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CSPG4 As a Target of Antibody-Based Immunotherapy For Malignant Mesothelioma

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Abstract

Purpose—Malignant mesothelioma (MM) is an aggressive cancer, resistant to current therapies. Membrane Chondroitin Sulphate Proteoglycan 4 (CSPG4), which has been successfully targeted in melanoma and breast cancer, was found highly expressed in MM, but not in normal mesothelium. Therefore, we explored CSPG4 as a suitable target for monoclonal antibody (mAb)-based immunotherapy of MM.

Experimental Design—We assayed adhesion, motility, invasiveness, wound-healing, apoptosis and anchorage-independent growth of MM cells on cell cultures. CSPG4 expression and signaling was studied by immunoblotting. The growth of MM SCID mice xenografts induced by PPM-Mill cells, engineered to express the luciferase reporter gene, was monitored by imaging, upon treatment with CSPG4 mAb TP41.2. Animal toxicity and survival were assayed in both tumor inhibition and therapeutic experiments.

Results—CSPG4 was expressed on 6 out of 8 MM cell lines and in 25 out of 41 MM biopsies, with minimal expression in surrounding healthy cells. MM cell adhesion was mediated by CSPG4-dependent engagement of extracellular matrix components (ECM). Cell adhesion was inhibited by mAb TP41.2 resulting in decreased phosphorylation of FAK and AKT, reduced expression of cyclin D1 and apoptosis. Moreover, TP41.2 significantly reduced MM cell motility, migration and invasiveness, and inhibited MM growth in soft agar. In vivo, treatment with mAb TP41.2 prevented or inhibited the growth of MM xenografts in SCID mice, with a significant increase in animal survival.

Conclusion—These results establish the safety of CSPG4 mAb-based immunotherapy and suggest that CSPG4 mAb-based immunotherapy may represent a novel approach for the treatment of MM.

Keywords

Mesothelioma; CSPG4; immunotherapy; neutralizing antibodies; xenografts

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Introduction

Malignant mesothelioma (MM) is an aggressive tumor of the pleura, peritoneum and, occasionally, pericardium and tunica vaginalis testis. Epidemiological and experimental studies have linked the development of MM with the exposure to asbestos or erionite fibers (1, 2). Genetics and co-factors influence the risk of developing MM following exposure to asbestos and erionite (3–5). About 3,000 cases of MM are diagnosed each year in the US, and median survival is 1 year from diagnosis. Five-year survival is unusual and limited to patients diagnosed in the early stages of the disease (6). More than 90% of MM are diagnosed at late stages, when the tumor is resistant to conventional therapy. Chemotherapy remains as the mainstay of MM treatment, although the standard chemotherapy for MM, pemetrexed/cisplatin, only extends survival by an average of 11 weeks (7). Given the recent major progress in the development of monoclonal antibody (mAb)-based immunotherapy for the treatment of some solid tumors, immunotherapy for MM is of interest (8). Targets for antibody-based treatment regimens for MM need to be defined.

CSPG4 consists of an N-linked glycoprotein of 280 kDa and a proteoglycan component of about 450 kDa (9) and plays an important role in melanoma cell proliferation, migration and metastasis (10). Neuron-glia antigen 2 (NG2), the rat homologue of CSPG4, binds directly to collagen types II, V and VI (CII, CV and CVI) and is critical for adhesion of glioma cells (11). CSPG4-specific mAbs have been shown to disrupt melanoma cell adhesion to collagen type I (CI), CVI and fibronectin (FN) (12, 13). Through its binding to extracellular matrix (ECM) components such as CI, CIV, CVI and FN, CSPG4 modulates cell polarization, adhesion, spreading and survival *via* activation of FAK, Src and ERK1/ERK2 (14, 15). Notably, MM cells are capable of adhering to CI, CIV and FN (16).

CSPG4 is over-expressed on melanoma cells and on triple negative breast cancer cells; in both types of malignancies CSPG4 has been successfully targeted in SCID xenografts by mAb-based immunotherapy, using several different CSPG4-specific mAbs that recognize distinct epitopes (17, 18).

Recent studies revealed common molecular alterations between mesothelioma and melanoma (5, 19). Thus, we investigated whether CSPG4 is over-expressed also in MM, and whether CSPG4 represents a useful target for mAb-based immunotherapy of MM.

Materials and Methods

Mice

Six week-old female NOD.CB17-Prkdc^{scid}/J SCID mice were purchased from Jackson Laboratory, Bar Harbor, ME.

