblotted with Abs against activated cSrc [anti-(phospho-cSrc) (Tyr⁴¹⁶)], activated FAK [anti-(phospho-FAK) (Tyr³⁹⁷/Tyr⁹²⁵)] and activated ERK [anti-(phospho-ERK) (Thr²⁰²/Tyr²⁰⁴)]. The membranes were stripped at 50°C for 30 min in stripping buffer [100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris/HCl (pH 6.8)] and reblotted with anti-cSrc, anti-FAK and anti-ERK Abs. Scion Image densitometry analysis of bands of activated cSrc (phospho-Tyr⁴¹⁶) (B), activated FAK (phospho-Tyr³⁹⁷) (C), activated FAK (phospho-Tyr⁹²⁵) (D) and activated ERK (phospho-Thr²⁰²/Tyr²⁰⁴) (E) in MCF-7/AZ cells after treatment with ET-18-OMe compared with treatment with ET-18-OMe and PP1. Results are means ± S.D.

and signal transducers (FAK, phospho-FAK, cSrc, phospho-cSrc, ERK and phospho-ERK) present in the GEM fractions and in the high-density soluble fractions (10–12) were identified further by SDS/PAGE (7.5% gels), followed by Western blotting as described in the Western blotting section of the Materials and methods section.

Immunoprecipitation of GSLs and signal transducers

Association of GSLs (Gb3, Gb5, MSGb5 or GM2) with signal transducers, FAK and cSrc, was analysed by immunopreci-

pitiation. When cell cultures reached 70% confluence they were treated for the indicated times with ET-18-OMe and then lysed as described in the Western blotting section. Lysates containing 800 µg of protein, were mixed with 50 µl of packed Protein G-Sepharose beads (Amersham Biosciences) and placed on a rotator for 30 min at 4°C, to pre-clear non-specific-binding proteins from the lysate. After centrifugation at approx. 70 g, the supernatant was collected and 2 µl of relevant Ab was added to the sample, which was then rotated at 4°C overnight. After incubation Protein G-Sepharose beads were added, rotated at 4°C for 2 h and centrifuged to collect the beads. The beads were then washed

Figure 3 Expression levels of activated cSrc (phospho-Tyr⁴¹⁶), FAK (phospho-Tyr³⁹⁷) and ERK (phospho-Thr²⁰²/Tyr²⁰⁴) in GEM (fractions 4–6) and non-GEM (fractions 10–12) of MCF-7/AZ cells

Cells were grown to 70 % confluence and treated for 0, 15 and 30 min with ET-18-OMe or ET-18-OMe and PP1. GEM were separated from postnuclear fractions by sucrose-gradient density centrifugation of approx. 2×10^7 cells, of which approx. 2 μ g of protein of each fraction was analysed by SDS/PAGE and immunoblotted with respective Abs. **(A)** Western blot analysis of cSrc, FAK and ERK in GEM fractions (4–6) and non-GEM fractions (10–12) in MCF-7/AZ cells after ET-18-OMe treatment. **(B)** Left-hand panel: Western blot analysis of activated cSrc (phospho-Tyr⁴¹⁶) in fractions 4–6 and 10–12. Right-hand panel: Scion Image densitometry analysis of bands of activated cSrc in fractions 4–6 (open bars) and 10–12 (closed bars) in MCF-7/AZ. **(C)** Left-hand panel: Western blot analysis of activated FAK (phospho-Tyr³⁹⁷) in fractions 4–6 and 10–12. Right-hand panel: Scion Image densitometry analysis of bands of activated FAK in fractions 4–6 (open bars) and 10–12 (closed bars) in MCF-7/AZ cells. **(D)** Left-hand panel: Western blot analysis of activated ERK (phospho-Thr²⁰²/Tyr²⁰⁴) in fractions 4–6 and 10–12. Right-hand panel: Scion Image densitometry analysis of bands of activated ERK in fractions 4–6 (open bars) and 10–12 (closed bars). Results are means $\pm$ S.D. Fr, fraction.

twice with lysis buffer and collected by centrifugation. SDS/PAGE sample buffer (100 μ l) was added to the immunoprecipitates and then heated to 95 °C for 5 min. The immunoprecipitates were subjected to SDS/PAGE (7.5 % gels), transferred electrophoretically on to PVDF membranes, incubated with relevant Abs and analysed and imaged as described in the Western blotting section.

