A cell surface 230 kDa protein from B16 murine melanoma is involved with malignancy.

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Abbreviations: MAb, monoclonal antibody; MCAM/MUC18, melanoma cellular adhesion molecule also known as MUC18; PBS, Phosphate Buffered Saline; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; i.p, intraperitoneal; i.t, intravenous.

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Abstract

Melanoma incidence increases at a dramatic rate. It is relevant to identify novel melanoma antigens for diagnosis and monoclonal antibodies helping identifying such molecules. Several mAbs raised against murine melanoma B16, identify molecules correlated with carcinogenesis and in diagnosis. Herein, we describe a novel murine melanoma-associated 230 kDa molecule, expressed only by tumorigenic lines. Moreover, its expression is higher by more metastatic than less metastatic cells. MAb G12F2, produced against this antigen, inhibited proliferation, migration and invasion of B16 cells and enhanced complement activity in vitro. It also affected growth and lung metastasis formation in vivo. This as yet undescribed 230kDa molecule represents an important target for experimental melanoma studies, and may become a potential diagnostic marker for malignancy as well as an useful tool for immunotherapeutic approaches.
Introduction

Melanoma is a highly invasive and metastatic tumor with increasing incidence and mortality in recent years \textsuperscript{1-7}. Its metastatic potential may be associated with molecules expressed on the cell surface whose level of expression contributes to distinct metastatic phenotypes \textsuperscript{8-10}. One approach to identify such molecules correlated with stepwise changes from normal skin melanocyte to melanoma is the production of monoclonal antibodies \textsuperscript{12-14}. Various studies described mAbs against known melanoma antigens that could be used as markers for diagnosis and prognosis or as targets for immunotherapeutic approaches \textsuperscript{15-24}. However, markers defining different phenotypes typical of unique populations of melanoma are still lacking.

B16 murine melanoma cells have served as a model for studying carcinogenesis due to their diversity as it brings insights into the metastatic process since it allowed the isolation of subpopulations with different metastatic capacities \textsuperscript{14, 26-28}. Previously, we obtained by \textit{in vitro} cloning and \textit{in vivo} selection, variants of B16 melanoma with different metastatic potential as well as \textit{in vitro} kinetics of growth \textsuperscript{29}. These subpopulations may differ in the protein expression patterns and cell surface components. Identification of molecules expressed by murine melanoma creates the perspective of studying tumoral heterogeneity and searching for homologues in the human melanoma system.

Here we report the production of an IgM monoclonal antibody (G12F2) directed against B16 melanoma that reacted with $M_r$ 230,000 protein on malignant murine cell extracts, but recognized no antigen in non-tumorigenic lines such as melan-a. Furthermore, G12F2 effectively inhibited cell proliferation, invasion, migration and enhanced complement activity against tumor cells \textit{in vitro}. It also, inhibited tumoral growth and development of lung metastasis in syngeneic mice.
Material and Methods

Animals

Six-weeks-old male C57BL/6 mice were obtained from the animal facilities of Federal University of São Paulo, Brazil. Male athymic BALB/C nude mice of the same age were purchased from the University of São Paulo, Brazil; both were maintained in a pathogen free environment, with free access to food and water in accordance with NIH Guide for Care and Use of Laboratory Animals. All experiments were approved by the Ethical Committee.

Cell culture

Melan-a murine melanocyte cell line was cultured in RPMI, pH 6.9 (Sigma, St. Louis, MO, USA), containing 5% fetal calf serum (FCS, Campinas, SP, Brazil) and 200 nM 12-o-tetradecanoyl phorbol 13-acetate (PMA; Tocris, MO, USA). Melanoma cell lines (3C, 4C and Tm5) derived from melan-a cells after cycles of adhesion impediment were similarly, cultured without PMA. 3T3 murine fibroblasts, B16 murine melanoma cell lines, high metastatic variant and low metastatic variant were cultured as monolayers in RPMI 1640 medium, pH 7.4, with 10% FCS. All cell lines were maintained in 5% CO₂ and 95% air humid atmosphere at 37°C.
B16 cells were used to immunize C57Bl/6 mice. Sub-confluent monolayer cultures of B16 melanoma cells were detached with 2.0 mM EDTA (Invitrogen, Carlsbad, CA, USA) in PBS, centrifuged and washed once in sterile PBS. Six-week-old male C57BL/6 mice were i.p. injected with $10^6$ cells in Complete Freund’s Adjuvant (GibcoBRL, Grand Island, NY, USA). Two weeks later, blood was collected from ocular plexus, serum was separated by centrifugation and screened for the presence of anti-B16 antibodies using whole cell ELISA technique. Mice were intraperitoneally boosted twice at two-week intervals with $10^6$ B16 cells in Incomplete Freund’s Adjuvant. Three days after the last injection, spleen cells were isolated and fused with murine mieloma cells according to Lopes & Alves (1983). Cells from positive cultures were cloned by limiting dilution and expanded for ascitic fluid production by injection of $10^7$ hybridoma cells into the peritoneum cavity of mineral oil-primed athymic Balb/C mice. Monoclonal antibody class and subclass obtained were determined by the Mouse Antibody Isotyping kit (BD Biosciences, San Diego, CA, USA) following the manufacturer’s instructions. For biological assays, G12F2 and an irrelevant IgM ascitic fluid were precipitated by 70% saturated ammonium sulfate.