Antibodies

The mouse mAbs 225.28, 763.74, TP32, TP41.2 and TP61.5 against distinct epitopes of CSPG4 were characterized as previously described (20). All the mAbs are IgG1, except mAb 225.28, (IgG2a). These antibodies do not cross-react with the CSPG4 mouse homolog NG2 (20, unpublished data) and unpublished results. The mouse mAb clone MF11–30 was the isotype matched control (IgG control). The following antibodies were purchased commercially: phospho-AKT (Ser473), AKT1/2/3, phospho-FAK (Tyr397) from Cell Signaling Technology (Beverly, MA); FAK, cyclin D1, goat anti-mouse IgG, goat anti-rabbit IgG from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); GAPDH monoclonal antibody from Chemicon International Inc. (Temecula, CA); Polyclonal Goat anti-mouse IgG/RPE, Goat F(ab')₂ from Dako North America, Inc. (Carpinteria, CA).

Reagents

Fibronectin, Collagen I, Collagen IV, Laminin, Osteopontin were purchased from BD Biosciences (San Jose, CA). MTS assay was purchased from Promega (Madison, WI). Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I was purchased from BD Pharmingen (San Jose, CA), and HEMA3 Protocol kit was purchased from Fisher Diagnostics (Kalamazoo, MO).

Cell lines

The MM cell lines Con, Gard, Gor, PPM-Mill, Phi and Rob were established from surgically resected human MM specimens and characterized for their mesothelial origin(21). Hmeso cell line was also established and characterized from human MM(22). The MM cell line Ren was provided by Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA) (23). The Burkitt's lymphoma Raji and the melanoma Colo38 cell lines were used as negative and positive controls, respectively. All cell lines were cultured in Dulbecco's modified Eagle's medium, DMEM (Gibco, Grand Island, NY), 10% FBS at 37°C in a 5% CO₂ atmosphere. Primary human mesothelial cells (HM) isolated from pleural effusions of seven patients with congestive heart failure were obtained from Queen's Medical Center, Honolulu, HI and cultured in DMEM supplemented with 20% FBS as described (24).

Western blotting

Cell lysates were prepared by using M-PER SDS-based lysing buffer (Invitrogen, Carlsbad, CA) and immunoblotting was performed as previously described (25), followed by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Rockford, IL).

Flow cytometry analysis

Subconfluent MM cells were incubated with 1 µg mAbs for 1 hour at 4°C. Cell surface CSPG4 expression was measured as previously described (26).

Immunohistochemistry (IHC)

IHC was performed on FFPE sections. Antigen retrieval and tissue section processing were performed by standard procedures.

siRNA transfection

Cells were transfected with CSPG4 Stealth RNAi siRNA from Invitrogen (Carlsbad, CA) using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA). Stealth RNAi siRNA with high GC content was used as a negative control ("scramble").

Cell viability assay

Cell viability of MM cells was measured after treatments with 10 µg/mL mAb TP41.2 or IgG control for 1 hour at 37°C, by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

Cell adhesion assay

Cells preincubated with mAb TP41.2 or IgG control, were plated onto dishes pre-coated with FN, CI, CIV, LN (10µg/ml) and cultured at 37 °C for 1 hour. Adherent cells were fixed with 2% glutaraldehyde and stained with 0.5% crystal violet. Dye was resolved in 10% acetic acid and 560nm absorbance was measured by a multi-well spectrophotometer (UV-VIS).

Migration and Invasion assays

MM cell motility and invasiveness were assayed in Transwell® plates as previously described (27) after pre-incubation with mAb TP41.2 or IgG control.

Wound healing assay

A distinct area was wounded by a micropipette tip on cultures of MM cells transiently transfected with CSPG4 siRNA, or pre-incubated with mAb TP41.2. Cell motility into wounded areas was evaluated by microscopy following a 24 and 48 hour incubation at 37°C. The number of cells migrated into the wound area was quantified by Image J software.

Apoptosis assay

MM cells were pre-incubated with mAb TP41.2 or IgG control and apoptosis was evaluated by Annexin V/7-AAD staining as previously described (27).

Anchorage-independence assay

MM cells were incubated with mAb TP41.2 or IgG control and the growth in soft agar was assayed as previously described (28). Briefly, MM cells (5×10^3) incubated with mAb TP41.2 or IgG control (10 µg/ml) were seeded on 0.3% agarose overlaid onto solidified 0.6% agarose in DMEM 10% FBS. Plates were fed every 48 h with mAbs. The growth in soft agar was assayed 17 days later. Colonies larger than 0.15 mm in diameter were scored using Biorad colony counter. Colonies were counted in 5 selected fields from representative of 3 wells per treatment. The experiments were repeated 3 times.

