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Development of a CA125-mesothelin cell adhesion assay as a screening tool for biologics discovery

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Abstract

Preventing peritoneal implantation of ovarian carcinoma cells could prolong patient remission and survival. CA125 is expressed on most ovarian cancer cells and was reported to be a ligand of mesothelin, a peritoneal protein. We developed a cell adhesion assay with CA125-expresser ovarian cancer cells and human mesothelin-transfected cells and we confirmed that CA125 and mesothelin mediate cell attachment. We also showed that this assay supplies a high-throughput screening system for reagents able to block CA125/mesothelin-dependent cell attachment with a sensitive quantitative readout. We finally demonstrated that a mesothelin chimeric protein and anti-CA125 antibodies block CA125/mesothelin-dependent cell attachment.

Keywords

CA125; Mesothelin; High-throughput screening; Cell adhesion model system; Ovarian cancer

1. Introduction

Attachment of ovarian cancer cells to the mesothelial lining of the peritoneum is necessary for the metastatic spread typical of recurrent ovarian cancer. Mesothelial cells normally express mesothelin on their surface while most ovarian cancer cells express CA125 [1]. Rump and colleagues demonstrated that CA125 binds to mesothelin in a specific manner [2]. Thus, mesothelin/CA125 interaction may facilitate peritoneal metastasis by initiating cancer cell attachment to the mesothelial epithelium.

Mesothelin is a 40 kDa membrane-bound protein [3] that results from the cleavage of a 69 kDa preproprotein encoded by the *MSLN* gene. The alternative splicing of the *MSLN* gene results in at least two transcript variants. MSLN1 represents the predominant transcript or variant (1) (accession NM_005823) and encodes isoform 1 that we used to build up our cell adhesion assay. The transcript variant (2) MSLN2 (accession NM_013404) uses an alternate splice site in the coding region, resulting in a longer transcript that includes a 21 bp insertion in position 1229 of variant 1. The cleavage of MSLN-encoded preproprotein at the cationic motif TILRPRFRREVE releases the megakaryocyte potentiating factor (MPF), a 31 kDa soluble

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protein [4,5] while mesothelin remains membrane-bound. However, mesothelin also exists as a soluble form and has been detected in sera of ovarian carcinoma and mesothelioma patients [6–8], possibly after cleavage of its hydrophobic glycosylphosphatidylinositol (GPI) anchor [8] or as a variant lacking GPI anchor motif due to a reading frame shift [6]. Mouse mesothelin is 55% homologous to its human counterpart. The protease target sequence TVIHP*R*F*RR*DAE is conserved, although the mouse sequence may not be optimal for proteolytic cleavage (mouse *R*×*RR* compared to human *R*×*R*×*RR*). Mesothelin knockout mice have no obvious phenotype [9] and the biological function of mesothelin remains to be elucidated, as does that of CA125.

CA125 is a mucin-like protein of high molecular mass, estimated from 200 to 20,000 kDa, although smaller subunits have been reported [10,11]. In spite of its size, CA125 carries only two major antigenic domains, leading to only two groups of anti-CA125 antibodies: the OC125-like (group A) and the M11-like (group B) [12]. The rarity of CA125 antigenic domains may be due to its unusual structure, which consists of more than 60 repeat units of 156 amino acids (AA) [13,14] and to its immunosuppressive effects [15] that may prevent immunized mice from developing a diversified population of anti-CA125 antibodies. CA125 cell surface expression is upregulated when cells undergo metaplastic differentiation into a Müllerian-type epithelium [16] but is also detected on normal epithelia of the female genital tract [17,18] and on fetal coelomic epithelium and derivatives. Like mesothelin, CA125 is found in human sera and ascites. The release of CA125 soluble proteolytic fragments into the extracellular space [19] appears to be triggered by serine/threonine and/or tyrosine-dependant phosphorylation within the cytoplasmic domain [20] and is affected by cell cycle, proliferation, various growth factors and cytokines [10,21] and the conversion from benign to malignant cells [22]. CA125 is the most extensively studied biomarker for possible use in the early detection of ovarian carcinoma [23–28]. However, although CA125 is conserved in some mammals [29,30] its study is hampered by its lack of conservation in rodents. The absence of CA125 in rodents may be due to ovarian biological differences and thus it is difficult to extrapolate from mouse to human.