Fluorescence immunostaining

Fluorescence microscopy

Cells were suspended in trypsin/EDTA and stained using indirect immunofluorescence. Briefly, cells were incubated on a rotator at 4 °C for 1 h with IgG₃ Ab against MSGb5, washed three times with CMF-HBSS (calcium- and magnesium-free Hanks balanced salt solution) and fixed with 3 % (w/v) paraformaldehyde in CMF-HBSS for 20 min. Fixed cells were washed three times with CMF-HBSS and incubated with FITC-labelled anti-mouse secondary Ab. Stained cells were mounted with a drop of

Glycerol mounting medium (Dako) containing 1 % (w/v) 1,4-diazabicyclo[2.2.2]octane (fluorescence stabilizer). Fluorescence was observed using an Olympus IX51 microscope with an attached Olympus U-CMAD3 camera.

Confocal microscopy

Cells were grown on 12-mm-diameter glass coverslips placed in the wells of a 24-well plate. The glass coverslips with cells attached were removed, washed three times with ice-cold PBS and fixed with 3 % (w/v) paraformaldehyde in PBS. Fixed cells were washed three times with PBS, incubated with Abs against MSGb5, Gb3, Gb5 or GM2 and mounted as described above. Confocal laser scanning was with a Leica DM IRBE fluorescence microscope and a Leica TCS NT confocal unit.

In-gel gelatinase assay

Gelatin zymography was performed as described [24]. Subconfluent cells were treated with ET-18-OMe and PP1 in

Figure 4 TLC of GSLs from whole-cell extracts (A) or GEMs (B) of MCF-7/AZ and MCF-7/6 cells with or without ET-18-OMe

(A) MCF-7/AZ and MCF-7/6 cells were treated for 1 h with ET-18-OMe or untreated. GSLs were extracted from cells with chloroform/methanol (2:1, v/v), then phospholipids were removed by alkaline treatment, and GSLs from equal numbers of cells were loaded in each lane of HPTLC plates. After developing in a solvent system of chloroform/methanol/0.2% aqueous CaCl_2 (5:4:1 by vol.), GSLs were visualized by spraying with 0.5% orcinol in 2 M sulfuric acid. CDH (lactosylceramide), Gb4, MSGb5, GM3, GM1, GD3 and GD1b were used as reference markers. (B) Immunostaining of various GSL structures present in GEM fractions (4–6) and non-GEM fractions (10–12) in MCF-7/AZ and MCF-7/6 cells after 1 h ET-18-OMe treatment, detected by nine Abs directed against the respective structures as described in the Materials and methods section. GSL fraction was prepared after elimination of sucrose by dialysis using C_{18} columns. Fr., fraction.

DMEM/Ham's F12 medium for 0, 1, 2, 4, 6, 8, 12 and 18 h at 37°C. The medium was collected, clarified by centrifugation, resolved in non-reducing gels containing 2 mg/ml gelatin and processed for activity by observing zones of gelatin degradation.

Statistics

All treatments were performed at least three times. The Student's *t*-test (95%) was used for statistical evaluation. Levels were quantified using Scion Image statistical software.

RESULTS

Invasiveness of MCF-7/AZ but not of MCF-7/6 cells

The two human breast cancer cell variants, MCF-7/AZ and MCF-7/6, are non-invasive into a collagen type I gel-layer (Figure 1). ET-18-OMe treatment, however, increased the invasiveness of MCF-7/AZ cells, while the same treatment had no effect on MCF-7/6 cells (consistent with previous observations [13]). Pre-treatment with PP1 blocked the enhanced invasiveness of MCF-7/AZ cells (Figure 1).

Expression levels of activated cSrc, FAK and ERK in invasive MCF-7/AZ cells compared with non-invasive MCF-7/6 cells