SCID human xenografts

PPM-Mill cells (1×10^6) engineered to express the luciferase reporter gene (PPM-Mill/luc) were injected into the peritoneal cavity of SCID mice as previously described (22). After luciferin injection, xenografts were visualized by luminescence using the In Vivo Imaging System (IVIS™, Xenogen Corp., Alameda, CA). In the experiments designed to inhibit MM tumor growth, five mice per group were injected with PPM-Mill/luc cells pre-incubated with mAb TP41.2 or IgG control (the treatment started at D0). Mice were treated with mAbs twice a week (0.2 mg mAb/mouse/injection) for four weeks. In experiments designed to evaluate the therapeutic efficacy of treatment with mAb TP41.2, PPM-Mill/luc were injected into mice. At day 5 (D5) after tumor challenge (when tumors were imageable), animals were divided in two groups to equate average tumor growth. Animals were injected with mAb TP41.2 or IgG control (200 µg/ml/mouse), twice a week for four weeks. Tumor growth was evaluated by IVIS every seven days.

Statistical Analysis

Data were collected from three independent experiments. Differences between treated groups were analyzed by Student t-test (unpaired), one-way or two-way Analysis of Variance (ANOVA). Differences were considered to be significant if $P < 0.05$. Survival was compared by the log-rank test. All statistical analyses were performed using the GraphPad Prism 5 software.

Results

CSPG4 is expressed in Malignant Mesothelioma cells

We examined the expression of CSPG4 on MM cells and on HM with mAbs 225.28, 763.74, TP32, TP41.2, and TP61.5 that recognize distinct epitopes of CSPG4 (20), by flow cytometry, Western blotting and immunohistochemistry (IHC). Cytofluorographic analysis

of Hmeso, PPM-Mill, and REN showed essentially identical staining patterns with the five mAbs: the cell surface expression of CSPG4 on PPM-Mill cells was very high, similar to that on Colo38 positive control, while it was lower on Hmeso cells and not detectable on REN cells, as in Raji Burkitt's lymphoma cells, the negative control. (Fig. 1A). By Western blotting the two CSPG4 components (450 kDa and 280 kDa) were detected in 6 out of the 8 MM cell lines examined, but not in HM, isolated from pleural exudates of five donors (Supplementary Fig. S1A and Fig. S1B). CSPG4 expression was neither detected on HM by flow cytometry (data not shown).

To determine the expression of CSPG4 in MM tissue, 30 epithelioid, 5 biphasic and 5 sarcomatoid FFPE biopsies were stained by IHC with the CSPG4-specific mAb D2.8.5-C4B8. Twenty-five out of the forty one biopsies stained positive for CSPG4. Expression in the surrounding non-tumor tissue and normal pleura was either not detectable or barely detectable. No expression of CSPG4 was found in a FDA Standard Frozen Tissue Array (90 tissue cores of 30 organs, 3 individual donors per organ). CSPG4 was expressed in 5/5 sarcomatoid and in 5/5 biphasic MM. The expression was positive in 15/31 epithelioid MM tested. In the 5 sarcomatoid lesions tested 100% of tumor cells were positive, with a homogenous and strong staining intensity (Fig. 1B, Table 1).

In summary, these data show that CSPG4 is expressed in 6/8 MM cell lines tested, 25/41 tumor biopsies, and was not detectable in HM from 5 different patients of congestive heart failure. The selective expression of CSPG4 in MM prompted us to investigate whether CSPG4 is a suitable target for therapeutic intervention in MM.

CSPG4-specific mAbs inhibit MM cell growth by blocking cell adhesion

To understand the possible role of CSPG4 in MM we analyzed the effect of CSPG4 gene silencing on MM cell viability. Transient transfection of PPM-Mill and Hmeso MM cells with human CSPG4 sequence-specific siRNAs resulted in a 40–60% down-regulation of CSPG4 expression on MM cells, as compared to cells transfected with scrambled control siRNA (Supplementary Fig. S2A). The viability of CSPG4-silenced PPM-Mill and Hmeso cells, was significantly ($P < 0.05$) reduced, compared to that of the same cells transfected with scramble siRNA. CSPG4-specific siRNA did not influence the viability of CSPG4-negative MM cells REN (Supplementary Fig. S2B). Together, these data indicate that CSPG4 may have a role in cell survival and suggest a potential therapeutic efficacy of CSPG4 targeting mAbs in MM.