We have developed an all-human cell adhesion assay with an ovarian cancer cell line that expresses CA125 (OVCAR-3) [31] and immortalized embryonic kidney (HEK 293F) cell lines [32] transfected with MPF, mesothelin and MSLN1 coding sequences. This assay supplies a high-throughput screening for reagents that block CA125-mesothelin mediated cell adhesion with a sensitive quantitative readout. We screened a number of reagents and we demonstrated that CA125/mesothelin-dependent cell attachment is partially blocked by mesothelin fused to an Ig protein [33] and cross-linked with an anti-human Ig mouse monoclonal antibody (mAb), and completely blocked by several anti-CA125 mAbs of group B.

2. Material and methods

2.1. Constructs (Fig. 1)

cDNAs encoding full-length human mesothelin (MSLN1), MPF (MSLN1 amino-terminal domain), mesothelin (MSLN1 carboxy-terminal domain) were amplified by PCR from the clone MGC:10273 IMAGE:3957372 (ATCC, Manassas, VA). The full-length MSLN1 was amplified with the primers MSLN1 forward (5′-aagcttttcgaagccgccatggccttgccaacggctcgacccc-3′) and MSLN1 reverse (5′-tctagattatcaggccagtgtggaggctaggagcagtgc-3′) and ligated to pCR®-TOPO® vector (Invitrogen Corporation, Carlsbad, CA). After verification by sequencing, MSLN1 was excised with *Hind* III and *EcoR* I and ligated into *Hind* III/*EcoR* I-cut pcDNA3.1/zeo(+) (Invitrogen) (Fig. 1(a)). To express mesothelin at the cell surface, mesothelin cDNA was amplified with the primers Meso forward (5′-gccaccggtgcagaagtggagaagacagcctgtccttc-3′) and Meso reverse (5′-gcctctagattatcaggccagggtggaggctaggagcagtgccaggacggtgag-3′) to create a sequence with flanking *Age* I and *Xba* I sites and ligated to the *Age* I/*Xba* I-cut vector pcDNA3.1/hygro (+)

(Invitrogen) modified by the insertion of a Kozak sequence [34] followed by the first 30 AA of HE4 leader [35] and an *Age* I site (Fig. 1(b)). To express MPF at the cell surface, MPF cDNA was amplified with the primers MPF forward (5′-ctagagatctatggccttgccaacggctcga-3′) and MPF reverse (5′-catgccgccggaggatggtccgttcaggctg-3′) to create a sequence flanked by *Bgl* II and *Sac* II sites and ligated to *Bgl* II/*Sac* II-cut pDisplay vector (Invitrogen). pDisplay-MPF encodes a protein fused to the PDGFR transmembrane domain and tagged with *c*-myc and HA (Fig. 1(c)).

To produce soluble chimeric proteins, mesothelin and MPF cDNAs were truncated for, respectively, their 3′ and 5′ end terminal sequences because they are predicted by TMpred [36] to encode hydrophobic stretches that could interfere with protein secretion (Fig. 1(d) and (e)). A mesothelin fragment was PCR amplified with Meso forward and Meso-Ig reverse (5′-gccagatctgccctgtagccccagccccagcgtgtccaggtcgtcctgccg-3′) to create a sequence with flanking *Age* I and *Bgl* II sites (Fig. 1(d)). A MPF fragment was amplified with the primers MPF-Ig forward (5′-gccaccggtgctggagagacagggcaggctg cgcccctg-3′) and MPF-Ig reverse (5′-gccagatctggcgaggatggtccgttcaggctgccgccaggatgg-3′) to create a sequence flanked by *Age* 1 and *Bgl* II sites (Fig. 1(e)). Mesothelin and MPF fragments were cloned into the modified *Age* I/*Bgl* II-cut pcDNA3.1/hygro(+) vector. A 696 bp fragment encoding a truncated human IgG1 was PCR amplified from human B lymphocytes with the primers huIgG1 forward (5′-gccagatctggagcccaaatcttgtgacaaaactcacacatgcccaccgtgccca-3′) and huIgG1 reverse (5′-gcctctagattatcatttacccggagacagggagaggctcttctgcgtgtag-3′) to create a sequence with flanking *Bgl* II and *Xba* I sites and ligated to *Bgl* II/*Xba* I-cut modified pcDNA3 vector in frame with mesothelin or MPF (Fig. 1(d) and (e)).