The invasion-promoting effect of ET-18-OMe on MCF-7/AZ cells suggests that ET-18-OMe initiates cSrc-mediated signalling in MCF-7/AZ cells, but not in the variant MCF-7/6 cells (Figure 1). Therefore the effect of ET-18-OMe on phosphorylation of Tyr³⁹⁷ of FAK, the autophosphorylation site of FAK, and Tyr⁴¹⁶ of cSrc kinase was examined. Expression levels of FAK and cSrc in both cell lines were unchanged, but kinase activity of cSrc (Figures 2A, left-hand panel and 2B) and FAK (Figures 2A, left-hand panel and 2C) were greatly enhanced in MCF-7/AZ cells 5–10 min after treatment. There was no such activation of cSrc and FAK in MCF-7/6 cells (Figure 2A, right-hand panel). The use of cSrc kinase inhibitor, PP1, blocked activation of cSrc but not Tyr³⁹⁷ phosphorylation on FAK, suggesting that the autophosphorylation of FAK promotes activation of cSrc. Next, the cSrc-dependent tyrosine phosphorylation sites on FAK (Tyr⁵⁷⁶, Tyr⁸⁶¹ and Tyr⁹²⁵) were assayed. Time-dependent phosphorylation of FAK on Tyr⁹²⁵ (Figures 2A, left-hand panel and 2D) but not Tyr⁵⁷⁶ and Tyr⁸⁶¹ (results not shown) was observed in ET-18-OMe-treated MCF-7/AZ cells, whereas no altered phosphorylation of FAK on Tyr⁹²⁵ (Figure 2A, right-hand panel) and no phosphorylation on Tyr⁵⁷⁶ and Tyr⁸⁶¹ was detected in MCF-7/6 cells (results not shown). We addressed whether inhibition of cSrc prevented phosphorylation of FAK on Tyr⁹²⁵ further. Treatment with ET-18-OMe and PP1 reduced Tyr⁹²⁵ phosphorylation in MCF-7/AZ cells (Figures 2A, left-hand panel and 2D). These results suggest that the MAPK (mitogen-activated protein kinase)-pathway might be involved, since phosphorylation of FAK on Tyr⁹²⁵ initiates downstream signalling resulting in activation of ERK (by phosphorylation of Thr²⁰² and Tyr²⁰⁴) [25,26]. Treatment with ET-18-OMe resulted in enhanced ERK activity in MCF-7/AZ cells after 10 min but not in MCF-7/6 cells (Figures 2A and 2E). p130^{Cas} and paxillin are known substrates of cSrc that are associated with phosphorylation of FAK on Tyr⁸⁶¹; in order to confirm the lack of phosphorylation on FAK Tyr⁸⁶¹ and Tyr⁵⁷⁶, we determined the kinase activity of p130^{Cas} and paxillin. Treatment with ET-18-OMe had no effect on phosphorylation of p130^{Cas} or paxillin in both MCF-7/AZ and MCF-7/6 cells (results not shown).

Expression levels of activated cSrc, FAK and ERK in GEMs of invasive MCF-7/AZ and non-invasive MCF-7/6 cells

FAK, cSrc and ERK are associated with GEMs (low-density fractions 4–6) and the soluble high-density fractions (10–12) in both MCF-7/AZ (Figure 3A) and MCF-7/6 cells (results not shown). In both cell-line variants, expression levels of FAK, cSrc and ERK were unchanged after ET-18-OMe treatment (Figure 3A). Activity levels of cSrc, FAK and ERK were found in GEM as well as in the non-GEM fractions and phosphorylation levels of FAK, cSrc and ERK were enhanced after 15 min and decreased after 30 min in MCF-7/AZ cells (Figures 3B, 3C and 3D). PP1 blocked activation of cSrc and, subsequently, blocked

Figure 5 Clustering of MSGb5 and association with cSrc and FAK in MCF-7/AZ cells

MCF-7/AZ cells were treated for the indicated times with ET-18-OMe. **(A)** The expression pattern of MSGb5 by fluorescence (a) and confocal (b) microscopy. Cells were suspended in trypsin/EDTA or grown on glass coverslips, and analysed using mouse IgG₃ against MSGb5 and detected with FITC-labelled anti-mouse Ab. (a) and (b) show the homogeneous distribution of MSGb5 in untreated MCF-7/AZ cells; arrows in (a) and (b) indicate the clusters of MSGb5 on the membrane of MCF-7/AZ cells 10 and 60 min after ET-18-OMe treatment. Expression pattern of Gb3 (c) and Gb5 (d) by confocal microscopy. Cells were grown on glass coverslips and analysed by mouse IgM 1A4 (E10) against Gb3 or mouse IgM SSEA-3 against Gb5, and detected with FITC-labelled anti-mouse Ab. Experiments were performed at least three times. Control staining was performed without primary Ab to measure possible cross-reaction or non-specific binding. All controls gave negative results (not shown). **(B)** Association of MSGb5 with cSrc and FAK and activated cSrc and FAK in MCF-7/AZ (left-hand panel) and MCF-7/6 cells (right-hand panel) after treatment with ET-18-OMe for the indicated times. MSGb5 was immunoprecipitated from whole-cell lysates with mouse IgG₃ anti-MSGb5 Ab. Aliquots of immunoprecipitates were immunoblotted with anti-FAK, anti-cSrc and anti-(phospho-FAK) (Tyr³⁹⁷) and anti-(phospho-cSrc) (Tyr⁴¹⁸). MSGb5 content in the different immunoprecipitates was determined by TLC. The lower panels show the Scion Image densitometry analyses of phospho-FAK, phospho-cSrc and total MSGb5. Results are means±S.D. * $P < 0.05$.

