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Monosialyl-Gb5 organized with cSrc and FAK in GEM of human breast carcinoma MCF-7 cells defines their invasive properties

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Abstract Two human mammary carcinoma cell variants, MCF-7/AZ and MCF-7/6, show the same composition in their glycosphingolipid-enriched microdomain (GEM) with regard to globo-series structures Gb3, Gb4, Gb5, monosialyl-Gb5, GM2, and cSrc and FAK. Both variants are non-invasive into collagen gel layer, and showed similar motility in wound migration assay. Whereas invasiveness and motility of MCF-7/AZ cells were enhanced greatly by treatment with mAb RM1 directed to monosialyl-Gb5, the same RM1 treatment had no effect on MCF-7/6. cSrc and FAK of MCF-7/AZ, but not MCF-7/6, were activated by RM1 treatment. Thus, malignancy of MCF-7 is highly dependent on monosialyl-Gb5, and its activation of cSrc and FAK in GEM.

Key words: Glycosphingolipid; Microdomain; Tumor cell motility; CD9; Signal transducer; Activation; Sensor; Collagen gel; Invasion

1. Introduction

Glycosphingolipids (GSLs) in tumors have been identified as tumor-associated antigens defined by specific monoclonal antibodies [1]. Essentially all GSLs, either in tumor cells or normal cells, are clustered and assembled with specific membrane proteins and signal transducers to form 'GSL-enriched microdomain' (GEM) or 'glycosignaling domain' (GSD) [2]. An interesting possibility is that tumor cell malignancy may be defined by organization of tumor-associated GSL antigens in GEM or GSD, since they are known to be involved in (i) GSL-dependent tumor cell adhesion, and (ii) signal transduction induced by stimulation of GSLs. Examples have been shown for GM3 in mouse melanoma B16 [3–6], and for disialyl-GalNAcLc$_4$ defined by mAb RM2, in renal cell carcinoma [7,8].

In this paper, we present data on functional role of monosialyl-Gb5 (MSGb5) in GEM of human mammary carcinoma variant cell lines MCF-7/AZ and MCF-7/6, which show differential invasiveness and motility in response to stimulation of MSGb5 by its mAb RM1.

2. Materials and methods

2.1. Cell lines, antibodies, and GSLs

MCF-7/AZ and MCF-7/6 cells [9] are variants of the human mammary carcinoma cell family MCF-7 [10]. They were cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 (50/50) (Cellgro, VA, USA) with penicillin (250 IU/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Cellgro). 7/AZ differ from 7/6 cells in that their morphology changes (fingerlike extensions), with increased membrane ruffling and penetration into collagen gel, upon treatment with ET-18-OMe (25 μg/ml; 50 μM) [11].

Anti-GSL antibodies used in this study were: mouse IgM MBr1 to globo-H, mouse IgM 1A4 (E10) to Gb3, mouse IgG3 DH2 to GM3, mouse IgM MK1-8 to GM2, mouse IgM 9G7 to Gb4, mouse IgM SSEA3 to Gb5 (see table 4 of ref. [12] for source and reference of each antibody), mouse IgM RM1 to MSGb5 [13], and mouse IgM 5F3 to disialyl-Gb5 [14]. Antibodies to cSrc, phosphorylated activated cSrc, FAK, and phosphorylated activated FAK (see Section 2.6).

Total GSLs were separated from phospholipids and other lipids by acetylation procedure [15], further purified by HPTLC using Iatrobeads 6RS-8010 in isopropanol/hexane/water system [16], and identified with thin-layer chromatography (TLC) immunostaining by specific mAbs as above. The procedure has been described in detail [8].

2.2. GSL composition in GEM and soluble fractions by TLC immunostaining

GEM and other fractions were separated from postnuclear fraction by sucrose gradient density centrifugation, and their content of GSLs, cSrc, and FAK was determined as described previously [5,17,18]. GSL components in GEM were identified by high-performance TLC (HPTLC) with immunostaining as above, following elimination of sucrose by dialysis or C18 column [18]. cSrc, FAK, and various other membrane proteins (integrins α1, α2, α3, CD9, MUC1, MUC4, E-cadherin, β-catenin) were further identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with Western blotting. Intensity of immunoblotted bands was quantified by densitometry, using the Scion Image program (Scion Corp., Frederick, MD, USA). TLC immunostaining was performed as described previously [8].