Previous reports have shown that CSPG4 binds to ECM components and that CSPG4-specific mAbs can disrupt these interactions (29). To verify the efficacy of CSPG4-specific mAbs in MM, PPM-Mill, Hmeso and REN cells were pre-treated with either mAb TP41.2 or with IgG control before plating on dishes pre-coated with CI, CIV and FN. Cell counting following a 30 minutes incubation revealed that the number of adherent PPM-Mill and Hmeso cells pre-incubated with mAb TP41.2 was significantly ($p < 0.05$) lower than that of cells treated with IgG control. In contrast, mAb TP41.2 had no effect on adhesion of CSPG4-negative REN cells (Fig. 2A). The CSPG4 specificity of the effect of mAb TP41.2 is supported by CSPG4 expression on PPM-Mill cells and by its lack of expression on REN cells also when they are cultured in ECM-coating culture conditions (Supplementary Fig. S3A).

We next analyzed whether the inhibition of the CSPG4-mediated interaction with ECM components by mAb TP41.2 affects MM cell viability. mAb TP41.2 or IgG control were added every 24 hours to PPM-Mill and REN cells cultured for three days on CI and FN in serum-free conditions. A significant ($P < 0.0001$) reduction of cell viability was observed in

PMM-Mill cells treated with mAb TP41.2, as compared to cells treated with IgG. In contrast mAb TP41.2 did not influence the viability of REN cells (Supplementary Fig. S3B).

These results indicate that CSPG4-specific mAb TP41.2 can prevent MM cell attachment on CI, CIV or FN ECM components. Prolonged cell treatment with CSPG4-specific mAb TP41.2 reduces cell adhesion, resulting in inhibition of MM cell viability. This suggests that CSPG4 is necessary to maintain cell viability.

Treatment with CSPG4-specific mAb TP41.2 inhibits cell adhesion mediated signaling

To evaluate the mechanism of the possible CSPG4-dependent cell survival, we evaluated the activity of AKT and FAK, and the expression of cyclin D1, in PPM-Mill cells by immunoblotting after pre-treating cells cultured on FN pre-coated dishes with CSPG4-specific mAb TP41.2 or IgG control. Cells were allowed to adhere for 15, 30, 60 or 120 minutes. Cells cultured on uncoated dishes were used as controls. The levels of FAK (Tyr397) and AKT (Ser473) phosphorylation, as well as the level of expression of Cyclin D1 transiently decreased within the first 15 minutes and then resumed the basal levels, when cells were treated with mAb TP41.2 but not with IgG control (Fig. 2B).

These data indicate that CSPG4 engagement with FN is involved in cell signaling following the adhesion of CSPG4-positive MM cells. Treatment with mAb TP41.2 blocks this interaction and reduces FAK and AKT activities, as well as the expression of cyclin D1. This suggests that the inhibition of the interaction of CSPG4 with ECM by mAb TP41.2 affects survival by interfering with FAK and AKT signaling and cell cycle, via enhanced levels of cyclin D1.

CSPG4-specific mAb TP41.2 reduces migration and invasion of MM cells

The aggressiveness of a malignant cell is determined by its potential to invade the ECM and metastasize to distant sites (30). To evaluate the effect of mAb TP41.2 on motility of MM cells, PPM-Mill, Hmeso, and REN cells were pre-incubated with mAb TP41.2 or with IgG control and plated on the top chamber of Transwell® in the presence of 10% serum and mAbs. Cell motility of CSPG4-positive cells PPM-Mill and Hmeso pretreated with mAb TP41.2 was reduced by two-fold, compared to cells treated with IgG control ($P < 0.0004$ for PPM-Mill and $P < 0.0009$ for Hmeso). Conversely, mAb TP41.2 did not influence the motility of CSPG4-negative REN cells (Fig. 3A). The specificity of the effect of mAb TP41.2 was further confirmed utilizing PPM-Mill cells in which CSPG4 expression was silenced by transfection with CSPG4-specific siRNAs. CSPG4 silencing led to at least 40% reduction of cell migration, compared to cells transfected with scrambled control siRNA (Supplementary Fig. S4).

A role in the induction of melanoma cell invasion and metastasis has been proposed for CSPG4 (31). Therefore, we next verified CSPG4 involvement in MM cell invasion. PPM-Mill and Phi MM cells, both of which express CSPG4 (see Supplementary Fig. S1A) were pre-treated with mAb TP41.2 or IgG control then cultured on the top chamber of the Transwell® coated with Matrigel® for 48 hours. The number of cells that invaded Matrigel was significantly reduced in both PPM-Mill and Phi cells treated with mAb TP41.2, compared to IgG control treated cells ($P < 0.0001$ for PPM-Mill; $P < 0.0032$ for Phi). On the other hand, treatment of REN cells with mAb TP41.2 or IgG control had no detectable effects on the invasion (Fig. 3B).

These results indicate that CSPG4 plays an important role in cell motility and invasion of MM cells and that mAb TP41.2 affects the invasive properties of MM cells.