activation of downstream ERK (Figures 3B and 3D). Activity levels in MCF-7/6 cells were essentially the same (results not shown).

GSL composition in MCF-7/AZ and MCF-7/6 cells

Major GSLs found in both MCF-7 cell lines treated with ET-18-OMe or untreated were identified as globo-series Gb3, Gb4, Gb5, MSGb5 and globo-H, and as the ganglio-series structure GM2. However, lacto-series structures were not detectable (Figure 4A). Composition of GSLs, as determined by their specific Abs, was compared for GEM fractions (4–6) and the soluble high-density fractions (10–12). Each GSL was present in fractions 4–6 but completely absent from fractions 10–12 (Figure 4B).

No differences between the two cell-lines were observed in the conditions tested. The presence of Gb3 and other globo-series structures similar to Gb5 and globo-H in MCF-7 cells was reported previously [27]. The presence of an abundance of GM3 and GM1 in MCF-7 cells has been observed previously [27]; however, the variants used in the present study lacked GM3 and GM1, which is in line with previous findings [11].

Clustering of MSGb5 and association with cSrc and FAK in invasive MCF-7/AZ cells compared with non-invasive MCF-7/6 cells

Clustering of MSGb5

Alterations in the pattern of MSGb5 were observed by immunofluorescence and confocal microscopy (Figure 5A). MSGb5 was

Figure 6 Activity and expression levels of MMPs in MCF-7/AZ and MCF-7/6 cells

MCF-7/AZ and MCF-7/6 cells were treated for 0, 1, 2, 4, 8 or 12 h with ET-18-OMe or ET-18-OMe plus PP1. (A) Whole-cell lysates (30 μ g) were analysed by SDS/PAGE and immunoblotted with Abs against MMP-2 and MMP-9. Scion Image densitometry analysis of bands of MMP-2 (B) and MMP-9 (C) in MCF-7/AZ cells after treatment with ET-18-OMe compared with ET-18-OMe and PP1. Results are means $\pm$ S.D. (D) Activity of MMP-2 in MCF-7/AZ cells compared with MCF-7/6 cells after 0, 1, 2, 4, 6, 8, 12 and 18 h of treatment with ET-18-OMe, analysed by gelatin zymography. The medium of subconfluent cultures were collected, resolved in non-reducing gels containing 2 mg/ml gelatin and the activity was measured by observing zones of gelatin degradation. The right-hand panel shows the Scion Image densitometry analysis of activity bands of MMP-2 in MCF-7/AZ compared with MCF-7/6 cells. Results are means $\pm$ S.D.

detected in both MCF-7/AZ and MCF-7/6 cells consistent with the above results. Fluorescence examination of MCF-7/AZ cells treated with ET-18-OMe revealed clustering of MSGb5 at the membrane 5–10 min after treatment compared with untreated MCF-7/AZ cells (Figure 5A, panels a and b). Clustering of MSGb5 in MCF-7/6 (results not shown), as well as clustering of the GSLs Gb3 and Gb5 in MCF-7/AZ cells after ET-18-OMe treatment was not observed (Figure 5A, panels c and d).