Glycoproteins and signal transducer proteins present in GEM or in high-density soluble fraction were determined by 8% SDS–PAGE followed by Western blotting on PVDF membrane (Immobilon-P, Milli-

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Abbreviations: GEM, glycosphingolipid-enriched microdomain; GSD, glycosignaling domain; GSL, glycosphingolipid; HPTLC, high-performance thin-layer chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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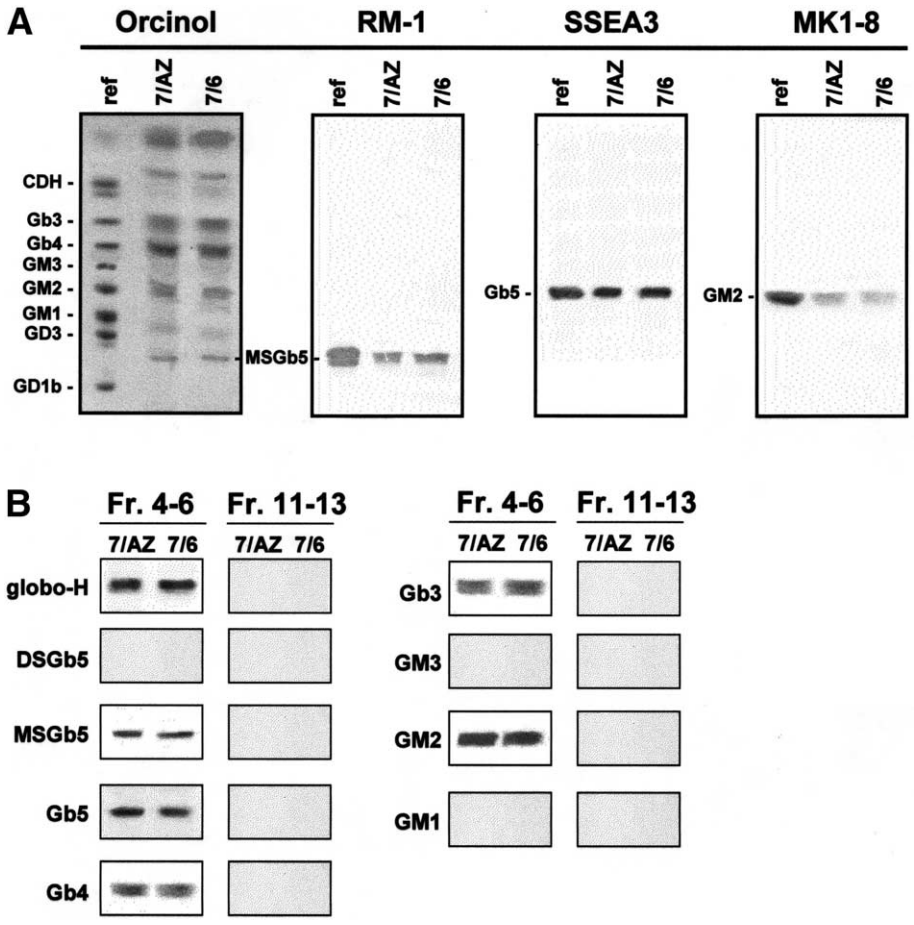

Fig. 1. TLC patterns of GSLs from whole cell extract (panel A) or present in GEM (panel B) of MCF-7/AZ and MCF-7/6 cells. Panel A: GSLs were extracted from cells of the same protein weight, spotted onto HPTLC plates, developed in a solvent system of chloroform/methanol/0.2% aqueous $CaCl_2$ (50:40:10), visualized by spraying with 0.5% orcinol in 2 N sulfuric acid or immunostaining of MSGb5 (RM1), Gb5 (SSEA3) and GM2 (MK1-8), and separated on TLC. Panel B: Immunostaining of various GSL structures present in GEM (Fr. 4–6) and Fr. 11–13 by nine antibodies directed to the respective structures. GSL fraction was prepared after elimination of sucrose by dialysis.

pore) with relevant antibodies vs. CD9, cSrc, p-cSrc, MUC1, MUC4, E-cadherin, FAK, p-FAK, α1, α2, and α3 (for sources, see Fig. 2 legend), followed by chemiluminescence method with a substrate kit (Super-Signal-CL-HRP, Pierce), as described previously [18].