2.2. Cell lines, transfections, cell culture and cell lysates

The constructs encoding cell-surface proteins and secreted Meso-Ig were stably transfected into HEK 293F cell lines (ATCC, Manassas, VA) with Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions and selected with hygromycin B (Invitrogen) or Geneticin® (Sigma–Aldrich, Corp. St Louis, MO). The constructs encoding secreted MPF-Ig was transiently transfected into HEK 293F cell lines with lipofectamin 2000. Cells were incubated at 37 °C with 5% CO_2 in a humidified atmosphere. HEK 293F cells and all transformants were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS(ATCC), 100 units penicillin–streptomycin (Invitrogen) and 0.2 mM-glutamine (Invitrogen).

The ovarian carcinoma cell line OVCAR-3 (ATCC) was grown in RPMI-1640 (Invitrogen) supplemented with 20% FBS 100 units penicillin–streptomycin and 0.2 mM-glutamine.

2.3. Protein secretion and purification

HEK 293F cells secreting chimeric proteins were incubated in serum-free medium for 48 h before harvesting the medium. The Ig-fusion proteins were purified from the media using Ultralink Protein A (Pierce, Rockford, IL) according to standard procedures, dialyzed against phosphate buffered saline (PBS) and stored at −80 °C.

2.4. Flow cytometry analysis and sorting

Cells transfected with MSLN1 or mesothelin constructs were analyzed by flow cytometry on Becton Dickinson FACScan Cytometer for their binding to 4H3, an anti-mesothelin mouse monoclonal antibody (4H3 mAb) that does not recognize MPF [6]. 4H3 was detected with Alexa Fluor® 488 F(ab′)2 fragment of goat anti-mouse IgG (H+L) (488 anti-mIg) (Invitrogen). In the absence of MPF-specific antibody, MPF-transfected cells were analyzed for their cell surface expression of *c*-myc tagged protein with an anti *c*-myc mAb (Santa Cruz biotechnology,

Santa Cruz, CA) and detected with 488 anti-mIg. The highest expressers were flow sorted with the Becton Dickinson FACS Vantage SE Cell Sorter and selectively grown.

2.5. Western blots

Purified chimeric proteins were mixed with 2× SDS loading buffer supplemented with 5% mercaptoethanol (Sigma–Aldrich), denatured by heating and separated by electrophoresis on a 4–12% NuPAGE Bis Tris gel (Invitrogen) using SDS running buffer (Invitrogen). The gels were transferred to a PVDF membrane (Invitrogen) in NuPAGE Transfer Buffer (Invitrogen) using an X-cell II blot module (Invitrogen). PVDF membranes were blocked with blocking buffer (Superblock, Pierce) supplemented with 0.05% Tween 20 (Fisher Biotech, Fair Lawn, NJ). The Ig-fusion proteins were detected with an HRP-conjugated F(ab′)2 fragment goat anti-human IgG (H+L) (HRP-anti-hIg) (Jackson Immuno Research Laboratory, Inc., West Grove, PA). Detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to standard procedures.

2.6. Heterotypic cell adhesion assay

Twenty-four to thirty-six hours prior to the assay, the adherent OVCAR-3 cells were distributed at 5×10^5 cells/ml into tissue culture sterile 96-well flat transparent bottom wells and black wall plates (Corning Costa3603, Corning, NY) and incubated at 37 °C to a confluence of 70–90%. On the day of the assay, wild-type HEK 293F cells or cells transfected with MPF, Mesothelin or MSLN1 constructs were fluorescently labeled with Vybrant® Cell Adhesion assay Kit (Invitrogen-GIBCO, Carlsbad CA) according to the manufacturer's instructions. OVCAR-3 cells immobilized in the wells were washed once with 0.2 ml of warmed DMEM medium and labeled HEK 293 cells were distributed in triplicate at a final concentration of 2.5 or 5×10^6 cells/ml and incubated with the OVCAR-3 cells at 37 °C for 1 h. Fluorescence was measured before and after washes with 0.2 ml of prewarmed PBS with FL×800™ Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc. Winooski, VT). For normalization, the after wash-fluorescent signals were divided by the fluorescent signal read before washing. The normalized values were averaged. Standard deviations were less than 4%.

