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Anti-tumor immunoglobulin M increases lung metastasis in an experimental model of malignant melanoma

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Abstract

Cancer metastasis involves distinct steps that depend on complicated tumor–host interactions. The hematogenous dissemination of tumor cells may be facilitated by factors that promote the arrest and adherence of cancer cells in capillaries. We examined whether anti-tumor monoclonal immunoglobulin M (IgM) antibodies promoted the hematogenous dissemination of B16 melanoma cells in syngeneic mice. IgM monoclonal antibodies were generated that selectively bind to B16 melanoma cells as compared to syngeneic fibroblasts, lymphocytes or Lewis lung carcinoma cells. Incubation of B16-BL6 or B16-F0 melanoma cells with these IgM anti-tumor antibodies significantly increased the number of lung colonies as compared with control antibodies. Moreover, intraperitoneal injection of specific antibody also significantly increased lung colonization. All anti-tumor antibodies promoted the aggregation of B16 melanoma cells. A chemically generated immunoglobulin G (IgG)-like fragment of an anti-tumor IgM antibody displayed greatly reduced tumor aggregation and, in contrast to intact IgM, did not significantly increase lung colonization of B16 melanoma cells. Neither intact IgM nor the IgG-like fragment enhanced the *in vitro* invasiveness of B16 melanoma cells across Matrigel-coated membranes. Our results, therefore, suggest that besides their beneficial anti-tumor effects, anti-tumor IgM antibodies may also promote the hematogenous dissemination of cancer cells.

Introduction

Cancer metastasis is a multiple step process that encompasses a coordinated program of events that include changes in cell adhesion, polarized proteolysis and migration, intravasation into the circulation, subsequent adhesion to endothelial cells followed by extravasation, invasion and induction of angiogenesis [1]. Although large numbers of tumor cells enter the circulation during the metastatic process [2, 3], only a small fraction successfully survive, adhere to endothelial cells and extravasate to initiate disseminated disease [4, 5].

Formation of tumor cell aggregates may increase the probability that hematogenous tumor cells successfully initiate metastatic lesions. Numerous studies have implicated interactions between tumor cells and platelets with increased hematogenous spread of cancer (reviewed in [6]). Homotypic aggregation of tumor cells also enhances retention of cancer cells in capillaries and promotes metastasis formation [7–9].

Natural immunoglobulin G (IgG) and immunoglobulin M (IgM) anti-tumor antibodies can be detected in the serum of cancer patients [10–12]. Since IgM antibodies can enhance homotypic aggregation of cells due to their multi-valent binding sites, we therefore examined the hypothesis that anti-tumor IgM antibodies may promote the hematogenous spread of cancer cells.

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Materials and methods

Cell lines

B16-F0 (CRL-6322) and B16-F10 (CRL-6475) melanoma cells, NOR-10 (CCL-197) fibroblasts, BLK CL.4 (TIB-81) fibroblasts, LL/2 (CRL-1642) Lewis lung carcinoma cells, and MLTC-1 (CRL-2065) Leydig tumor cells were obtained from the American Type Culture Collection (Manassas, Virginia). B16-BL6 melanoma cells were from the DCT Tumor Repository, NCI Frederick Cancer Research and Development Center (Frederick, Maryland). All cells were cultured at 37 °C, 5% CO_2 in Delbecco's modified Eagle's medium (Gibco BRL, Grand Island, New York) supplemented with 5% heat-inactivated bovine serum, 2.98 g/l Hepes, 2 g/l $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were free of mycoplasma as determined by a PCR-based mycoplasma detection kit (American Type Culture Collection).

Animals

C57BL/6 and BALB/c mice were obtained from the animal center at the Institute of Biomedical Sciences. Mice were allowed free access to food and water. All animal experiments were carried out with ethical committee approval.