2.3. Immunofluorescence detection of CD9

Cells were suspended in trypsin/EDTA and stained by an indirect immunofluorescence technique. Cells were incubated at 4°C for 1 h with primary antibody against CD9, followed by washing with PBS and incubation in the dark at 4°C for 1 h with an FITC-labeled goat anti-mouse secondary antibody. Then cells were washed, fixed with 2% paraformaldehyde in PBS at room temperature for 20 min, and analyzed by flow cytometry with a Beckman Coulter with Expo32 software.

2.4. Invasion through collagen type I gel

This was determined as described previously [19]. Briefly, neutralized type I collagen (0.09%) was incubated for 1 h at 37°C to allow gelification. Single-cell suspensions in DMEM/Ham's F12 were prepared with trypsin/EDTA, mixed with or without antibodies (for dilution of antibodies and concentration of cSrc kinase inhibitor, see Fig. 3, legend), placed on top of collagen gel, and cultured at 37°C for 24 h. Numbers of cells penetrating into gel or remaining at the surface were counted in 12 fields of 0.157 mm^2, using an inverted microscope controlled by a computer program. The invasion index expresses the percentage of penetrating (invading) cells divided by total number of cells.

2.5. Wound migration assay

The 7/AZ and 7/6 cells were cultured in 24-well Corning tissue culture plates. After 40 h at 37°C, the cells were wounded, a line was scratched with a plastic scriber, debris was removed by washing, and culture medium was added with or without antibody against MSGb5 (MR-1), cSrc kinase inhibitor Pp1, or the combination of the two. The distance between the borders was measured (t_0) along the well, using a grid in a Nikon phase-contrast inverted microscope

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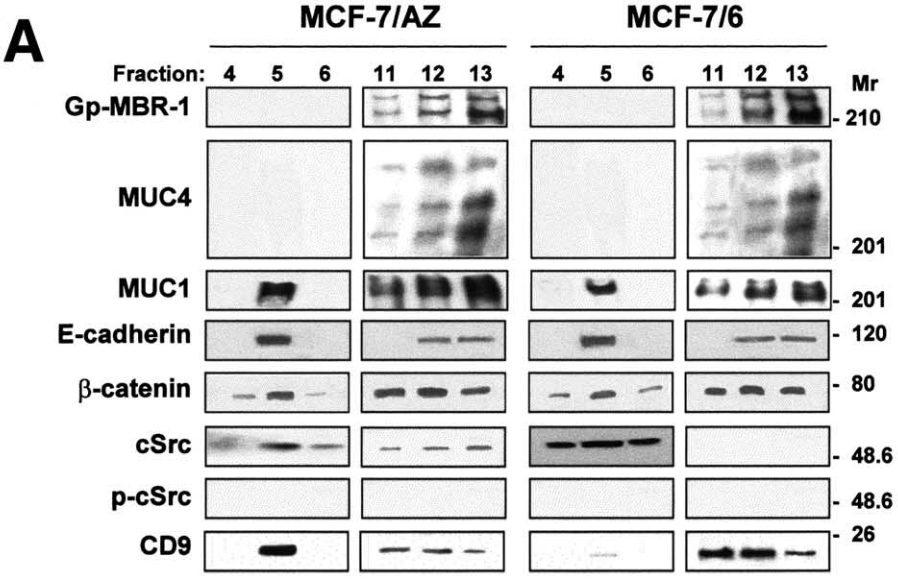
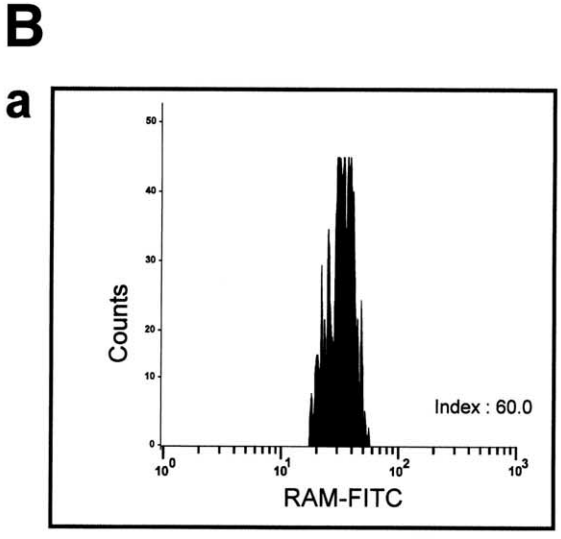
Fig. 2. Panel A: Detection of glycoproteins and signal transducers in GEM (Fr. 4–6) and Fr. 11–13 from 7/AZ and 7/6 cells by SDS–PAGE followed by Western blotting with respective antibodies as indicated at left. Glycoprotein carrying MBr1 epitope (Gp-MBR-1) was blotted by MBr1 [29]. Mouse IgG 21404 to MUC1 was donated by Dr. John Hilkens (Dutch Cancer Institute, Amsterdam, The Netherlands). Rabbit polyclonal antibodies to MUC4 were donated by Dr. Carme de Bolos (Institut Municipal d'Investigacio Medica, Barcelona, Spain). mAb HECD-1 (mouse IgG) directed to E-cadherin was purchased from Takara Bio Inc. (Otsu, Shiga, Japan). Mouse IgG mAb to β-catenin was from Sigma. Mouse IgG mAb to CD9 was from Becton-Dickinson (Lincoln Park, NJ, USA). mAbs directed to α1, α2, and α3 integrin receptors were from Chemicon (Temecula, CA, USA). Panel B. Left: CD9 expression in 7/AZ (A) and 7/6 cells (B) determined by flow cytometry. CD9 fluorescence index was 60.0 for 7/AZ and 27.3 for 7/6 cells. Right: CD9 expression in GEM fraction of 7/AZ (white column) and 7/6 (black column), quantified by Scion image program.