**Screening of Monoclonal Antibodies by whole cell ELISA**

For screening, B16 cells in RPMI 1640 FCS-free were added (2x10^4/0.1 ml/well) to 96-well tissue culture plates and allowed to attach for 12 hours in a humidified atmosphere of 5% CO₂ at 37°C. Cells, washed with sterile PBS were fixed with 0.5% glutaraldehyde in PBS (0.05 ml/well) for 15 minutes. Plates were again washed and blocked with 0.1% glycine (0.1 ml/ well)
in PBS for 15 minutes. Peroxidase endogenous activity was blocked with 3% hydrogen peroxide in PBS (0.2 ml/well), followed by washes with PBS. Next, wells were blocked with 5% fat-free milk in PBS (0.2 ml/well) for one hour at 37°C. Hybridoma supernatants were added to the wells, incubated for one hour at 37°C and washed three times with 2.5% milk, 0.25% Tween-20 (Sigma) in PBS. Peroxidase-labeled goat anti-mouse IgM (Zimed, San Francisco, CA, USA) was added, 50 µl/well in PBS, and incubated or one hour at 37°C. Plates were again washed and the substrate (1.0 mg of o-phenylenediamine in 5.0 ml of 0.1 M citrate-phosphate buffer [pH 5.0] plus 10 µl of 30% H₂O₂) was added. The reaction was interrupted with 4N H₂SO₄. Optical density at 492 nm was measured in an automatic MCC/40 plate reader (Labsystem Multiscan Dynatech, Chantilly, VA, USA).

Western Blotting

Cells were lysed in NP-40 lysis buffer, containing a protease inhibitor mixture (Sigma) according to the manufacturer’s instructions, and protein content was determined by the Bradford method (1976). Lysates were centrifuged at 12,000 rpm at 4°C for 20 minutes and separated by electrophoresis in a 10% polyacrylamide gel containing SDS (SDS-PAGE). Proteins were stained with Coomassie Brilliant Blue (Sigma) or electrotransferred to nitrocellulose (NC) membranes whose non-specific sites were blocked by 5% free milk in PBS. Membranes were washed with 0.05% Tween-PBS and incubated with G12F2 hybridoma supernatants or diluted ascitic fluid (1:100) for one hour at room temperature (RT). Bound antibodies were detected with peroxidase-labeled goat anti-IgM antibody for one hour followed by diaminobenzidine (DAB; Sigma) addition in the presence of H₂O₂. Controls were realized with normal mouse serum.
FACScan Analysis

Quantitation of G12F2 ligation to cell surfaces was analysed by FACS. 3T3, melan-a, 3C, 4C, Tm5, B16 (10^6 cells) lineages were detached, washed in PBS, centrifuged at 2000 rpm at 4°C and incubated with G12F2 or irrelevant IgM ascitic fluid (1:3 dilution) for 1h at 4°C. After washes with PBS, samples were incubated with phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-conjugated anti-murine IgM (Sigma) for 1h in ice and protected from light. Cells were fixed in 1% paraformaldehyde (Merck, Darmstadt, Germany) in PBS and examined by CellQUEST program using FACSCalibur (Becton Dickinson Immunocytometry Systems).