As the Transwell® assay measures directional migration towards a chemoattractant gradient, we also performed a wound-healing assay to compare non-directional motility. Following a 24 and 48 hour incubation mAb TP41.2 markedly inhibited wound closure as compared to the IgG control. The effect was mediated by the interaction of mAb TP41.2 with CSPG4, since the wound healing of the CSPG4-negative REN cells was not affected (Supplementary Fig. S5). These findings were corroborated by the inhibition of wound closure of PPM-Mill cells, but not of REN cells, by CSPG4 siRNA-mediated silencing (Supplementary Fig. S6).

CSPG4-specific mAb TP41.2 reduces anchorage independent growth of MM cells

To determine whether treatment of MM cells with mAb TP41.2 suppresses anchorage independent growth, we analyzed the formation of colonies in soft agar. PPM-Mill and REN cells were treated with mAb TP41.2 or with IgG control before plating in soft agar and cultured for further 17 days supplementing antibodies daily. The number and size of colonies were markedly reduced when PPM-Mill cells were pre-incubated with mAb TP41.2 compared to IgG control treated cells (Fig. 4A). On the contrary, no significant differences in the number or size of colonies were observed in REN cells treated with IgG control or mAb TP41.2 (Fig. 4B).

These results indicate that mAb TP41.2 antagonizes anchorage-independent growth of MM cells and support the role of CSPG4 in the maintenance of the malignant phenotype in MM.

CSPG4-specific mAb TP41.2 induces apoptosis of MM cells

We analyzed whether mAb TP41.2 induced apoptosis of MM cells. To this end PPM-Mill and REN cells were treated with mAb TP41.2 or IgG control for 24 hours and apoptosis was measured by flow cytometry using Annexin V and 7-Amino-Actinomycin (7-AAD) double staining. The percentage of apoptotic cells after treatment with mAb TP41.2 was 35.17% as compared to 17.17% in the IgG control treated group ($P < 0.0001$; Fig. 5). The percentage of apoptotic cells found in the negative control was similar to that found in the untreated MM cell cultures. No induction of apoptosis above background was detected in CSPG4 negative REN cells incubated with mAb TP41.2. These data indicate that mAb TP41.2 elicits apoptotic cell death of CSPG4-positive MM cells.

CSPG4-specific mAb TP41.2 inhibits the growth of MM cells in SCID mice

We assessed the in vivo anti-tumor activity of mAb TP41.2 against MM xenografts by using two approaches: 1) Inhibition of MM growth and 2) Therapy of MM, as described in Material and Methods. In the experiment aimed at inhibiting tumor growth, the treatment with TP41.2 monoclonal antibody was initiated in parallel with intraperitoneum cell injection. In the experiment on the possible therapeutic effect of CSPG4 targeting, tumors were allowed to grow, before initiating the treatment with mAb TP41.2. In this model, 5 days after injection the tumors were 3 mm in diameter. We established that this size was clearly measurable by IVIS and in previous papers we showed that tumors of this size were responsive to anti-tumor treatments (27, 32). In the tumor inhibition experiment a delay in growth was observed in the group treated with mAb TP41.2 compared to the control treated group (Fig. 6A). Tumor growth was significantly ($P < 0.0001$) inhibited by mAb TP41.2 within 4 weeks, as compared to IgG control treated mice. The rate of tumor growth in the treated group was lower than that in the IgG control treated group even after the end of treatment, and the growth delay was maintained until the termination of the study (Fig. 6B). Analysis of survival showed a significant ($P = 0.001$) increase in median survival of mAb TP41.2 treated mice, as compared to IgG control treated animals (Fig. 6C).

In the MM therapy experiment mAb TP41.2-mediated control of tumor growth was observed by day 14 after tumor challenge (Fig. 6D). Treatment with mAb TP41.2

significantly reduced average tumor volume as compared to IgG control for the entire duration of treatment ($P<0.0001$) (Fig. 6E). Thereafter, tumors resumed growth by maintaining a slower pace. Analysis of survival showed a significant ($P=0.03$) increase in median survival of mAb TP41.2 treated mice, compared to IgG control treated animals (Fig. 6F). Thus, mAb TP41.2 caused a significant increase in survival.

In both survival experiments mice were euthanized and necropsied when tumor development caused severe ascites limiting the animal's mobility, according to IACUC regulations.

No signs of toxicity were detected in mice treated with mAb TP41.2. The experiments were terminated 9 months after tumor challenge. Animals treated with the IgG control died within 120 days in both experiments. In the inhibition of tumor growth experiment (Fig. 6A–C) 4 of the 5 mice treated with mAb TP41.2 survived for 140 days and one was still alive day 270. In the MM therapy experiment (Fig. 6D–F) 3 out of the 5 mice treated with mAb TP41.2 died within 130 days, leaving 2 surviving on day 270.