Antibodies

Three-week-old female C57BL/6 mice were i.p. injected with 10^6 mitomycin-treated B16-BL6 cells. Mice were boosted twice at three-week intervals with 5×10^5 mitomycin C-treated B16-BL6 cells. Spleen cells were isolated three days after the last injection and fused with FO myeloma cells as described [13]. Hybridoma supernatants were screened by ELISA. Selected hybridomas were cloned and cultured in DMEM (Gibco BRL, Grand Island, New York) supplemented with 15% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All antibodies, including the control antibodies RH1 [14] and 1A2 [15], were produced as ascites in pristane-primed BALB/c mice. Monoclonal antibody isotypes were determined using the Mono-Ab-ID-EIA kit (Zymed Lab, Berkeley, California) according to the manufacturer's instructions. IgM antibodies were purified by precipitation with 5% PEG 6000 (Merck Taiwan Ltd, Taipei) followed by gel filtration on a 2.5 × 100 cm Sephacryl S-300 column (Amersham Biosciences Asia Pacific, New Territories, Hong Kong). An IgG-like fragment of IgM was produced according to the procedure of Miller and Metzger [16]. HRP-conjugated goat anti-mouse Ig, FITC-conjugated goat anti-mouse Ig, biotin-labeled goat anti-mouse IgM μ-chain specific antibody and streptavidin-FITC were from Organon Teknika (Durham, North Carolina).

ELISA

B16-BL6 or NOR-10 fibroblasts that were coated in the wells of 96-well microtiter plates were employed for screening hybridomas as described [17]. In brief, plates that had been pretreated with polylysine were coated with 10^5 cells/well and fixed with 0.5% gluteraldehyde. Wells were blocked with 0.1 M glycine, 0.01% BSA in PBS followed by 5% skim milk in PBS. Graded concentrations of antibodies were added to the wells for 1 h at 37 °C. The plates were washed 3 times with PBS containing 0.05% Tween 20 and specific HRP-conjugate (1:1000) was added for 1 h. The plates were washed and bound peroxidase activity was measured by adding 100 μl/well ABTS solution (0.4 mg/ml 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, 100 mM phosphate-citrate, pH 4.0) for 30 min. Absorbance (405 nm) of wells was measured in a Molecular Devices microplate reader. B16-BL6 coated plates were also employed to measure serum concentration of anti-B16 antibodies as well as measure the binding of purified antibodies and fragments.

Flow cytometric assay

B16 cells (1×10^6) were first incubated with ascites (1:500) or the indicated concentrations of purified antibodies or fragments followed by FITC-conjugated goat anti-mouse Ig for ascites or biotin-labeled goat anti-mouse IgM μ-chain specific antibody and streptavidin-FITC for purified antibodies and fragments. The mean fluorescence (MF) of 10,000 cells was measured on a FACScaliber flow cytometer (Becton Dickinson, Mountain View, California) and was quantified with FlowJo 3.2 software (Tree Star, Inc., San Carlos, California). The specific fluorescence ratio was calculated as:

$$\mathrm{SFR} = \frac{\mathrm{MF_T}}{\mathrm{MF_C}}$$

where $\mathrm{MF_T}$ and $\mathrm{MF_C}$ represent the mean fluorescence of cells stained with test or control antibody, respectively.

Aggregation assay

B16-F0 or B16-BL6 cells (5×10^5) in 50 μl PBS were dispensed in low protein binding 96-well microtiter plates previously blocked with 2% skim milk. 200 μl of hybridoma culture supernatant or 10 μg/ml purified antibody in PBS was added to the wells at 37 °C for 2 h under gently rotation. Wells were then individually photographed under microscopic magnification.

In vitro *invasion assay*

Tumor cell invasion was examined in a 96-well membrane invasion culture system. A polycarbonate membrane with 8 μm pores (Nucleopore Corp., Pleasanton, California) was coated with 2 mg/ml BD Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, Massachusetts) in PBS. B16-F0 cells (4×10^4) and 5 μg/ml antibodies were added to the upper wells ($n = 7$). The cells that invaded through the coated membrane in 48 h were counted as previously described [18].