2.7. Blocking agents for cell adhesion assay

HEK 293F cells were preincubated with 10 μg/ml of anti-mesothelin antibody in 0.1 ml DMEM medium for 30 min at 37 °C. Alternatively, OVCAR-3 cells were preincubated with 10 μg/ml of anti-CA125 antibody in 0.1 ml DMEM medium for 30 min at 37 °C or with 10 μg/ml of chimeric proteins alone or cross-linked. To cross-link the proteins, an equal amount of anti-human Ig mAb (Jackson ImmunoResearch Laboratories, Inc.) was added [37] and incubated at a final concentration of 10 μg/ml in DMEM medium for 30 min before the incubation with OVCAR-3 cells. Anti-mesothelin mAb 4H3 was acquired from the Hellström laboratory [6]. Anti-CA125 mAb were acquired from Fujirebio Diagnostics, Inc (FDI, Malvern, PA) (OC-125 and M11), Fitzgerald Industries International, Inc. (FIII, Concord, MA) (M8072320, M8072321, M002201 and M002203) and Research Diagnostics, Inc. (RDI, Flanders, NJ) (X306 and X52). M8072320, M002201 and X306 epitopes are OC-125-like (group A) while M8072321, M002203 and X52 are M11-like (group B) [17,38].

3. Results

3.1. Validation of HEK 293F transfected cells

Cells transfected with MSLN1 or mesothelin constructs were incubated with 4H3 and analyzed by flow cytometry (Fig. 2(a) and (b)). In the absence of MPF-specific antibody, HEK 293 cells transfected with MPF construct were incubated with anti-*c*-myc mAb to detect the transfected

tagged-protein (Fig. 2(c)). The purified supernatants of HEK 293 cells transfected with Meso-Ig or MPF-Ig constructs were analyzed by western blot (Fig. 2(d) and (e)).

3.2. Development of a CA125/mesothelin-dependant cell adhesion assay

Fluorescent-labeled HEK 293F cells were incubated with an adherent monolayer of OVCAR-3 cells in 96-well plates. Gentle washing quickly decreased the fluorescent signal given by wild type HEK 293F cells, but mesothelin-transfected cells were resistant to washing (Fig. 3(a)). We observed that although the fluorescent signals were clearly proportional to the quantity of labeled cells, they were also variable from one staining to another (from 1000 to 3000 for 2×10^6 cells), hence the necessity to normalize our readings. Fig. 3(b) shows that after normalization of the fluorescent signals, mesothelin- and MSLN1-trans-fected HEK293F adhere equally well to OVCAR-3 cells and more strongly than wild type cells or MPF-transfected cells. This confirms that mesothelin mediates cell attachment to OVCAR-3 and demonstrates that CA125/mesothelin interaction can be assessed by an assay compatible with high-throughput screening.

3.3. Identification of agents able to block CA125/mesothelin-dependant cell adhesion

Identifying agents that block CA125/mesothelin interaction could help design treatments to prevent peritoneal spreading of ovarian carcinoma cells. Using the CA125/mesothelin cell adhesion assay, we tested several agents for their ability to interfere with cell attachment. First, we tested chimeric proteins. Fig. 4(a) shows that Meso-Ig cross-linked with an anti-Ig antibody interfered with CA125/mesothelin-dependant cell attachment while Meso-Ig alone did not; neither MPF-Ig nor cross-linked MPF-Ig affected the cell attachment. We also tested several antibodies directed against CA125 and mesothelin. Fig. 4(b) shows that all anti-CA125 mAb tested interacted with the cell adhesion and decreased the fluorescence signal by 30% or more. Three out of the four M11-like anti-CA125 mAb decreased the fluorescence signal by 85% or more, which suggested that anti-CA125 antibodies of group B are more efficient that antibodies of group A for blocking CA125/mesothelin-dependant cell adhesion. The addition of the anti-mesothelin mAb 4H3 during the incubation did not block the MSLN1-transfected 293F cell adhesion to OVCAR-3 cell line (data not shown).

4. Discussion

Spreading and implantation of ovarian carcinoma cells to adjacent organs through peritoneal fluid is characteristic of ovarian carcinoma and contributes to the grim prognosis of the disease. Blocking the CA125/mesothelin interaction is a potential therapeutic target, but the absence of a CA125 homolog in rodents makes it difficult to test therapeutic approaches in vivo.