Necropsy was performed on each death and the tumors were removed and macroscopically and histologically evaluated. In both experiments, the tumors collected from mice treated with mAb TP41.2 were consistently smaller than those collected from mice treated with the IgG control, in all five paired animal groups tested (data not shown).

Together, these data indicate that mAb TP41.2 is a well tolerated and promising anti-tumor agent that is capable of inhibiting MM and prolonging survival.

Discussion

CSPG4 is an attractive target for mAb-based immunotherapy for melanoma and breast cancer (18) and a number of CSPG4-specific mAbs, characterized for their specificity (20), are now available. We show here that most MM cell lines and 60% of MM biopsies express high levels of CSPG4, while HM and normal pleura contain very low levels of CSPG4. The highest CSPG4 expression was found in 5 out of 5 tested sarcomatoid tumors, a histological subtype that is completely resistant to present therapies (6), suggesting that CSPG4 could be a potential target also for therapy of those patients that currently have no available therapeutic options.

Our data indicate that CSPG4 influences MM cell viability and survival through the interaction with ECM. Cell adherence was higher on FN suggesting the occurrence of a positive feedback loop of FN that induces the expression of CSPG4, which has high binding affinity for FN. Moreover, CSPG4 is known to interact with $\alpha 4 \beta 1$ integrin, characterized as the FN cell receptor. This mechanism can enhance MM cell adhesion to FN in a CSPG4-dependent manner. In support to this hypothesis, in melanoma cells a fragment of FN has been shown to bind CSPG4 directly to promote melanoma cell adhesion in a CSPG4-dependent manner (29) and to promote melanoma cell spreading on FN (33).

The link between CSPG4 and melanoma progression has been well documented by studying integrin-mediated adhesion and spreading, and the subsequent signaling involving CDC42, ACK1 and p130CAS, which in turn activate FAK, Src and ERK1/ERK2 (14, 15). In MM cells adhered to FN we found that FAK phosphorylation induced by integrin engagement was decreased in CSPG4-expressing cells treated with mAb TP41.2. This result matches the observation that CSPG4 enhances FAK activation in melanoma cells (14). Moreover, in breast cancer cells CSPG4 inhibition reduced Akt activity (26). In accordance with these results, treatment of CSPG4 expressing MM cells with the mAb TP41.2 led to Akt activity inhibition, a signal for apoptosis and reduction of cell motility and spreading (34).

Our results show a reduction in size and growth rate of mAb TP41.2 treated MM colonies in anchorage-independent growth conditions, suggesting that in MM cells, as in melanoma cells (35), CSPG4 over-expression allows MM tumor survival in the absence of adhesion.

The inhibition by CSPG4-specific mAbs of tumor growth, metastasis and tumor recurrence was demonstrated in melanoma and breast cancer xenografts (17, 18, 26). The therapeutic potential of mAb TP41.2 targeting CSPG4 in MM was validated here in MM xenografted mice. Treatments with this mAb were well tolerated and caused no toxicity. Tumor growth was delayed in two separate in vivo experiments. Studies in different tumor models (17, 18, 36) observed only a partial reduction in tumor growth rate in vivo by CSPG4 targeted immunotherapy. The lack of complete destruction of MM xenografts may be explained either by the emergence of cell subpopulations heterogenous for CSPG4 expression or by the relative inability of mAb TP41.2 to penetrate MM tumors completely. An additional and not exclusive possibility is represented by the insufficient dose of mAb administered. Experiments using higher doses of mAb TP41.2 will be conducted to verify the latter possibility.

The previous characterization of the CSPG4 specific monoclonal antibody 225.28 (26) showed that the effects of a CSPG4 antibody on MM might be comparable to those of trastuzumab, widely used in breast cancer (37). Both mAbs target cell surface receptors (CSPG4 and HER2, respectively) and consequently the downstream PI3K/Akt pathway(26), which is critical for cell migration and survival. Moreover, trastuzumab targets the tumor vasculature of the tumor microenvironment controlling neo-angiogenesis (38). Similarly, CSPG4-specific mAbs have been shown to reduce vascular density in melanoma (36) and breast cancer (26) tumor microenvironments. The efficacy of the combination of trastuzumab and several chemotherapeutic drugs has been demonstrated in breast cancer (39). Similarly, it is conceivable that mAb TP41.2 may also enhance the sensitivity towards chemotherapeutic drugs such as cisplatin and pemetrexed, currently used in MM as first-line treatment (7).