Experimental metastasis

B16-F0 or B16-BL6 cells were incubated with the indicated concentrations of hybridoma ascites, culture supernatant or purified antibodies for 30 min on ice. Male C57BL/6 mice aged 4–6 weeks old were i.v. injected with 100 μl of the B16 cell mixture via the lateral tail vein. Different cell numbers were examined to ensure that observed effects applied over a range of conditions. Mice were killed 14 to 18 days later and lung colonies were enumerated under a dissecting microscope. Lungs containing > 500 colonies were defined as containing 500 colonies for statistical analysis.

Statistical analysis

Statistical significance of differences between mean values was estimated with Microsoft Excel using the independent *t*-test for unequal variances.

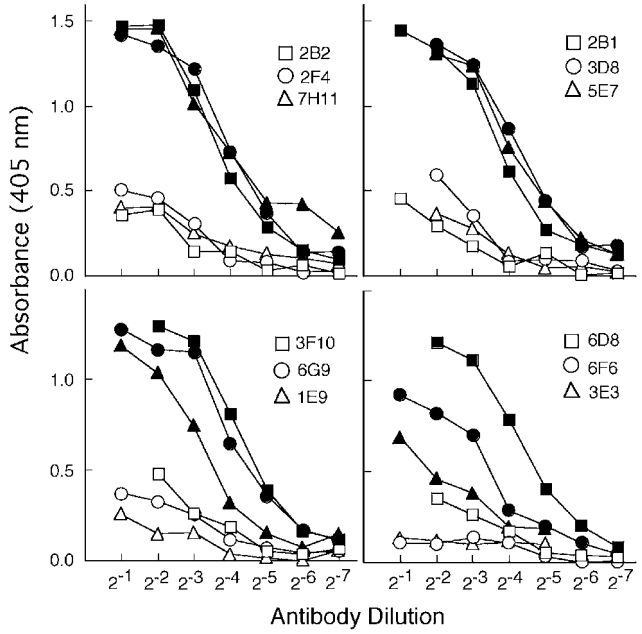

Figure 1. ELISA analysis of anti-B16-BL6 antibodies. Graded dilutions of hybridoma culture supernatant were assayed for binding to microtiter plates coated with B16-BL6 melanoma (solid symbols) or NOR-10 muscle (open symbols) cells. The absorbance (405 nm) of wells was determined 30 min after the addition of ABTS substrate.

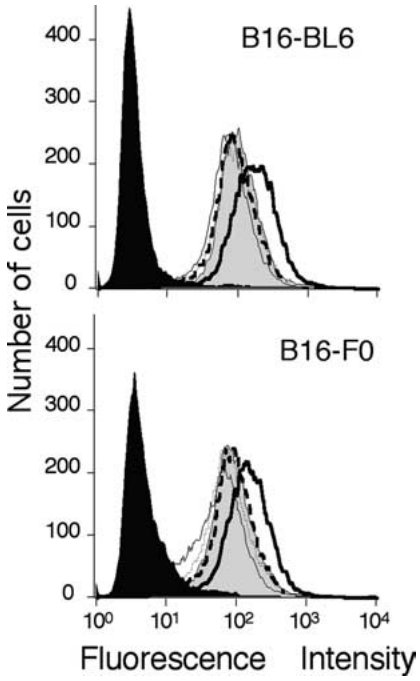

Figure 2. Comparison of anti-B16 antibody binding to B16 melanoma variants. B16-BL6 (upper panel) or B16-F0 (lower panel) cells were stained with 1E9 (heavy dashed line), 3D8 (shaded curve), 3F10 (thin solid line), 6D8 (heavy solid line), 7H11 (dotted line) or control 19BF (filled curve) ascites and FITC-labeled second antibody before the immunofluorescence of 10,000 cells was determined by flow cytometry.

