Cancer Therapy: Preclinical

CSPG4 as a Target of Antibody-Based Immunotherapy for Malignant Mesothelioma

Zeyana Rivera1,2, Soldano Ferrone4, Xinhui Wang4, Sandro Jube1, Haining Yang1,3, Harvey I. Pass5, Shreya Kanodia1, Giovanni Gaudino1, and Michele Carbone1,3

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 for 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 severe combined immunodeficient (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 ECM. Cell adhesion was inhibited by mAb TP41.2 resulting in decreased phosphorylation of focal adhesion kinase (FAK) and AKT, reduced expression of cyclin D1 and apoptosis. Moreover, mAb 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. Clin Cancer Res; 18(19): 5352–63. ©2012 AACR.

Introduction

Malignant mesothelioma (MM) is an aggressive tumor of the pleura, peritoneum, and, occasionally, pericardium and tunica vaginalis testis. Epidemiologic and experimental studies have linked the development of MM with the exposure to asbestos or erionite fibers (1, 2). Genetics and cofactors 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 United States, 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.

Chondroitin sulphate proteoglycan 4 (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-glial antigen 2, 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 ECM components such as CI, CV, and FN, CSPG4 modulates cell polarization, adhesion, spreading, and survival.

Authors' Affiliations: 1University of Hawai'i Cancer Center, 2Department of Molecular Biosciences and Bioengineering, University of Hawai'i; 3Department of Pathology, John A. Burns School of Medicine, Honolulu, Hawaii; 4Departments of Surgery, Immunology, and Pathology, University Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania; and 5Department of Cardiothoracic Surgery, NYU School of Medicine, New York, New York

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Michele Carbone, University of Hawai'i Cancer Center, 651 Ilalo Street, Honolulu, HI 96813. Phone: 808-440-4596; Fax: 808-587-0790; E-mail: mcarbone@hcc.hawaii.edu

doi: 10.1158/1078-0432.CCR-12-0628

©2012 American Association for Cancer Research.
Targeting CSPG4 for Mesothelioma Therapy

Translational Relevance
There is an urgent need for effective therapies for malignant mesothelioma (MM), a very aggressive cancer, with mortality rates estimated to constantly increase in the next decade. 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 preclinical 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.

via activation of focal adhesion kinase (FAK), Src, and ERK1/2 (14, 15). Notably, MM cells are capable of adhering to CI, CIV, and FN (16).

CSPG4 is overexpressed on melanoma cells and on triple negative breast cancer cells; in both types of malignancies CSPG4 has been successfully targeted in severe combined immunodeficient (SCID) mice 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 overexpressed also in MM, and whether CSPG4 represents a useful target for mAb-based immunotherapy for MM.

Materials and Methods

Mice
Six-week-old female NOD.CB17-Prkdcscid/J SCID mice were purchased from Jackson Laboratory.

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 Neuron-glial antigen 2 (20, unpublished data). The mouse mAb clone MF11-30 was the isotype-matched control [immunoglobulin G (IgG) control]. The following antibodies were purchased commercially: phospho-AKT (Ser473), AKT1/2/3, phospho-FAK (Tyr397) from Cell Signaling Technology; FAK, cyclin D1, goat anti-mouse IgG, goat anti-rabbit IgG from Santa Cruz Biotechnology Inc.; glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody from Chemicon International Inc.; Polyclonal Goat anti-mouse IgG/RPE, Goat F(ab’2) from Dako North America, Inc.

Reagents
FN, CI, CIV, laminin, and osteopontin were purchased from BD Biosciences. MTS (cell viability) assay was purchased from Promega. Fluorescein isothiocyanate Annexin V Apoptosis Detection Kit I was purchased from BD Pharmigen, and HEMA3 Protocol kit was purchased from Fisher Diagnostics.

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), 10% FBS at 37°C in a 5% CO₂ atmosphere. Primary human mesothelial cells (HM) isolated from pleural effusions of 5 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 lysis buffer (Invitrogen) and immunoblotting was conducted as previously described (25), followed by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate).

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
Immunohistochemistry (IHC) was conducted on formalin-fixed, paraffin-embedded (FFPE) sections. Antigen retrieval and tissue section processing were carried out by standard procedures.

siRNA transfection
Cells were transfected with CSPG4 Stealth RNAi siRNA from Invitrogen using Lipofectamine 2000 Reagent (Invitrogen). Stealth RNAi siRNA with high guanine-cytosine 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 Titre 96 Aqueous One Solution Cell Prolif-eration Assay (Promega).

Cell adhesion assay
Cells preincubated with mAb TP41.2 or IgG control, were plated onto dishes precoated with FN, CI, CIV, LN (10 μg/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 560 nm absorbance was measured by a multiwell spectrophotometer (IVIS-VIS).

Migration and invasion assays
MM cell motility and invasiveness were assayed in Trans-well plates as previously described (27) after preincubation 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 preincubated 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 preincubated with mAb TP41.2 or IgG control and apoptosis was evaluated by annexin V/7-ami-no-actinomycin (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 hours 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.). In the experiments designed to inhibit MM tumor growth, 5 mice per group were injected with PPM-Mill/luc cells preincubated for 1 hour with mAb TP41.2 or IgG control (the treatment started at day 0). Mice were treated with mAbs twice a week (0.2 mg mAb/mouse/injection) for 4 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 (DS) after tumor challenge (when tumors were imageable), animals were divided in 2 groups to equate average tumor growth. Animals were injected with mAb TP41.2 or IgG control (200 μg/mL/mouse), twice a week for 4 weeks. Tumor growth was evaluated by IVIS every 7 days.