Overall, our data provide preclinical data for the translation of an antibody-based therapeutic regimen to clinical trials for MM. Based on the encouraging data we have obtained so far in this preclinical work, we are planning to chimerize the mouse version of mAb TP41.2 for future clinical application. Given the lack of effective therapies to treat patients with MM, the development of new therapeutic regimens is of critical urgency. Of particular interest is the ability of CSPG4-specific mAbs therapy to target sarcomatoid MM, a histological variant comprising about 30% of MM that are associated with 6–9 months survival, because it is completely resistant to current therapies. We do not expect potential side effects of mAb TP41.2 because of the lack of expression of CSPG4 in normal tissues as demonstrated by our through screening of a large FDA Standard Frozen Tissue Array.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

There is an urgent need of effective therapies for malignant mesothelioma (MM), a very aggressive cancer, with mortality rates estimated to constantly increase in the next decade. The patients' median survival of 1 year from diagnosis is only extended by an average of 11 weeks with the current standard pemetrexed/cisplatin regimen. We report here that Chondroitin Sulphate Proteoglycan 4 (CSPG4) is a successful target for antibody-based immunotherapy for MM. The CSPG4 specific mAb TP41.2 inhibits the growth of human xenografts and delays the growth of established MM in immunodeficient mice. The treatment was not toxic and significantly extended animal survival. Given the lack of efficacious therapies available to treat patients with MM, these results provide the pre-clinical data necessary to translate to clinical trials an antibody-based therapeutic regimen, which would have the potential to bring hope to patients who currently have no recourse for treatment.

Figure 1. CSPG4 is expressed in Malignant Mesothelioma

A. Mesothelioma cells PPM-Mill, Hmeso and REN (5×10^4) were harvested with 1mM EDTA and then incubated with CSPG4-specific mAbs 225.28, 763.74, TP32, TP41.2 and TP61.5 for 1 hour at 4°C and with RPE-conjugated goat anti-mouse secondary antibodies for 30 minutes at 4°C. Cells were then analyzed with the FACScan flow cytometer. Results are expressed as percentage of stained cells and as mean fluorescence intensity. The CSPG4-positive melanoma Colo38 cells and the CSPG4-negative Burkitt's lymphoma cells Raji were used as positive and negative controls, respectively. **B.** Sections of formalin fixed, paraffin embedded tissues. A CSPG4-positive melanoma lesion was used as a positive control, normal pleura, sarcomatoid MM, biphasic MM and epithelioid MM were sequentially incubated with CSPG4-specific mAb D2.8.5 overnight at 4°C and with peroxidase-conjugated goat anti-mouse IgG antibodies for 1 hour at room temperature. "Epithelioid (CSPG4+)" indicates the epithelioid tumors that stained positive for CSPG4 and "Epithelioid (CSPG4-)" indicates the same type of tumors that stained negative for CSPG4. (Magnification 400X).

Figure 2. CSPG4-specific mAbs inhibit MM cell growth by blocking cell adhesion

A. CSPG4-positive MM cells were plated in serum-free condition on dishes pre-coated with CI, CIV and FN and cultured for 30 minutes. Then cells were stained with crystal violet. Acetic acid was then added to solubilize the cells and the absorbance was measured at 560nm to determine the extent of cell adhesion. Values represent the mean $\pm$ SEM of triplicates from three independent experiments. Asterisks indicate $P < 0.05$. **B.** PPM-Mill MM cells were grown on fibronectin-coated dishes for up to 2 hours. Cell lysates were tested in Western blotting with phosphoFAK-, FAK-, phosphoAKT-, AKT- and Cyclin D1- specific antibodies. GAPDH was used as a loading control.

Figure 3. CSPG4-specific mAb TP41.2 reduces migration and invasion of MM cells

A. PPM-MIII and Hmeso MM cells were pre-incubated with mAb TP41.2 or with IgG control for 1 hour. Cells were then plated on the membrane of the upper chamber of a Transwell® plate and grown for 48 hours in the presence of 10% FBS. Cells grown in serum-free conditions were used as a negative control. The cells migrated to the lower surface of the membrane were stained with Giemsa staining for microscopical observation. Then cells were solubilized and the absorbance was measured at 560nm to determine the extent of cell migration. The graph indicates the mean $\pm$ S.D. from three separate experiments. The statistical differences represent comparisons versus untreated cells using Student's t test. $P < 0.05$. **B.** PPM-MIII, Phi and REN MM cells were pre-incubated with mAb TP41.2 or with IgG control for 1 hour. Cells were then plated on the membrane of the upper chamber of a Matrigel®-coated Transwell® plate and grown for 48 hours in the presence of 10% FBS. Cells grown in serum-free conditions were used as a negative control. The cells migrated to the lower surface of the membrane were stained with Giemsa for microscopical observation. The graph indicates the mean $\pm$ S.D. from three separate experiments. The statistical differences represent comparisons versus untreated cells using Student's t test. $P < 0.05$.