Results

Syngeneic C57/BL6 mice were employed to generate monoclonal antibodies against B16-BL6 melanoma cells to enhance the probability of isolating antibodies against tumor-associated antigens. All hybridomas secreted immunoglobulins of the IgM class. Interestingly, we were unable to isolate a single hybridomas that secreted anti-B16-BL6 IgG antibodies over the course of several fusions. A panel of 12 monoclonal antibodies was selected based on their preferential binding to B16-BL6 melanoma cells as compared to CCL197 normal muscle cells (Figure 1). The specificity of several of the antibodies (1E9, 3D8, 3F10, 6D8 and 7H11) was further investigated by indirect immunofluorescence and flow cytometric analysis (Table 1). All of the antibodies bound B16-BL6 cells at levels that were 12 to 51 times above the background staining of the control antibody (19BF). None of the antibodies specifically bound to normal BLK CL.4 or NOR-10 fibroblasts. Interestingly, all the antibodies bound to MTLC Leydig cell tumor cells. Figure 2 compares the binding of these antibodies to B16-BL6 and the low metastatic variant B16-F0 cells. The binding of antibodies to B16-F0 and B16-BL6 cells was comparable, suggesting that the antibodies do not recognize metastasis-specific antigens. All the 12 antibodies also bind to B16-F10 melanoma cells but not to normal mouse lymphocytes (results not shown).

The effect of anti-BL6 antibodies on the lung colonization of B16 melanoma cells was examined by incubating the cells with antibody *in vitro* before i.v. injection of the tumor cells into the tail vein of C57BL/6 mice. The number of lung colonies in each mouse was then counted after 14–18 days. Figure 3A shows that 3D8 significantly ($P \leqslant 0.005$) increased the number of lung colonies as compared

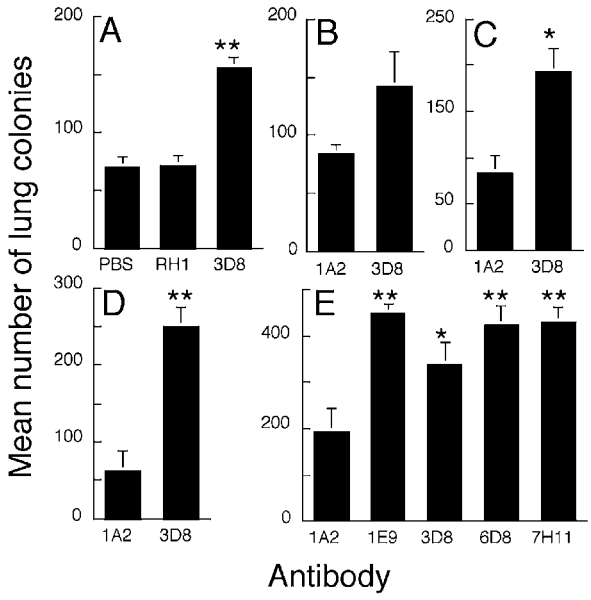

Figure 3. Anti-B16 melanoma antibodies increase melanoma lung colonization. The indicated number of cells were incubated with antibodies before i.v. injection into C57BL/6 mice (n per group). Lung colonies were enumerated 14–18 days later. A, B16-BL6 (1.3×10^5) cells were incubated with PBS, RH1 or 3D8 ascites (1:200) ($n = 3$). B, B16-F0 (2.3×10^5) cells were incubated with 1A2 or 3D8 culture supernatant ($n = 5$). C, B16-F0 (7.5×10^4) cells were incubated with 1A2 or 3D8 culture supernatant ($n = 5$). D, B16-F0 (5×10^5) cells were incubated with 1A2 or 3D8 culture supernatant ($n = 4$). E, B16-F0 (2.5×10^5) cells were incubated with 1A2, 1E9, 3D8, 6D8 or 7H11 culture supernatant ($n = 6$). Significant differences between mean colony numbers of specific and control antibody-treated mice are indicated; $^*P \leqslant 0.05$; $^{**}P \leqslant 0.005$.

Table 1. Antibody binding to cell lines. Viable cells were incubated with the indicated antibodies and FITC-conjugated second antibodies. The immunofluorescence of 10,000 cells was measured.