Confocal Microscopy

B16 cells (5x10^4) were cultivated on glass coverslips in RPMI 1640 containing 10% FCS for 48 hours. Cells were washed three times with PBS, fixed in cold methanol and incubated with G12F2 or irrelevant IgM ascitic fluid (1:8 dilution) for 1h. Samples were washed, incubated with fluorescein isothiocyanate (FITC)-conjugated antimurine-IgM (Sigma) with 50 μM DAPI (4’,6’-diamidino-2-phenylindole; Sigma) for nuclei staining plus 0.01% saponin (Sigma) in PBS-0.5% skim milk for 1 h at RT, light protected, and sealed on glass slides. Images of stained cells were analyzed in a Bio-Rad 1024 UV confocal system attached to a Zeiss Axiovert 100 microscope, using a 40x numerical-aperture 1.2 planapochromatic differential interference contrast water immersion objective.
**In vitro proliferation assay**

B16 cells were detached after exposure to 2mM PBS-EDTA and counted on a Neubauer chamber. Viability was evaluated by Trypan blue exclusion. Then, 2x10^3 cells were added per well of 96-wells plates and incubated for 24 hours at 37ºC. After incubation, G12F2 or control mAb were added in different concentrations (1 and 5 µg/ml) in RPMI 1640 FCS-medium. In order to measure the daily proliferation rate, 10 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] per 100 µl of culture medium were added and incubated for 90 minutes at 37ºC. Medium was discarded and 100 µl of cool isopropanol was added for spectrophotometry reading at 570 nm.

**Inhibition of tumor growth**

B16 cells in exponential growth were harvested by exposure to 2 mM EDTA in PBS. Cells were washed, counted and viability determined by Trypan blue exclusion (v/v). Only viable cells (2x10^5) pre-incubated with 100 µg G12F2 or irrelevant IgM or PBS only were used for injection in the scapular region. C57Bl/6 mice (n=5) were treated intravenously with G12F2, irrelevant IgM or PBS two days before tumor injection and once weekly for four weeks. Growth of subcutaneous tumors was measured with calipers every 2 days.

**Cytolytic activity mediated by complement**

B16 cells (10^4 cells/well) were cultivated in 96-well plates during 24 hours at 37ºC. Next, adherent cells were incubated with guinea-pig complement (Gibco) and G12F2 or control IgM at
0.5, 1.0, 2.5 and 5.0 µg/ml concentrations for 24 hours and viability was measured by MTT (5 mg/ml). Cells incubated with complement or mAb only were used as controls.

Migration and Invasion inhibition assay

Chemotaxis assay used uncoated 24-well cell culture inserts with 8µm-porosity polyethylene-terephylate membrane (Transwell®; Corning Costar Corporation, NY, USA). Cells were removed by non-enzymatic method and resuspended to a concentration of 2x 10^5 cells in 200 µl of serum free RPMI 1640 medium. Cells were pretreated for 30 minutes with G12F2 or a control IgM (5 µg/ml) and added to the upper chambers compartments. Lower chambers were filled with culture medium containing 10% FCS. Chambers were assembled by placing the uncoated membrane between the lower and upper compartments according to the manufacturer’s instructions. Migration assay was carried out at 37ºC for 24 hours, after which filters were removed, washed in PBS, fixed and stained with 1%toluidine blue. Cells on the upper surface of the filters were removed and only stained cells in the lower chamber were measured by spectrophotometry reading at 570 nm. To evaluate whether G12F2 could interfere with invasion, B16 cells were plated in 24-well cell culture inserts with a similar membrane coated with Matrigel (BD Biosciences) according to the manufacturer’s directions and the assay realized as described.

Inhibition of metastasis

To check the effect of G12F2 on metastasis of B16 melanoma, 10^5 cells plus G12F2 or irrelevant IgM in PBS were injected intravenously (IT) into C57Bl/6 mice (n=5). IT treatment
with mAbs (100 µg/ml) was made a week for three weeks. Mice were sacrificed 23 days after injection and lung metastatic colonies counted.

Statistics

Analysis of variance and the Tukey-Kramer test were used for statistical comparisons. All values were reported as the mean ± standard error of the mean.
Results

*Generation of a monoclonal antibody that recognizing a Mr 230,000 antigen on B16 cells*

The obtained hybridoma G12F2 produced an IgM\_k immunoglobulin that recognized a single sharp band of approximately 230 kDa by Western blotting analysis of B16 cell extract (Figure 1A). G12F2 also recognized B16 cells by flow cytometry (99%) as compared with control IgM (20%) and positivity higher than with anti-B16 serum (59%) (Figure 1B). Results suggested that the antigen recognized was localized on B16 cell surface. Confocal microscopy confirmed is expression on the cell surface as shown by peripheral pattern of FITC labeling (Figure 1C). No labeling was seen with irrelevant IgM.