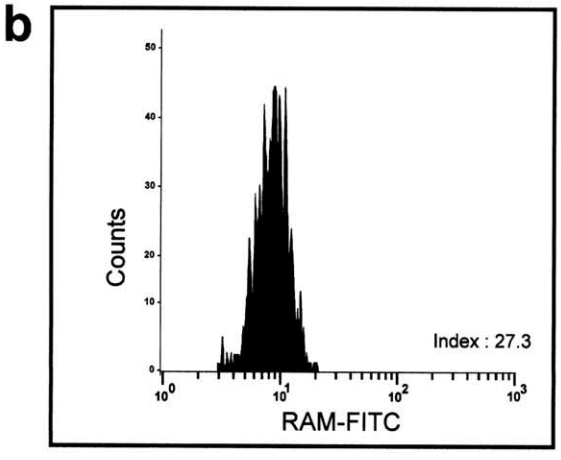
(Model 95023) with 40× magnification. Experiments were done in triplicate and the distance was measured in five fields of each well. After 8 h incubation at 37°C, the distance between the borders in each field was measured again (t_{final}). Migration was calculated as the distance moved by the advancing cells of both borders, divided by 2, times migration hours, and expressed as μm/h [20].

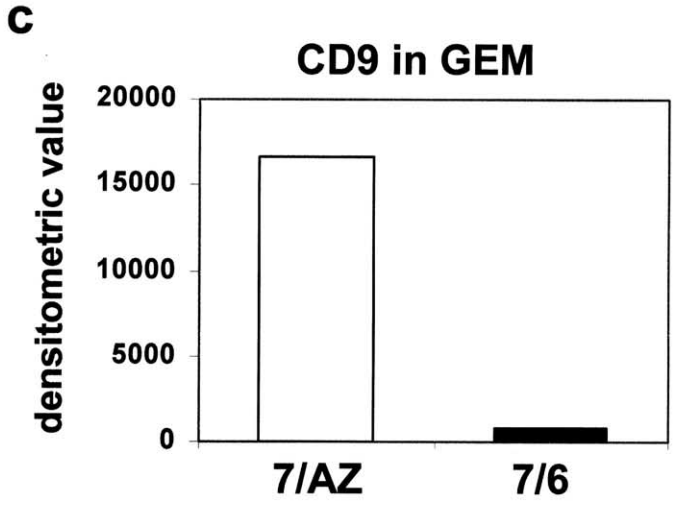
2.6. Levels of cSrc and FAK, and their activation state through tyrosine phosphorylation