Antibody	Specific fluorescence ratio[a]				
	B16-BL6	BLK CL.4	LL/2	MTLC-1	NOR-10
19BF	1.0	1.0	1.0	1.0	1.0
1E9	12.8	1.3	2.6	8.5	1.6
3D8	26.6	1.7	3.1	14.4	1.9
3F10	18.5	1.7	3.1	12.8	1.5
6D8	51.0	2.0	3.5	14.7	2.1
7H11	17.0	1.4	2.9	9.1	2.0

[a]Specific antibody mean fluorescence divided by control antibody (19BF) mean fluorescence.

to untreated B16-BL6 cells or cells incubated with control IgG antibody (RH1). Figure 3B shows that 3D8 also increased the number of B16-F0 lung colonies as compared with 1A2, a control IgM antibody, but the difference did not show statistical significance ($P = 0.058$). Additional experiments, however, demonstrated that 3D8 significantly increased the number of B16-F0 colonies in the lungs of C57/BL6 mice as compared to cells incubated with control IgM (Figures 3C and D). To determine whether other antibodies in the panel could also increase lung colonization, B16 melanoma cells were incubated with control 1A2 antibody or anti-B16 antibodies 1E9, 3D8, 6D8 or 7H11 before i.v. injection. Figure 3E shows that all the tested antibodies significantly increased the lung colonies of B16-F0 cells as compared with control IgM.

The ability of 3D8 to increase the metastasis of B16 melanoma cells *in vivo* was also examined. Maximum serum concentration of 3D8 was achieved about 1 h after i.p. injection of 3D8 ascites into C57BL/6 mice (Figure 4A). Therefore, the mice were injected intraperitoneally with 3D8 ascites or the control 1A2 ascites 1 h before i.v. injection of B16-F0 melanoma cells in the tail vein. The circulating 3D8 significantly ($P \leqslant 0.01$) increased the number of melanoma lung colonies as compared to control IgM (Figure 4B).

One possible mechanism by which anti-B16 antibodies could increase lung colonization is by promoting homotypic aggregation of tumor cells. Figure 5 shows that the anti-B16 antibodies (1E9, 7H11, 6D8 and 5E7) did indeed induce the aggregation of B16-BL6 cells. In contrast, control 1A2 IgM did not increase aggregation of B16 cells above background (PBS) levels. To further evaluate the effect of tumor cell aggregation by multivalent anti-tumor IgM, an IgG-like fragment of 1E9 was produced by limited reduction of 1E9 IgM. The IE9 fragment had a molecular weight of about 180–200 kDa on a non-reduced SDS-PAGE (Figure 6A), corresponding to the expected molecular weight of an IgG-like fragment; the heavy chain of IgM contains an additional domain as compared to IgG as can clearly be seen on SDS PAGE under reducing conditions (Figure 6A). The 1E9 fragment retained antigen-binding activity as determined by indirect immunofluorescence (Figure 6B) and by ELISA (Figure 6C). As expected, the 1E9 fragment displayed reduced ability to induce the aggregation of B16 melanoma cells as compared with 1E9 IgM (Figure 6D).

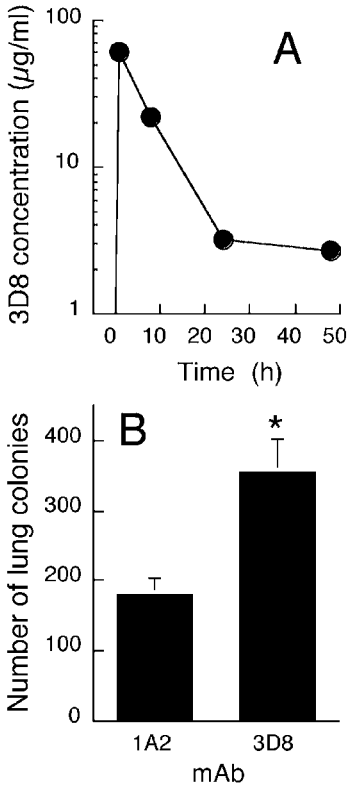

Figure 4. Circulating 3D8 increases B16-F0 lung colonization. (A) Serum levels of 3D8 after i.p. injection of 1 ml 3D8 ascites in C57BL/6 mice were determined by ELISA after 0, 1, 8, 24 and 48 h. (B) Groups of 6 C57/BL6 mice were i.p. injected with 1 ml control 1A2 or specific 3D8 ascites 1 h before i.v. injection of 2.5×10^5 B16-F0 cells. Lung colonies were enumerated 18 days later. $^*P \leqslant 0.05$ compared to control antibody.