Here, we present a cell adhesion assay using human cell lines that can measure 100s of conditions in a single experiment and thus supplies a high-throughput screening system with a sensitive quantitative readout for reagents that block CA125-mesothelin mediated cell adhesion. Using this assay, we confirmed that CA125 and human mesothelin mediate cell attachment and we showed that cells transfected with MSLN1, full-length or carboxy-terminal domain (mesothelin), bind equally well to CA125-expresser cells. This was not surprising because mesothelin results from the cleavage of a preproprotein encoded by MSLN1. It would be of interest to study the pathways that trigger the preproprotein cleavage since interfering with it might prevent CA125/mesothelin-dependant cell adhesion.

We showed that the CA125/mesothelin-dependent cell adhesion could be partially blocked by a cross-linked chimeric mesothelin protein. The need to cross-link mesothelin to disrupt cell attachment hints at the importance of the cooperativity of blocking compounds. Soluble mesothelin is detected in patient sera and ascites and a recent publication demonstrates the

existence of a humoral immune response to mesothelin in mesothelioma and ovarian cancer patients [39]. The presence of soluble mesothelin in patients does not seem to be linked to a better prognosis or survival (observation based on 33 ovarian cancer cases followed for more than 2 years—Urban N, unpublished data) but to our knowledge the presence of both soluble mesothelin and anti-mesothelin-autoantibodies has not been correlated to patient outcome. Addressing whether mesothelin/anti-mesothelin mAb complexes could interfere with ovarian cancer cell attachment to the peritoneum is clinically relevant. A positive correlation between favorable prognosis and presence of both circulating mesothelin and anti-mesothelin auto-antibodies may be an incentive for the development of anti-mesothelin antibodies as well as vaccination strategies against mesothelin aimed to prevent metastastatic disease.

Although 4H3, an anti-mesothelin antibody, could not block CA125/mesothelin-dependent cell adhesion, we believe that other antibodies directed against different mesothelin epitopes might demonstrate some activity in this model system. In contrast, CA125/me-sothelin-dependent cell attachment could be completely blocked with three anti-CA125 mAb of group B and partially blocked with five other anti-CA125 mAbs. Although the two anti-CA125 antibody groups do not overlap, the epitopes of both groups lie within the 156 AA repeat units of CA125. This suggests that CA125 binds to mesothelin through a site located in the 156 AA repeat units and that the mesothelin binding site shares more homologies with M11 epitope than with OC-125 epitope.

Metastasis remains a major challenge in the clinical management of ovarian cancer [40–42]. Various approaches to maintenance therapy are being evaluated but they rely on continued treatment with highly toxic chemotherapeutic agents. Biologics that are better tolerated by the patients, like Herceptin, an anti-Her2/Neu used for breast cancer therapy [43,44] are urgently needed as a way to maintain remission. We developed a robust and high throughput cell adhesion assay that makes it possible to test small molecules and new generations of biologics for their ability to disrupt the cell attachment that underlies ovarian cancer metastasis. Our results suggest that developing therapeutic agents such as humanized or recombinant antibodies against CA125 or mesothelin may help to prevent the implantation of ovarian carcinoma cells in the peritoneum.