Cells stimulated for 0 min, 10 min, 30 min, or 48 h with antibody against MSGb5 (RM1) were lysed in 0.5 ml lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 75 U/ml aprotinin, 1% Triton X-100). Aliquots of lysate containing the same quantity of









protein (~100 μg) were boiled for 5 min in SDS–PAGE sample buffer containing 5% 2-mercaptoethanol, electrophoresed on 8% SDS–PAGE, transferred to PVDF membranes, and Western blotted.

To determine levels of cSrc and FAK and their activation state, they were isolated from cell lysate by immunoprecipitation, followed by Western blotting with specific antibodies defining tyrosine phosphate for cSrc and FAK. Briefly, 400 μl cell lysate (containing 40 μg protein) was mixed with 10 μl protein G-Sepharose beads (Amersham Pharmacia, Sweden) and rotated for 2 h at 4°C. After centrifugation (900×*g* for 5 min), the supernatant was added with 2 μl of 100 μg/ml mouse anti-FAK mAb (Transduction Laboratories) or 2 μl of rabbit anti-Src SRC2 (Santa Cruz Biotech, CA, USA), rotated overnight at 4°C, and then incubated with 10 μl protein G-Sepharose beads for 2 h at 4°C. After centrifugation at 900×*g* for 5 min and washing of beads with TBS, 0.05% Tween 20, samples were prepared for SDS–PAGE followed by Western blotting with antibodies defining PY416 (Cell Signaling, Beverly, MA, USA) for cSrc, and PY397 (Bioscience Int., Camarillo, CA, USA) for FAK. The membranes were probed with either horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, and developed as described above.

3. Results

3.1. Composition of GSLs in MCF-7/6 and MCF-7/AZ cells, in GEM and in soluble fraction (Fr.) 11–13

Major GSLs found in these two MCF-7 variants were identified as globo-series Gb3, Gb4, Gb5, and α1→2 fucosyl-Gb5 (globo-H), defined by TLC immunostaining with mAbs 1A4, 9G7 [21], MC631, and MBr1, respectively. A major slow-migrating ganglioside was identified as MSGb5, defined by mAb RM1 (Fig. 1A). Ganglio-series structures (each defined by specific mAb; see Section 2) such as GM3 and GM1 were absent, but GM2 was present as a major component, overlapping with Gb5 (Fig. 1A). Lacto-series GSLs were not detectable. Compositions of GSLs as above were compared for Fr. 5 (GEM) vs. soluble high-density Fr. 11–13. Each GSL was present in Fr. 5 but completely absent in Fr. 11–13 (Fig. 1B).

Presence of Gb3, and other globo-series structures similar to Gb5 and fucosyl-Gb5, in MCF-7 cells was reported previously [22]. This previous paper also reported the abundant presence of GM3 and GM1 in MCF-7 cells, but the variant used in the present study lacked GM3 and GM1.

3.2. Other components found in GEM

Various membrane proteins and glycoproteins associated with GEM (low-density Fr. 4–6) or associated with soluble high-density Fr. 11–13 are shown in Fig. 2A,B. Levels of integrins α1, α2, α3, and cSrc were essentially the same between 7/6 and 7/AZ. CD9 in 7/AZ was present at high level in GEM, whereas CD9 in 7/6 was virtually absent in GEM. All other membrane-associated proteins, i.e. MUC1, E-cadherin, β-catenin, cSrc, and CD9, were found in GEM as well as in Fr. 11–13. Levels of MUC1, E-cadherin, and β-catenin were essentially identical between 7/6 and 7/AZ. All these components in GEM of various types of cells are known to be involved in cell adhesion coupled with signal transduction.

Because CD9 showed a major difference between 7/6 and 7/AZ, we compared cell surface expression patterns by flow cytometry. 7/AZ showed much higher expression than 7/6; expression index was 60.0 vs. 27.3 (Fig. 2C). However, this was much less than the difference observed in CD9 level in GEM, which was very low in 7/6 but high in 7/AZ (Fig. 2B, left lower panel).