*Mr 230,000 antigen was not expressed on non-tumorigenic cell lines*

G12F2 did not react by Western blotting against non-tumorigenic cell lines as melan-a melanocytes and 3T3 fibroblasts (Figure 2). Moreover, Mr 230,000 molecule was recognized by G12F2 on Tm5 and B16 tumorigenic lines, but not on 3C and 4C non-tumorigenic lines, all derived from melan-a (Figure 3A), data corroborated by FACS analysis (Figure 3B). These results suggest that Mr 230,000 molecule is tumor-specific.
Mr.230,000 antigen showed expression correlated with metastatic capacity

Previously, we described isolation of variants of B16 cell line with different in vivo metastatic patterns and in vitro kinetics of growth. Mr. 230,000 could be a differential marker of B16 subpopulations. Western blotting assay showed that this molecule was expressed in higher amounts on high metastatic B16 than low metastatic B16 extracts (Figure 4), suggesting that it could be associated with its metastatic phenotype.

G12F2 mAb inhibited proliferation of B16 cells

Next, we investigated whether Mr. 230,000 neutralization by G12F2 could affect biologic functions shown by B16 cells. Was evaluated, in vitro, G12F2 capacity to inhibit cell growth showing that addition at 1 and 5 µg/ml concentrations inhibited melanoma growth, as compared with control IgM (Figure 5), significantly in the fourth day. After five days, B16 cells cultivated with mAb G12F2 resumed growth tendency, suggesting that it was not cytotoxic.

G12F2 protects against tumor growth

Was investigated G12F2 capacity to affect tumor establishment in C57Bl/6 mice. Greater tumor growth was observed with irrelevant IgM or PBS treated groups (Figure 6) while growth was arrested in G12F2-treated mice until the last treatment day, suggesting that this mAb was protective in this model.
**G12F2 boosted cytolytic effect of complement against B16**

Since B16 cells are susceptible to complement lysis, we evaluated the cytotoxicity of G12F2 mAb plus complement *in vitro* and observed an increased cytotoxic effect. Complement and mAb reduced cellular viability when added alone, but this activity was amplified with both (Figure 7), suggesting that this mechanism could be responsible for growth inhibition *in vivo*.

**Inhibition of migration and invasion by G12F2**

Addition of G12F2 inhibited the migration of melanoma cells toward lower chambers of Transwell® systems, a finding not observed with control IgM (Figure 8A). Moreover, after 24 hours of incubation, cells previously treated with G12F2 exhibited significantly less invasion capacity through Matrigel-coated filters than control IgM-treated or untreated cells (figure 8B). Our data indicate that blockade of *M*<sub>r</sub> 230,000 molecule by G12F2 also decreased cellular migration and invasion functions.

**G12F2 inhibits metastasis**

*In vivo* anti-metastatic potential of G12F2 was evaluated in C57BL/6 mice treated IT with this mAb an experimental lung metastasis assay. Number of metastatic nodules was significantly smaller in G12F2-treated than in irrelevant IgM- or PBS- treated control mice (Figure 9A). Also, G12F2-treated mice developed metastases smaller in size than those seen in the control groups (Figure 9B). These results show that treatment with G12F2 decreased metastasis development by B16 cells.
Discussion

Herein we describe a mAb, G12F2, recognizing a high molecular weight antigen, of approximately 230 kDa, present on the cell surface of B16 murine melanoma, but not expressed by non-tumorigenic cells. Also, more malignant melanoma cells, as seen by the number of lung colonies, showed higher expression of the molecule as compared with less malignant cells. G12F2 mAb demonstrated biological activities against B16 cells in vitro, inhibiting proliferation, migration, invasion through Matrigel and by increasing cytolytic activity by complement. In vivo, G12F2 inhibited tumor nodule growth and lung metastases.

Several investigations suggested that changes in glycoproteins on the cell surface could be important in cancer metastasis, making the Mr 230,000 antigen a target for studies on carcinogenesis and metastases in the murine melanoma model (Fig. 1). The possibility of expression of a homologue molecule by human melanoma cells is being investigated.

The ideal target antigen is expressed in a tumor-specific manner. MAb G12F2 did not bind and/or affect normal cells as fibroblasts and melanocytes (Fig. 2) and instead, recognized only tumorigenic cells such as B16 and Tm5. On the other hand, 3C and 4C melan-a-derived cell lines, but not tumorigenic in vivo, were not recognized by G12F2, suggesting that Mr 230,000 may represent a novel marker of highly malignant murine melanoma (Fig. 3). Phenotypic markers that discriminate melanocytes from melanoma are particularly useful for stage differentiation in melanocyte transformation.