Association of MSGb5 with cSrc and FAK

MSGb5 association with cSrc and FAK and phosphorylated-cSrc and -FAK was shown by immunoprecipitation. Aliquots of cell lysates were immunoprecipitated by incubation with anti-MSGb5 Ab and capture with protein G-Sepharose beads as described in the Materials and methods section. Both FAK and cSrc were detected in MSGb5 immunoprecipitates (Figure 5B, left-hand panel). In addition, kinase activity of FAK (phospho-Tyr³⁹⁷) and cSrc (phospho-Tyr⁴¹⁶) was observed in the MSGb5-immunoprecipitates in MCF-7/AZ cells 5–10 min after ET-18-OMe treatment (Figure 5B), which is in line with the kinase experiments described above. No association of FAK and cSrc with Gb3, Gb5 or GM2 was observed (results not shown). In MCF-7/6 cells, cSrc and FAK were associated with MSGb5, and as expected no kinase activity was observed upon ET-18-

OMe treatment (Figure 5B, right-hand panel). Equal levels of MSGb5 were detected in the immunoprecipitates of both cell lines (Figure 5B).

Activity and expression levels of MMPs in invasive MCF-7/AZ and non-invasive MCF-7/6 cells

Expression level of MMPs in MCF-7/AZ and MCF-7/6 cells

To address whether active ERK promotes MMP processing, we determined the expression levels of MMP-2, MMP-9 and MT1 (membrane type 1)-MMP. In MCF-7/AZ cells, expression of MMP-2 was greatly enhanced after 1 h and decreased 4 h after ET-18-OMe treatment (Figures 6A and 6B), whereas the level of MMP-9 increased after 1 h (Figure 6A and 6C). MT1-MMP expression was unaffected (results not shown). PP1 blocked expression of both MMP-2 and MMP-9 in MCF-7/AZ cells (Figure 6A, left-hand panel). ET-18-OMe had no effect on any of the MMPs tested in MCF-7/6 cells (Figure 6A, right-hand panel).

Activity level of MMPs in MCF-7/AZ and MCF-7/6 cells

Since ET-18-OMe treatment resulted in increased expression of MMPs, we determined further the activity of MMP-2 in both cell-line variants after ET-18-OMe treatment. The results shown in Figure 6(D) revealed that MMP-2 activity increased significantly in MCF-7/AZ cells compared with MCF-7/6 cells.

Figure 7 Integrin expression in MCF-7/AZ and MCF-7/6 cells and expression levels of integrin $\alpha 1$ and $\beta 1$ in GEM fractions (4–6) and non-GEM fractions (10–12) of MCF-7/AZ cells

MCF-7/AZ and MCF-7/6 cells were grown to 70% confluence and treated for 0, 1, 2, 4, 8, 12 h with ET-18-OMe. **(A)** Whole-cell lysates (30 μ g) were analysed by SDS/PAGE and immunoblotted with Abs against integrins $\alpha 1$ and $\beta 1$. Scion Image densitometry analysis of bands of integrin $\alpha 1$ **(B)** and integrin $\beta 1$ **(C)** in ET-18-OMe treated MCF-7/AZ cells. Results are means $\pm$ S.D. **(D)** MCF-7/AZ cells were grown to 70% confluence and treated for 0, 15 and 30 min with ET-18-OMe. GEM were separated from postnuclear fraction by sucrose-gradient density centrifugation from approx. 2×10^7 cells and approx. 2 μ g of protein from each fraction was analysed by SDS/PAGE and immunoblotted with Abs against integrin $\alpha 1$ and integrin $\beta 1$ in GEM fractions (4–6) and non-GEM fractions (10–12). The lower panels show the Scion Image densitometry analysis of bands of integrin $\alpha 1$ (left-hand panel) and integrin $\beta 1$ (right-hand panel) in fractions 4–6 (open bars) and 10–12 (closed bars). Results are means $\pm$ S.D. Fr, fraction.

Differences in integrin expression levels

Integrin expression in MCF-7/AZ compared with MCF-7/6 cells

Tumour-cell invasion includes not only secretion and activation of proteases but also alteration in integrin expression levels. To analyse the involvement of integrins, we determined the expression levels of integrins $\alpha 1$, $\alpha 2$ and $\beta 1$. Expression of integrins $\alpha 1$ and $\beta 1$ decreased over time in MCF-7/AZ cells (Figures 7A, left-hand panel, 7B and 7C), whereas no changes in expression of either integrin was observed after treatment of MCF-7/6 cells (Figure 7A, right-hand panel). In both cell lines integrin $\alpha 2$ was not detectable (results not shown).