3.3. Anti-MSGb5 mAb RM1 strongly enhances motility and invasiveness of 7/AZ but not 7/6 cells

Both 7/AZ and 7/6 cells are non-invasive through type I collagen gel layer as determined by a method using a specific assembly [19], and motility of the two variants as determined by wound migration assay is essentially the same. However, when 7/AZ cells were treated with anti-MSGb5 mAb RM1, their invasiveness and motility were greatly enhanced (Fig. 3A, columns 'RM1'). Control IgG or IgM antibodies from normal mouse serum, and antibodies to other GSLs (anti-Gb3 1A4, anti-Gb5 SSEA-3, anti-GM2 MK1-8), did not enhance invasiveness or motility of either variant (Fig. 3A, all other columns). When 7/AZ cells were pretreated with cSrc kinase inhibitor Pp1, the enhancement of invasiveness and motility as above was blocked (Fig. 3A, columns 'Pp1' and 'RM1+Pp1'). 7/AZ cell motility determined by wound migration assay, indicating enhancement in the presence of RM1, and its inhibition in the presence of Pp1, are shown in Fig. 3B (top). 7/6 cell motility was not affected by RM1 or Pp1 (Fig. 3B, bottom). Quantitative evaluation of 7/AZ cell motility change in wound migration assay is shown in Fig. 3C.

3.4. RM1-dependent enhancement of cSrc and FAK activation in 7/AZ cells, and absence of such effect in 7/6 cells

The motility/invasion-promoting effect of RM1 on 7/AZ cells as above suggests that RM1 initiates signaling in 7/AZ but not in 7/6 cells. We therefore examined effect of RM1 on activity of cSrc kinase and FAK. Chemical levels of cSrc and FAK in both variants were unchanged by RM1 treatment, but cSrc kinase activity and FAK activity in 7/AZ were greatly increased after 10–30 min RM1 treatment (Fig. 4, left panel). There was no such activation of cSrc or FAK by RM1 treatment in 7/6 cells (Fig. 4, right panel).

4. Discussion

Metastatic and invasive properties of tumor cells are of central importance for understanding tumor malignancy. Any mechanism revealed could be a target for inhibition or prevention of tumor progression. Many recent studies have revealed that tumor cell penetration into extracellular matrix (ECM), based on ECM-dependent enhanced motility coupled with ECM-destructive activity, is often associated with high metastasis (e.g. [23–25]). The present study is focused on: (i) composition of GSLs, signal transducers, and other membrane receptors in GEM fraction of two variants of breast carcinoma cell line MCF-7, 7/AZ vs. 7/6; (ii) stimulation of major ganglioside MSGb5 by its antibody RM1 enhances motility and invasiveness through activation of cSrc and FAK in 7/AZ but not 7/6 cells. These results suggest a close connection of MSGb5 with cSrc and FAK in GEM, although co-immunoprecipitation was difficult to perform since RM1 is IgM. In contrast, GM3 was successfully co-immunoprecipitated with cSrc, RhoA, and FAK in B16 melanoma cells in our previous studies [5,6], since anti-GM3 antibody is IgG3. Nevertheless, it is plausible that MSGb5 in GEM, stimulated by RM1, may initiate a signal leading to strong enhancement of tumor cell penetration into collagen gel, and of cell motility as determined by wound migration assay. This connection was observed only in 7/AZ, but not in 7/6. The connection was evidenced by tyrosine kinase activation of cSrc and FAK following stimulation of MSGb5 by its mAb RM1 in 7/AZ,