Western blotting investigation demonstrated that the 230 kDa molecule has enhanced expression on B16 high metastatic variant extract than on low metastatic variant (Fig. 4), a typical situation in which the Mr 230,000 molecule could be a differential antigen among
subpopulations. These data, plus the fact that normal melanocytes do not express the protein, offer a new possibility for investigation in the human melanoma model, in which characterization of a $M_r$ 230,000 murine antigen homologue protein could be very useful if expressed by human melanomas.

The major characteristic of malignant cells is their ability to invade tissues and form metastatic foci at distant locations in the host, a process which requires cellular proliferation, attachment to matrix proteins followed by migration through the surrounding stroma. G12F2 affected tumor cell proliferation \textit{in vitro}. Also, despite inhibiting B16 growth, the mAb was not cytotoxic since it did not totally abolish proliferation (Fig. 5).

The anti-proliferative activity of G12F2 was confirmed \textit{in vivo}, since tumor growth in G12F2 treated mice was slowed during treatment but was resumed after its interruption, while IgM or PBS control treatments did not change tumor growth (Fig. 6).

MAbs may affect cellular biologic functions directly inhibiting growth, adhesion, migration and invasion, or indirectly via immune system, trigging complement and cellular cytotoxicity \cite{33}. Inhibition of B16 growth by G12F2 could have resulted from complement activation, since its addition to B16 culture plus complement significantly increased cell lysis (Fig. 7).

Migration and invasion are events necessary to metastasis development, and several mAbs may avoid both processes. Possibly, anti-proliferative activity of G12F2 may have contributed for the poor migration and invasion by B16 cells \textit{in vitro}.

Malignant cells are not inherently metastatic but probably develop this capacity during tumor progression as they acquire or express new characteristics through a process of selection from continuously arising variants \cite{28,34}. MAb G12F2 suppressed lung metastasis of B16 cells in
mAb-treated mice. Three weeks after injection, control groups developed a greater number of lung colonies with bigger diameter; G12F2 treatment, however, hindered installation of B16 large colonies (Fig. 9). According to our data, $M_r$ 230,000 expression correlated with the metastatic phenotype of murine melanoma and may become a novel target for immunodiagnostic approaches and should lead to investigations to determine whether it has an equally relevant human homologue. Moreover, G12F2 mAb can be of help to better understand some crucial events along the metastatic cascade that characterize both, this melanoma model as well as the human cancer.
References


LEGENDS

**Figure 1.** Reactivity of G12F2 monoclonal antibody with B16 cells. (A) B16 cell extract (45 µg) was separated on a 10% polyacrylamide gel, transferred to NC membrane and incubated with mAb G12F2 (1:100 dilution). **Arrow** points to $M_r$ 230,000 band recognized by mAb. (B) FACS of B16 cells ($10^6$) marker with control IgM G12F2 (20% reactive), polyclonal serum (59% reactive) and mAb G12F2 (99% reactive). (C) Confocal microscopy of B16 melanoma showing that the epitope is localized on cell surface; (a, c) Phase contrast microscopy of B16 cells; (b) cells incubated with fluid ascitic irrelevant IgM (1:10 dilution) and anti-IgM FITC; (d) cells incubated with G12F2 ascitic fluid (1:10 dilution) and anti-IgM FITC Blue: nuclei marked by DAPI, 400x.

**Figure 2.** G12F2 monoclonal antibody recognized B16 cell antigen but did not recognize normal cell lines by Western Blotting. 3T3 fibroblast, melan-a melanocytes and B16 melanoma extracts (45 µg) were separated on a 10% polyacrylamide gel and stained with Coomassie blue or transferred to NC membranes and incubated with mAb G12F2 (1:100 dilution). $M_r$ 230,000 band was observed on B16 cell extract only.

**Figure 3.** Western Blotting analysis of $M_r$ 230,000 expression on melanocyte-derived cell lines recognized by G12F2. Non-tumorigenic melan-a melanocytes were isolated after adhesion-nonadhesion cycles in agarose generating 3C and 4C non-tumorigenic and Tm5 tumorigenic cell line. (A) Melan-a, 3C, 4C, Tm5 and B16 cellular extracts were submitted to electrophoresis and stained with Coomassie blue or transferred to NC membranes and incubated with G12F2 ascitic fluid (1:100 dilution). 230 kDa band was recognized by G12F2 on Tm5 and B16 extracts, but not
on melan-a, 3C and 4C. (B) Melan-a, 3C, 4C, Tm5 and B16 cells \((10^6)\) were analyzed by flow cytometry after reaction with mAb G12F2 (1:10 dilution), confirming results observed by Western blotting.