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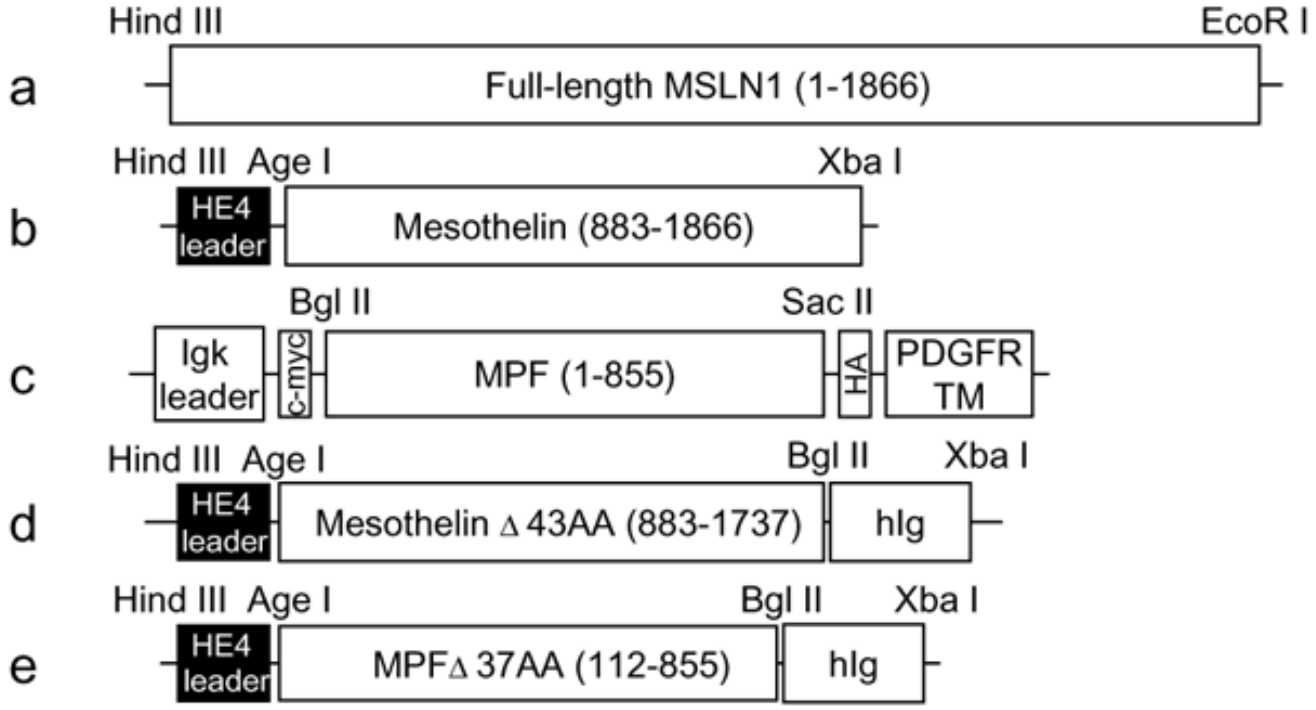

Fig. 1.

Constructs. (a)–(c) Constructs for cell surface expression: (a) full-length MSLN1 cDNA (1866 bp) was ligated to pcDNA3.1/−zeo(+) vector in between *Hind* III and *EcoR* I sites; (b) the N-terminal domain of MSLN1 cDNA that encodes mesothelin (984 bp) was ligated to a modified pcDNA3.1/hygro(+) vector in between *Age* I and *Xba* I sites and in frame with an HE4 leader sequence; (c) the C-terminal domain of MSLN1 cDNA that encodes MPF (855 bp) was ligated to pDisplay vector between *Bgl* II and *Sac* II sites and in frame with Igk leader sequence, *c*-myc tag, HA tag and PDGFR transmembrane domain. (d)–(e) Constructs for secreted chimeric proteins: truncated forms of (d) mesothelin (855 bp) and (e) MPF (744 bp) were ligated to a modified pcDNA3.1/hygro(+) vector between *Age* I and *Bgl* II sites and in frame with HE4 leader and human Ig sequence. The beginning and end positions of the inserts relative to the sequence of MSLN1 clone IMAGE no. 3957372 are shown in parenthesis. The number of truncated amino acids (AA) follows the Δ symbol.

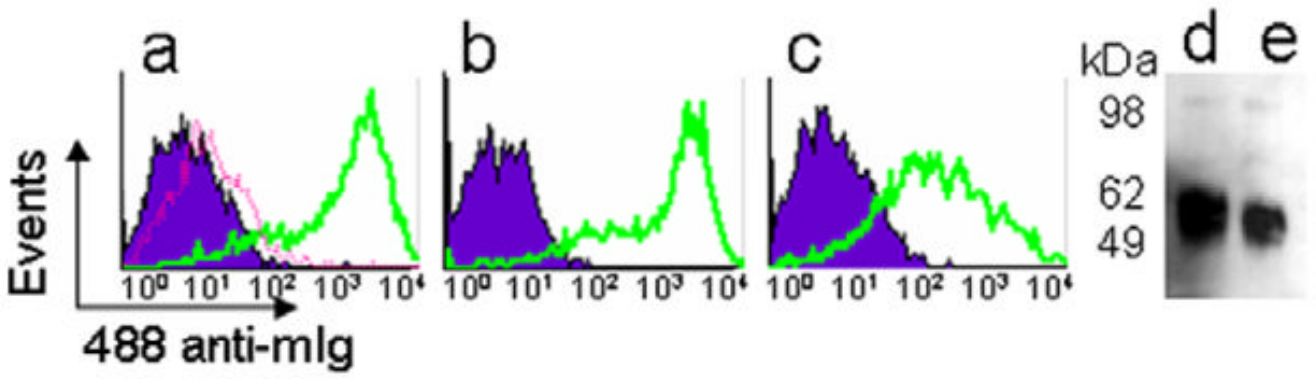

Fig. 2.