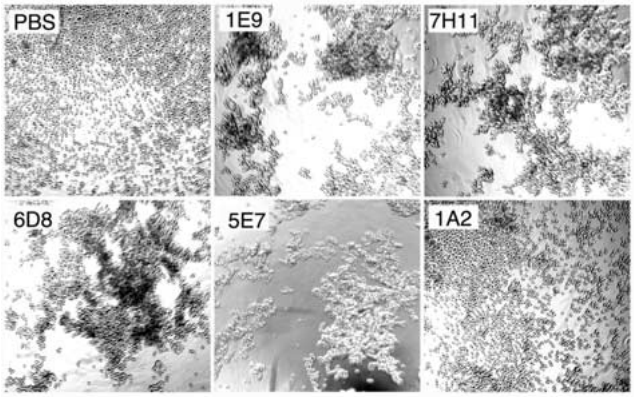

Figure 5. Anti-B16-BL6 antibodies induce B16 cell aggregation. B16-BL6 cells (5×10^5) were incubated with culture supernatants of the indicated hybridomas for 2 h at 37 °C.

The effect of 1E9 and its IgG-like fragment on the invasiveness of B16 melanoma cells was examined in a transwell invasion chamber. Figure 7A shows that neither 1E9 IgM nor 1E9 fragment significantly affected the invasion of B16-F0 cells through Matrigel-coated polyacrylamide filters. In contrast, 1E9 IgM significantly ($P \leqslant 0.005$) increased the number of melanoma lung colonies compared to the control 1A2 IgM and 1E9 fragment (Figure 7B).

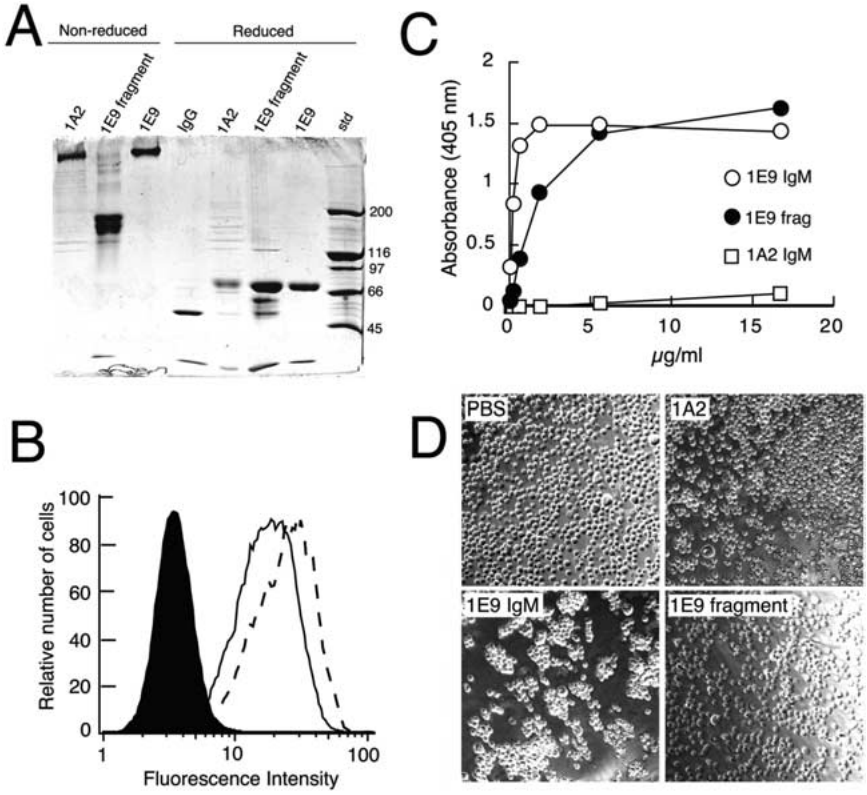

Figure 6. Generation and activity of an 1E9 Ig-like fragment. (A) The indicated proteins were separated by SDS-PAGE under non-reducing (left) or reducing (right) conditions. (B) Indirect immunofluorescence staining of B16-F0 cells by 1E9 (dashed line), 1E9 fragment (solid line) or 1A2 (100 μg/ml) as measured on a flow cytometer. (C) Binding of 1E9, 1E9 fragment or 1A2 to B16-F0 melanoma cells as determined by ELISA. (D) B16-F0 cells (5×10^5) were incubated for 2 h at 37 °C with PBS or 10 μg/ml 1A2, 1E9 or 1E9 fragments.