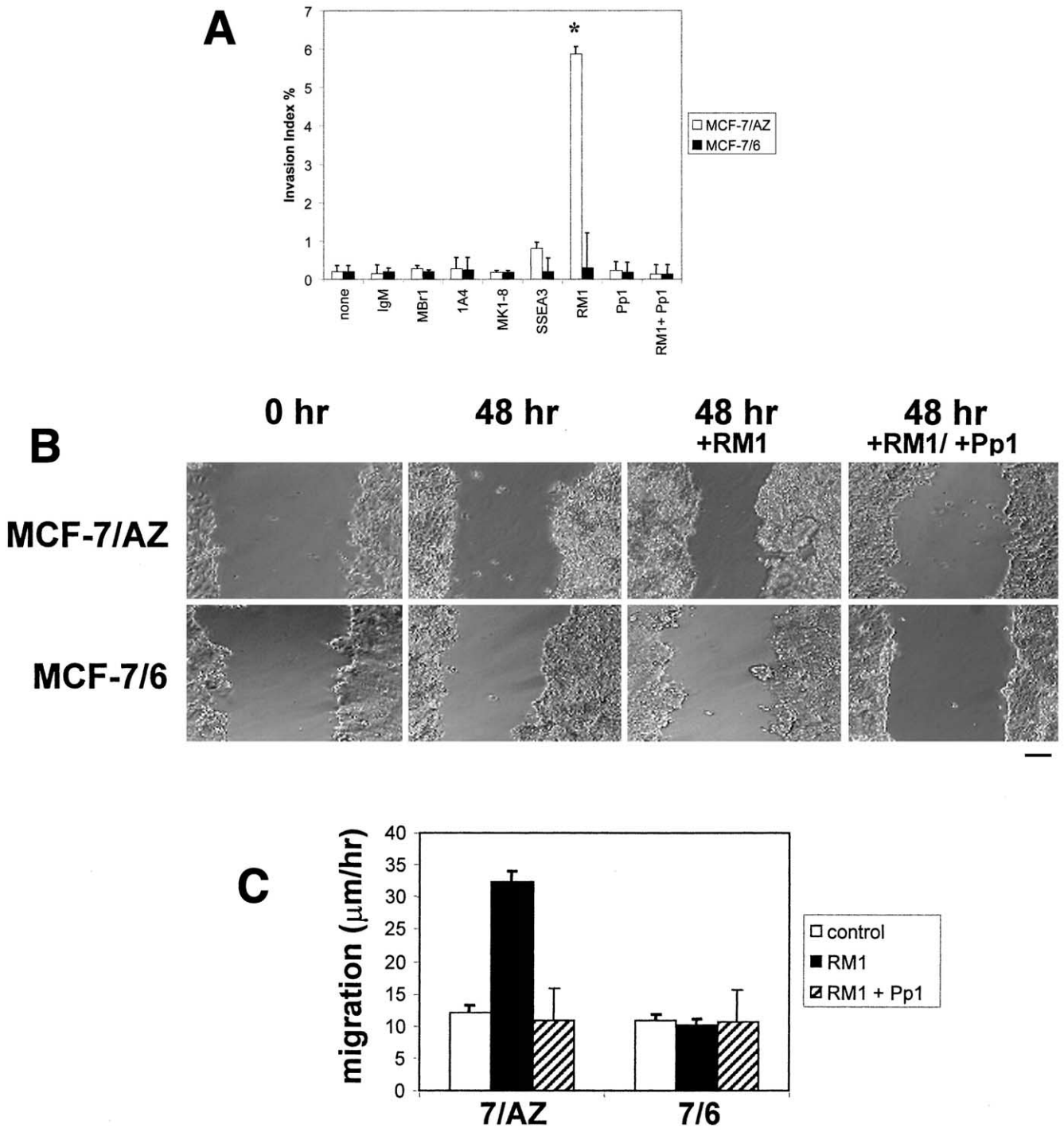


Fig. 3. Panel A: Effect of mouse IgM or antibodies against Gb3 (1A4), Gb5 (SSEA3), globo-H (MBr1), MSGb5 (RM1), GM2 (MK1-8), cSrc kinase inhibitor (Pp1), or RM1 plus Pp1, on invasion into collagen type I of 7/AZ (white columns) and 7/6 cells (black columns). Bars indicate standard deviation; asterisks indicate statistical difference from controls (7/AZ and 7/6 without antibodies). 1:10 dilution was used for 1A4, SSEA3, and RM1 since they are hybridoma culture supernatants; 1:1000 dilution was used for MBr1 and MK1-8 since they are ascites or purified samples; 1:100 dilution was used for control purified IgM. At these dilutions, approximate mouse IgM concentration was 1.5–3.0 μg/ml. Final concentration of Pp1 was 20 μM. Panel B: Effect of anti-MSGb5 antibody (RM1; dilution 1:10) or cSrc kinase inhibitor (Pp1; 20 μM) on 7/AZ and 7/6 migration in wound migration assay. Cells were plated in 24-well plates. After 40 h, cells were wounded, and allowed migration for 8 h in the presence of RM1, Pp1, or both. Scale bar = 250 μm. Panel C: Migration (expressed in μm/h) of 7/AZ and 7/6 cells in wound migration assay (see text). Open bars, controls (mouse IgM). Black bars, cultured in presence of RM1 (dilution 1:10). Striped bars, cultured in presence of RM1 plus Pp1 (20 μM).

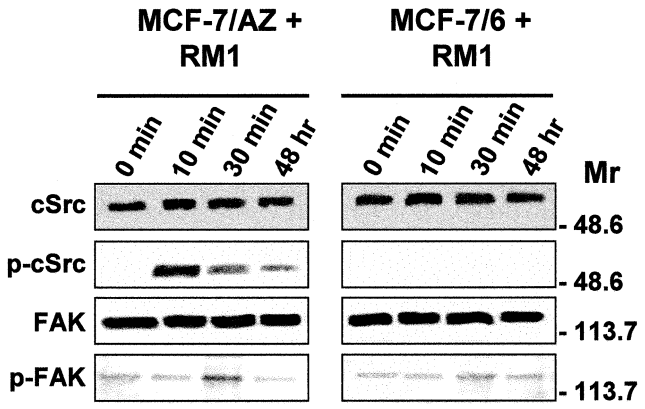


Fig. 4. Time course effect of anti-MSGb5 mAb RM1 on activity of cSrc and FAK kinases in 7/AZ vs. 7/6 cells, determined by blotting with antibodies defining PY416 for cSrc, and PY397 for FAK. Quantity of cSrc was determined using antibody SRC2. Notations at left margin: cSrc, quantity of cSrc; p-cSrc, level of PY416; FAK, quantity of FAK; p-FAK, level of PY397, at times indicated at top. Notations at right margin: molecular mass (kDa).