Validation of transfected HEK293F cells. (a)–(c) Flow cytometry analysis: HEK293F cells transfected with (a) MSLN1 or (b) mesothelin constructs were incubated with 10 μg/ml of 4H3 mAb (solid lines); (c) HEK293F cells transfected with MPF construct were incubated with 10 μg/ml of anti-*c*-myc tag mAb (solid lines). As negative controls, wild type (dotted line) or transfected cells (shade areas) were incubated with the secondary antibody only. (d)–(e) Western blot: (d) Meso-Ig and (e) MPF-Ig chimeric proteins were detected with an HRP-anti-hIg mAb.

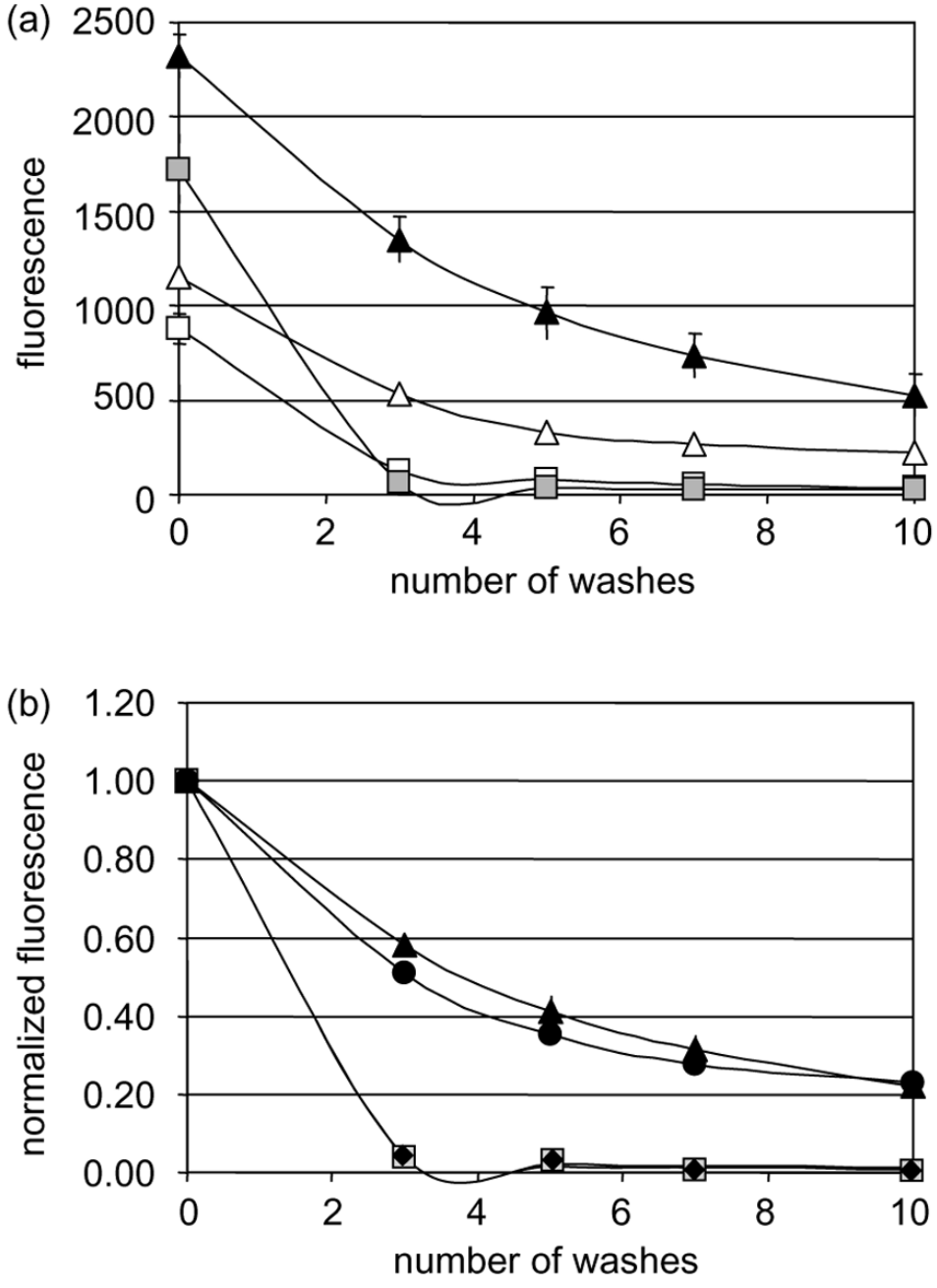

Fig. 3.