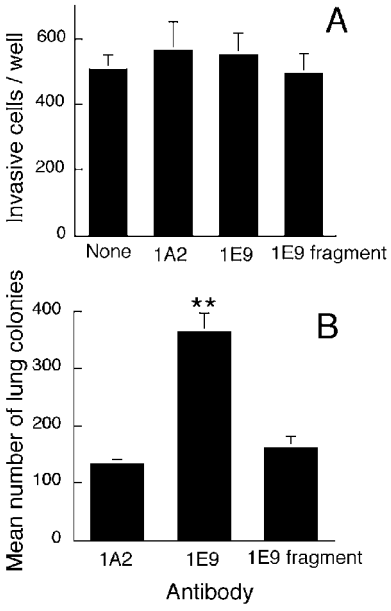

Figure 7. Effect of antibodies on the invasion and colonization of B16-F0 cells. (A) 4×10^4 B16-F0 cells were added to the top well of a 96-well invasion chamber with no antibody (none) or 5 μg/ml 1A2, 1E9 IgM or1E9 fragment. Invasion through a polycarbonate membrane (8 μm pores) coated with 2 mg/ml Matrigel was determined 72 h later. (B) Groups of 5 C57 mice were i.v. injected with 2.5×10^5 B16-F0 cells that had been incubated *in vitro* with 5 μg/ml purified 1A2, 1E9 IgM or1E9 fragment. Lung colonies were counted 18 days later. $^{**}P \leqslant 0.005$ compared with 1A2 and 1E9 fragment.

Discussion

The generation of a humoral response against tumors as well as the induction of anti-tumor antibody responses after the administration of tumor vaccines is generally regarded as beneficial to patients. We found, however, that several monoclonal antibodies against B16-BL6 melanoma cells promoted the formation of B16 melanoma metastases. Our results suggest that besides controlling tumor growth, induction of a specific anti-tumor IgM response may promote the hematogenous dissemination of tumor cells.

A limiting step of cancer metastasis is the arrest of circulating cancer cells on the endothelial cells in capillaries. Established tumors shed large numbers of cancer cells into the blood. Quantification of circulating tumor cells in animal models has shown that from hundred of thousands to more than a million cancer cells are shed from established tumors into the blood every day [2, 3]. Circulating tumor cells can also be readily detected in the circulation of cancer patients [19, 20]. Although large numbers of cells can be present in the circulation of advanced-stage cancer patients, most circulating tumor cells fail to form metastases because they are rapidly destroyed [4, 5]. Factors that facilitate the arrest or increase the time of tumor cell contact with endothelial cells would be expected to enhance hematogenous metastasis. Indeed, numerous studies have demonstrated that both heterotypic interactions of tumor cells with platelets

(reviewed in [6]) and homotypic aggregation of tumor cells [7–9] promote the frequency of metastasis.

Our results are consistent with the hypothesis that aggregation of tumor cells by specific anti-tumor IgM can promote the hematogenous dissemination of cancer. Incubation of tumor cells with intact anti-tumor IgM enhanced the lung colonization of both B16-F0 and B16-BL6 melanoma variants. Similar enhancement was found when the tumor cells entered the circulation of mice with anti-tumor antibody in the serum. Although we employed a relatively high level of circulating anti-tumor IgM *in vivo* (50–60 μg/ml), it should be noted that lower concentrations of anti-BL6 IgM (5–10 μg/ml) promoted cell aggregation (Figure 6D) and metastasis (Figure 7B), suggesting that promotion of metastasis may occur over a range of antibody concentrations. Conversion of the multivalent IgM molecule to an IgG-like fragment abrogated the ability of anti-tumor IgM to enhance lung metastasis. This correlated with reduced ability of the IgG-like fragment to induce homotypic aggregation of the melanoma cells, suggesting that enhanced homotypic aggregation of tumor cells by anti-tumor IgM may have been responsible for promoting hematogenous metastasis. Aggregation of tumor cells may also protect them from host immune defenses. For example, IgM-mediated aggregation of neutrophils protects them from complement lysis in the circulation [21]. Similarly, aggregation of trypanosomes is believed to play a role in the defense of parasites against the host immune system [22].