and lack of such response in 7/6. A search for any differential level of GSLs, transducers, or membrane receptors (MUC1, MUC4, E-cadherin, integrins α1, α2, α3) in 7/AZ vs. 7/6 was unsuccessful. The only difference between the two variants was in CD9 level, which cannot be logically correlated with RM1-induced cSrc or FAK activation, nor with induction of invasiveness/motility. Since CD9 level was high in GEM fraction of 7/AZ but essentially zero in GEM of 7/6, there is a possibility that CD9 in 7/AZ GEM may mediate connection of stimuli caused by RM1-induced clustering of MSGb5 with activation of cSrc and FAK. However, co-immunoprecipitation was not observed between CD9 and cSrc or FAK under conditions used in this study. The connection may be mediated by a yet-unexplored lipid such as cholesterol or sphingomyelin, which are enriched in GEM fraction, as is CD9. This idea is plausible in view of the recent identification of CD9 as a proteolipid, a highly lipophilic component.

MSGb5 and FucGb5 (globo-H) are tumor-associated antigens. The current results suggest that stimulation of MSGb5 through its antibody RM1 activates cell motility and invasiveness, although the pathobiological implications of this phenomenon remain to be studied. It is plausible that MCF-7 cell adhesion to target cells is mediated by binding receptor (e.g. siglec) or complementary carbohydrate (through carbohydrate-to-carbohydrate interaction), and that this step is followed by transducer activation leading to enhanced invasiveness of MCF-7 cells. Such process, in general, is consistent with our predictions for "Type 1 glycosynapse" [26], and is analogous to melanoma cell binding to endothelial cells through melanoma-associated GM3 antigen followed by transducer activation [4,27,28].

An alternative possibility is that MSGb5 in both 7/AZ and 7/6 cells has an inherent inhibitory effect on cell motility, through inhibition of cSrc and FAK. RM1 may block such inhibitory effect, and the extent of such blocking may differ between 7/AZ and 7/6 cells because of their different CD9 level.

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