Figure 4. CSPG4-specific mAb TP41.2 reduces the size and growth rate of MM colonies in soft agar

A. CSPG4-positive PPM-Mill and, **B.** CSPG4-negative REN MM cells were pre-incubated with IgG control or mAb TP41.2 then plated in soft agar. Thereafter, mAb TP41.2 or IgG control mAb were added to the cultured cells every 48 hours. The rate of cells growth was evaluated by phase-contrast microscopy 17 days later. The average number of colonies was quantified by colony counter using Quantity One software analysis and is reported in the bar graph. The size of the colonies was determined in 5 random optical fields from each plate.

Figure 5. CSPG4-specific mAb TP41.2 induces apoptosis of MM cells

CSPG4-positive PPM-Mill and CSPG4-negative REN MM cells were pre-incubated with mAb TP41.2 or IgG control for 1 hour and then cultured for 24 hours on uncoated dishes. Then the extent of apoptosis was evaluated by flow cytometry using Annexin V and 7-AAD. The percentages of live cells, of early and late apoptotic cells and of necrotic cells are indicated in the cytograms. The total percentage of apoptosis is reported in the bar graph. Asterisks indicate $p < 0.0001$.

Figure 6. CSFG4-specific mAb TP41.2 inhibits human MM cell growth in xenografts
 CSFG4-positive PPM-Mill/luc cells (1×10^6 /mouse) were pre-incubated with mAb TP41.2 or IgG control for 1 hour and injected i.p. in SCID mice, treatment started at D0 (**A**) or injected i.p. in SCID mice to induce tumor establishment, treatment started at D5 (**D**). mAb TP41.2 was administered i.p. twice a week for four weeks. The size of tumors was monitored weekly by IVIS™ after injection of luciferin. The luminescence signals are expressed as total flux of photons/sec (**A: inhibition of MM growth, and D: therapy of MM**). Quantitative analysis of the whole-body total photon counts of IgG control and mAb TP41.2 treated mice at doses of 0.2 mg in 200ul per 20g mouse (**B: inhibition of MM growth, and E: therapy of MM**). Kaplan-Meier survival plots of mice with MM xenografts. Survival curves of xenograft-bearing mice treated with CSFG4-specific mAb TP41.2 or isotype IgG control. In both survival experiments mice were euthanized and necropsied when tumor development caused severe ascites limiting the animal's mobility, according to IACUC regulations. No animals were censored in both Kaplan-Meier plots (**C: inhibition of MM growth, and F: therapy of MM**).

Table 1

CSPG4 staining of MM biopsies

Sample ID	Subtype	IHC Results	Intensity of Staining
0301	E	POSITIVE	Strong
0302	E	NEGATIVE	None
0303	E	NEGATIVE	None
0304B	E	POSITIVE	Strong
0305	E	POSITIVE	Strong
0308A	E	NEGATIVE	None
0308B	E	POSITIVE	Weak
0309	E	POSITIVE	Strong
0310	E	NEGATIVE	None
0311	E	NEGATIVE	None
0312	S	POSITIVE	Very Strong
0313	E	NEGATIVE	None
0314	B	POSITIVE	Very Strong
0316	E	NEGATIVE	None
0317	E	POSITIVE	Strong
0318	E	POSITIVE	Very Strong
0319	B	POSITIVE	Strong
0321	E	NEGATIVE	None
0329A	B	POSITIVE	Very Strong
502B	S	POSITIVE	Very Strong
504	B	POSITIVE	Very Strong
505	B	POSITIVE	Very Strong
511	E	NEGATIVE	None
512	S	POSITIVE	Very Strong
513	S	POSITIVE	Very Strong
514	S	POSITIVE	Very Strong
515	E	POSITIVE	Weak
516	E	NEGATIVE	None
518 B	E	POSITIVE	Weak
B9-3B	E	POSITIVE	Strong
B11-2A	E	POSITIVE	Strong
B13-2C	E	POSITIVE	Strong
04514697D	E	POSITIVE	Very Strong
97-283 (5)	E	NEGATIVE	None
ME99	E	NEGATIVE	None
T01	E	POSITIVE	Very Strong
NYU 47	E	NEGATIVE	None
NYU 517	E	NEGATIVE	None
NYU 754	E	POSITIVE	None

Sample ID	Subtype	IHC Results	Intensity of Staining
NYU 809	E	NEGATIVE	None
NYU 1017	E	NEGATIVE	None

E: Epithelioid; B: Biphasic; S: Sarcomatoid

Intensity of Staining: Weak: 1+; Strong: 3+; Very Strong: 4+