**Figure 4.** *M* 230, 000 is expressed in higher amounts on B16 high metastatic variant than on its low metastatic counterpart. Parental B16, low metastatic B16 and high metastatic B16 variants extracts were submitted to electrophoresis and stained with Coomassie blue or transferred to nitrocellulose membrane and reacted with G12F2 ascitic fluid (1:100 dilution). *M* 230,000 expression was greater on high metastatic B16 than low metastatic B16 variant.

**Figure 5.** *G12F2 inhibited B16 proliferation.* B16 \((10^4 \text{ cells/well})\) were incubated for 24 hours and after adherence, mAb G12F2 or control IgM \((1.0 \text{ and } 5.0 \mu\text{g/ml})\) were added to determinate proliferation rate during 5 days. Daily, MTT was added to one triplicate to evaluate cellular viability. (A) MAb G12F2 and control IgM added at 1.0 \(\mu\text{g/ml} \); (B) MAb G12F2 and control IgM added at 5.0 \(\mu\text{g/ml} \). Both concentrations of G12F2 inhibited growth, but not control IgM.** \(p<0.001\)

**Figure 6.** *In vivo effect of G12F2 mAb on tumor development.* C57Bl/6 male mice, 6-weeks-old were injected with \(2\times10^5\) B16 cells with G12F2, irrelevant IgM or PBS. Animals were previously treated with G12F2, irrelevant IgM or PBS and during three weeks after challenge. Growth of subcutaneous tumors was measured with calipers every 2 days. The data represent the mean volume observed in five animals per group.\(*p <0.05\) versus controls PBS and irrelevant IgM.
Figure 7. *G12F2 increased cytotoxicity by complement against B16 cells in vitro*. B16 \((10^4\text{ cells/well})\) were incubated 24 hours with G12F2 \((1.0, 2.5\text{ and } 5.0 \mu\text{g/ml})\), in the presence or absence of guinea pig complement diluted 1:80. At the end of incubation period, viable cells were measured with MTT. Controls were done with cells incubated with complement or G12F2 alone and cells incubated in the absence of mAb and complement. \(^*p<0.05\) versus B16 cells incubated with mAb alone.

Figure 8. *Inhibition of migration and invasion of B16 cells in vitro by G12F2*. (A) Motility assay by B16 cells *in vitro*. Metastatic melanoma resuspended in RPMI 1640 were placed in the upper compartment of the Transwell® unit and incubated either with G12F2 or control IgM antibody \((5 \mu\text{g/ml})\) for 24 h at 37°C. Filters were then removed and cells that migrated to the lower compartment were fixed and stained with 1% toluidine blue. Absorbance was read by spectrophotometry at 570 nm \(^*p<0.05\) versus RPMI only and \(^{**}p<0.001\) versus control IgM antibody. (B) Invasion assay in Matrigel by B16 cells. Cells were cultivated on 8µm-porosity membrane coated with Matrigel according to the manufacturer’s directions. Results were determined by cells that had trespassed Matrigel to the lower side as seen by the motility assay. \(^*p<0.05\) versus control IgM antibody and culture medium only.

Figure 9. *Suppression of B16 lung metastases by G12F2*. (A) B16 cells \((10^5\) ) were injected IT into C57Bl/6 mice treated with antibodies or PBS as described in Material and Methods. After 21 days, animals were sacrificed by cervical dislocation, and number of lung metastases determined. (B) Lungs from C57Bl/6 mice bearing melanoma, treated with PBS, control IgM or
G12F2. **$p<0.001$** versus PBS treated animals and *$p<0.05$* versus control IgM treated animals, as determined by Tukey-Kramer test.
FIGURES

Figura 1.

(A) B16 extract

(E) B16+ control IgM  B16+ polyclonal serum

(B) 20%

(C) B16+ mAb G12F2

(D) 59%

(E) 50%
Figura 2.
Figura 3.

(A)

(B)
Figura 4.
Figure 5.

(A)

(B)
Figure 6.

* $p < 0.05$
Figure 7.
Figure 8.

(A)

(B)