Integrins in GEMs of MCF-7/AZ compared with MCF-7/6 cells

Additionally, we found integrins $\alpha 1$ and $\beta 1$ associated with GEMs in both cell-lines. However, 15 min after treatment with ET-18-OMe, the two integrins were no longer found in the GEM fractions, but were present in the non-GEM fractions from MCF-

7/AZ cells (Figure 7D). Moreover, immunoprecipitation revealed that both integrins were not associated with MSGb5, FAK and cSrc (results not shown). Similar treatment had no effect on the distribution of these integrins in MCF-7/6 cells (results not shown).

DISCUSSION

Tumour-cell invasion is a complex process controlled by several factors, many of which operate at the cell-surface membrane. This process includes secretion and activation of proteases [28], alteration of integrin receptor expression levels [29] and changes in GSL expression patterns [2,4]. In addition, GSLs and transmembrane glycoproteins, as well as signal transducers, are assembled in multiple types of GEMs, which are involved in signal transducers, thereby initiating signals that result in the modification of the cellular phenotype [30]. This has been demonstrated for GM3 in mouse melanoma B16 cells [23,31] and in human bladder KK47 cancer cells [6], for DSGG (diasyl-GalNAcLc₄) in renal cell carcinoma [16,32] and for GD3 (disialoganglioside 3) in melanoma cells [33].

MSGb5, described originally as SSEA/4 [8], is highly expressed in human renal cell carcinomas and correlates with metastatic potential [9,10]. Stimulation of MSGb5 by the mAb RM1 was suggested to be associated with invasiveness of MCF-7 human breast carcinoma cells, along with activation of FAK and cSrc [11]. However, no other studies demonstrate a clear functional role for MSGb5 in invasion or a role in signal regulation leading to invasion.

In the present study, we have used two breast carcinoma cell variants MCF-7/AZ and MCF-7/6 that show similar composition in their GEM with regard to globo-H, Gb3, Gb4, Gb5, MSGb5 and GM2, and associated signalling molecules. Both variants are non-invasive into a collagen type I gel-layer; however, treatment with ET-18-OMe caused the phenotypic conversion of MCF-7/AZ cells from non-invasive to invasive, whereas no effect was observed in MCF-7/6 cells. This result enabled us to identify the key players in the activation of the signalling pathway that initiates invasion.

Next, enhanced activity levels of cSrc were detected in MCF-7/AZ but not in MCF-7/6 cells after treatment with ET-18-OMe. Suppression of cSrc with the tyrosine kinase inhibitor PP1, demonstrated clearly the prominent role for cSrc in invasion induced by ET-18-OMe. A probable mechanism by which cSrc promotes invasive behaviour is through formation of a transient complex with the protein tyrosine kinase, FAK [34,35]. A previous study [25] has described FAK association with a variety of cell surface molecules, resulting in its autophosphorylation at Tyr³⁹⁷ and thereby creating a high-affinity-binding site for cSrc. This specific organization has been shown to play a crucial role in cSrc-dependent phosphorylation of distinct tyrosine residues on FAK [36], leading to specific downstream signalling events. Our results support the formation of a FAK–cSrc complex upon FAK (Tyr³⁹⁷) activation and the subsequent cSrc-dependent phosphorylation of FAK on Tyr²⁵, and the downstream phosphorylation of ERK, which is consistent with previous studies [25]. The results of the present study appear to be similar with the observations made by Steelant et al. [11] and suggested the implication of GSLs, in particular MSGb5, in the initiation of downstream signalling events. Although both cell lines displayed no differences in their GSL composition upon treatment with ET-18-OMe, the possibility arose that changes in organization or clustering of their GSLs was responsible for the initiation and activation of associated signal transducers. Such an activation model

Figure 8 Changes in GEM leading to cancer cell invasion

In non-invasive MCF-7/AZ cells integrin subunits $\alpha 1$ and $\beta 1$ form a stable complex with tetraspanin CD9 and GSLs, associated with non-active signal transducers, FAK and cSrc. In invasive MCF-7/AZ cells clustering of MSGb5 and loss of integrin subunits $\alpha 1$ and $\beta 1$ disorganize glycosynapse frameworks, resulting in activation of downstream signalling to invasion. EC, extracellular; IC, intracellular; TSP, tetraspanin.