CA125/mesothelin-dependant cell adhesion assay. Ninety six-well plates containing OVCAR-3 adherent cells and fluorescently-labeled HEK 293 cells, wild type at 2.5×10^6 cells/ml (white squares) or 5×10^6 cells/ml (gray squares); or transfected with mesothelin at 2.5×10^6 cells/ml (white triangles) or 5×10^6 cells/ml (black triangles); MPF at 5×10^6 cells/ml (black diamonds); MSLN1 at 5×10^6 cells/ml (black circles) were washed up to 10 times. (a) Remaining fluorescence was plotted after each wash or (b) normalized to the original fluorescence for each well. The assay was performed in triplicates and the values were averaged. All standard deviations were less than 4%.

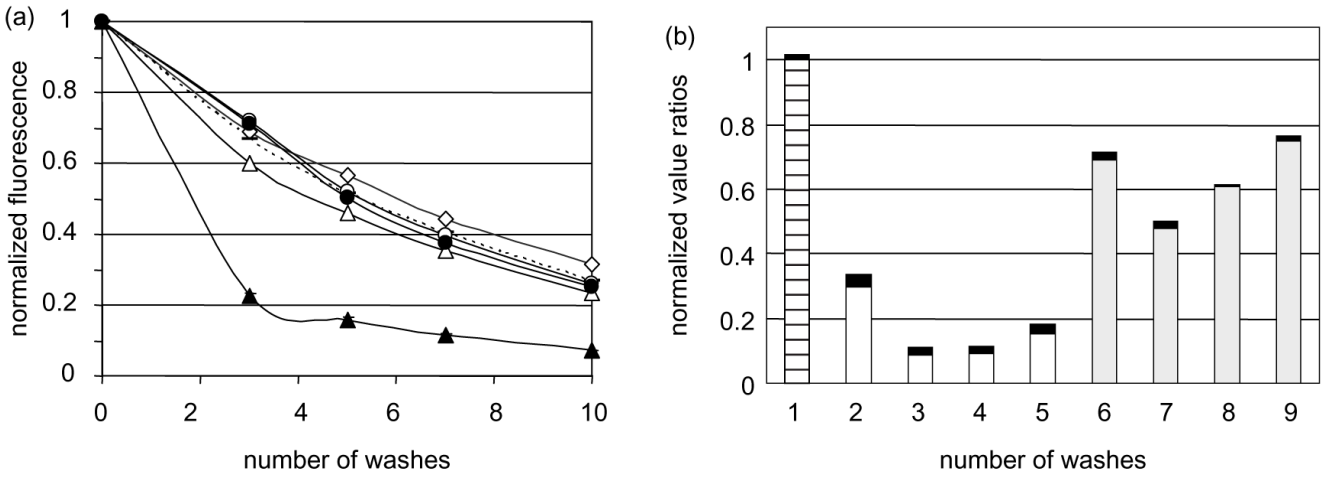

Fig. 4.

Cross-linked Meso-Ig and anti-CA125 mAb block cell adhesion (a) The cell adhesion assay was conducted in presence of 10 μg/ml of MPF-Ig alone (white circles) or cross-linked with anti-Ig mAb (black circles), or Meso-Ig alone (white triangles) or cross linked (black triangles). As a positive control for cell attachment, the assay was performed in medium only (white diamonds) or in medium supplemented with anti-hIg (dotted line). The assays were performed in triplicates. All standard deviations were less than 4%. (b) The cell adhesion assay was conducted after preincubation of OVCAR3 cells with 10 μg/ml of anti-CA125 antibodies (2=M002203; 3=M11; 4=M8072321; 5=X52; 6=M002201; 7=OC-125; 8=M8072320; 9=X306). The normalized fluorescent signals after five washes were divided by the fluorescent signal of the adhesion assay performed in medium only (1). The gray bars correspond to the anti-CA125 mAb of group A and the white bars to the group B. The black boxes represent the standard deviations and the stripped bar is the positive control for cell attachment.