Other mechanisms could also explain the enhanced formation of melanoma metastases afforded by the anti-tumor antibodies. Antibody cross-linking or activation of receptors on the tumor cells could transduce signals that increase their ability to form metastases. Although we cannot rule out this possibility, it should be noted that at least one of the antibodies had no effect on the *in vitro* invasiveness of B16 melanoma cells (Figure 7A). It is also possible that the antibodies promoted heterotypic interactions between tumor cells and other cells such as platelets or endothelial cells. Minimal binding of the antibodies to normal cells, however, is predicted since the antibodies were generated by immunizing syngeneic C57BL/6 mice with B16-BL6 melanoma cells. Thus, these antibodies should recognize tumor-associated antigens since the mice are tolerant to self-antigens. It is interesting to note that although, as expected, none of the antibodies bound to syngeneic fibroblasts or Lewis lung carcinoma cells, significant binding to MTLC-1 Leydig cells was observed. Many tumor-associated antigens including melanoma antigen (MAGE) and Melan-A (MART-1) genes have been found to be cancer-testis antigens; they are expressed in tumor cells but display a restricted pattern of normal tissue expression that is limited to the testis [23, 24]. Although we were unable to detect the antigens recognized by our antibodies on immunoblots, possibly due to the low affinity of IgM, the binding profile of the antibodies suggests that they may recognize cancer-testis antigens expressed on the surface of B16 melanoma cells.

The clinical significance of circulating anti-tumor IgM is unclear. Many tumors induce a humoral response in patients [10–12]. In fact, the basis of a technique to identify tumor antigens termed the serological analysis of recombinant cDNA expression libraries (SEREX) relies on the fact that autologous sera contains anti-tumor antibodies [25]. Anti-tumor humoral immune responses induced by vaccination can be clearly beneficial [26, 27]. In addition, naturally occurring anti-tumor antibodies have been associated with increased disease-specific survival in cancer patients [28, 29].

On the other hand, anti-tumor antibodies may sometimes be detrimental. For example, host antibody responses can favor tumor progression and metastasis by allowing selective expansion of high metastatic variants [30]. Antibodies have also been shown to promote the formation of metastases. An anti-melanoma IgM antibody enhanced the lung colonization of B16-F1 cells [31] and immune serum collected from rats bearing established lung metastases effectively enhanced the formation of lung metastases in transfer experiments [32]. Similarly, depletion of B lymphocytes resulted in a lower incidence of spontaneous lung metastasis in mice [33]. In addition, IgM has been casually linked to metastasis in some cancers. Increased serum IgM was found in neuroblastoma patients with metastases [34] and the presence of IgM-positive but not IgG-positive lymphoid cells in lymph nodes or tumors has been associated with high grade tumors and lymph node metastasis [35–37]. Elevated concentrations of IgM in serum has also been related to more rapid relapse in stage II malignant melanoma patients [38].

In summary, our results suggest that, in some instances, circulating anti-tumor IgM may promote the hematogenous dissemination of cancer cells. Whether anti-tumor antibodies enhance or control the formation of cancer metastases may be related to a balance between the ability of the antibodies to promote tumor cell aggregation versus the anti-tumor activity of the antibodies, i.e., efficacy of complement activation or ability to activate effector cells. Efficient activation of complement by IgM requires high epitope densities [39, 40], suggesting that weak humoral immune responses against sparse antigens, as typified by the IgM antibodies employed in our study, may favor metastasis formation.

Acknowledgements

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