was reported for disialylgalactosylgloboside in the renal cell carcinoma cell-line TOS-1 [32] and for GM3 in B16 melanoma cells [23,31]. In the present study, we observed prompt clustering of MSGb5 in MCF-7/AZ cells and no changes in organization in MCF-7/6 cells upon treatment with ET-18-OMe. A close connection of FAK and cSrc with MSGb5 was suggested, since activated FAK and cSrc were found in GEMs of MCF-7/AZ cells, which was confirmed further by co-immunoprecipitation experiments that revealed the association of both FAK and cSrc, as well as their activated forms, with MSGb5 after ET-18-OMe treatment in MCF-7/AZ cells, whereas no association of the active forms of the proteins with MSGb5 was found in MCF-7/6 cells. Furthermore, we could exclude the involvement of other GSLs present in the GEM, since no clustering and no activation of these signal transducers were observed upon treatment with ET-18-OMe.

Another factor controlling tumour cell invasion is the secretion and activation of MMPs [28]. Several studies [34,35] have shown that FAK–cSrc signalling complexes are associated with an invasive phenotype. In the present study, the results obtained using ET-18-OMe and PP1 support the importance of the downstream activation of ERK in the MAPK-pathway, in response to activation of cSrc and FAK (phospho-Tyr⁹²⁵), as being necessary for secretion and activation of MMP-2 and MMP-9. This is consistent with studies using MEK1AA (dominant negative MAPK/ERK kinase) expression or specific MEK1-inhibitor (PD98059) where secretion of MMP-2 and MMP-9 was suppressed dramatically [37,38]. Other signalling pathways that link the FAK–cSrc complex with MMPs were excluded, for example, via the activation of the distinct cSrc-dependent tyrosine residues on FAK and subsequent phosphorylation of the FAK-binding proteins p130^{Cas} and paxillin [39,40], since no phosphorylation was observed on the cSrc-dependent tyrosine

residues on FAK, and was confirmed by the lack of p130^{Cas} and paxillin activation.

Since integrin receptors are crucial in cancer cell invasion [29], we searched for differences in integrin expression levels. Our results showed decreased expression levels of integrin subunits $\alpha 1$ and $\beta 1$ after ET-18-OMe treatment of MCF-7/AZ cells compared with MCF-7/6 cells. These findings are in line with previous observations [41]. Both receptor subunits associated initially with GEM, were undetectable in the GEMs of MCF-7/AZ cells, which suggests that they have been translocated, whereas the expression levels in MCF-7/6 cells remained unchanged. Moreover, co-immunoprecipitation experiments revealed that FAK is not associated with either integrin, which is in contrast with data reported by Schaller et al. [42]. This confirmed the role of MSGb5 in the activation of downstream signalling events further.

In the present study, the multiple factors regulating the complex process of invasion can all be placed in the 'concept' of the glycosynapse [43]. This new concept proposes the particular interactions and organization of integrins with tetraspanins and gangliosides, and has recently been extended to phenotypic conversion induced through the deletion or addition of a single component, resulting in a disorganized glycosynapse framework and initiating altered signalling events [6,43]. In the present study, we conclude that phenotypic conversion from non-invasive to invasive MCF-7/AZ breast cancer cells is induced by: (i) an aberrant MSGb5 pattern; (ii) loss of integrin receptor subunits $\alpha 1$ and $\beta 1$; and (iii) high tetraspanin (CD9) expression levels [11], all of which are responsible for the formation of disorganized glycosynapse framework interfaces, thereby inducing activation of FAK, cSrc and downstream ERK, with consequent enhanced secretion and activity of MMP-2 and MMP-9, and thus leading to invasion (Figure 8).

In conclusion, our studies are an extension of previous work on the glycosynapse [43], re-formulating the classic concept of integrin-dependent invasion of tumour cells and providing evidence that phenotypic conversion can be explained by differences in composition and organization of crucial molecules in the glycosynapse. At present, only a few studies have appeared that focus, in particular, on GM3 [6,44]. The present study reveals a novel insight into the composition and organization of the glycosynapse in MCF-7/AZ breast cancer cells, which explains phenotypic changes. We are currently investigating differences in the composition and organization of GSLs and associated molecules in other cancer cell-lines where similar phenotypic conversion has been observed. Further studies along this line are necessary to understand the complex interplay of distinct molecules in invasion, as well as other basic cellular mechanisms, and their implications on disease processes, which will be expected to lead to novel therapeutic approaches.

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