Statistical analysis
Data were collected from 3 independent experiments. Differences between treated groups were analyzed by Student t test (unpaired), 1-way or 2-way ANOVA. Differences were considered to be significant at \( P < 0.05 \). Survival was compared by the log-rank test. All statistical analyses were conducted using the GraphPad Prism 5 software.

Results
CSPG4 is expressed in MM 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 IHC. Cytofluorographic analysis of Hmeso, PPM-Mill, and REN showed essentially identical staining patterns with the 5 mAbs: the cell surface expression of CSPG4 on PPM-Mill cells was very high, similar to that on Colo38 positive control, although 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 2 CSPG4 components (450 and 280 kDa) were detected in 6 out of the 8 MM cell lines examined, but not in HM, isolated from pleural exudates of 5 donors (Supplementary Fig. S1A and S1B). CSPG4 expression was also not detected on HM by flow cytometry, Western blotting, or IHC. CSPG4 expression was detected in 5/5 sarcomatoid and MM with 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). To determine the expression of CSPG4 in MM tissues, 31 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 41 biopsies stained positive for CSPG4. Expression in the surrounding nontumor tissue and normal pleura was either not detectable or barely detectable. No expression of CSPG4 was found in a U.S. Food and Drug Administration (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
Transient transfection of PPM-Mill and Hmeso MM cells with human CSPG4 sequence-specific siRNAs resulted in a 40% to 60% downregulation of CSPG4 expression on MM cells, as compared with 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 with 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 pretreated with either mAb TP41.2 or with IgG control before plating on dishes precoated with CI, CIV, and FN. Cell counting following a 30 minutes incubation revealed that the number of adherent PPM-Mill and Hmeso cells preincubated 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 specificity of mAb TP41.2 effect is supported by CSPG4 expression on PPM-Mill cells and by its lack of expression on REN cells when cultured in ECM-coating culture conditions (Supplementary Fig. S3A).

We next assessed 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 3 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 with 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 pretreating cells cultured on FN precoated 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 Cyclin D1 expression transiently decreased within the first 15 minutes and then resumed to 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 preincubated with mAb TP41.2 or with IgG control and plated on the top chamber of Transwell in the presence of 10% serum

### Table 1. CSPG4 staining of MM biopsies

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Subtype</th>
<th>IHC results</th>
<th>Intensity of staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>0301</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>0302</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0303</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0304B</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>0305</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>0308A</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0308B</td>
<td>E</td>
<td>Positive</td>
<td>Weak</td>
</tr>
<tr>
<td>0309</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>0310</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0311</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0312</td>
<td>S</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>0313</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0314</td>
<td>B</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>0316</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0317</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>0318</td>
<td>E</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>0319</td>
<td>B</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>0321</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>0329A</td>
<td>B</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>502B</td>
<td>S</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>504</td>
<td>B</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>505</td>
<td>B</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>511</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>512</td>
<td>S</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>513</td>
<td>S</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>514</td>
<td>S</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>515</td>
<td>E</td>
<td>Positive</td>
<td>Weak</td>
</tr>
<tr>
<td>516</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>518 B</td>
<td>E</td>
<td>Positive</td>
<td>Weak</td>
</tr>
<tr>
<td>B9-3B</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>B11-2A</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>B13-2C</td>
<td>E</td>
<td>Positive</td>
<td>Strong</td>
</tr>
<tr>
<td>04514697D</td>
<td>E</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>97–283 (5)</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>ME99</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>T01</td>
<td>E</td>
<td>Positive</td>
<td>Very strong</td>
</tr>
<tr>
<td>NYU 47</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>NYU 517</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>NYU 754</td>
<td>E</td>
<td>Positive</td>
<td>None</td>
</tr>
<tr>
<td>NYU 809</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
<tr>
<td>NYU 1017</td>
<td>E</td>
<td>Negative</td>
<td>None</td>
</tr>
</tbody>
</table>

NOTE: Intensity of staining: Weak, 1++; Strong, 3++; Very strong, 4++. Abbreviations: B, biphasic; E, epithelioid; S, sarcomatoid.
and mAbs. Cell motility of CSPG4-positive cells PPM-Mill and Hmeso pretreated with mAb TP41.2 was reduced by 2-fold, compared with 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 using 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 with 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 pretreated 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 the Matrigel was significantly reduced in both PPM-Mill and Phi cells treated with mAb TP41.2, compared with IgG control treated cells (P < 0.0032 for Phi). In contrast, 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 toward a chemoattractant gradient, we also conducted a wound-healing assay to compare nondirectional motility. Following a 24- and 48-hour incubation, mAb TP41.2 markedly inhibited wound closure as compared with the IgG control. The effect was mediated by the interaction of mAb TP41.2 with CSPG4, because 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 preincubated with mAb TP41.2 compared with IgG control treated cells (Fig. 4A). In contrast, 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 Hmeso MM cells were preincubated with mAb TP41.2 or 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 that migrated to the lower surface of the membrane were stained with Giemsa staining for microscopical observation. Then, the cells were solubilized and the absorbance was measured at 560 nm to determine the extent of cell migration. The graph indicates the mean ± SD from 3 separate experiments. The statistical differences represent comparisons versus untreated cells using Student t test. P < 0.05.

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

We assessed the in vivo antitumor activity of mAb TP41.2 against MM xenografts by using 2 approaches: (i) inhibition of MM growth and (ii) therapy for MM, as described in the Materials and Methods section. 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 studies we showed that tumors of this size were responsive to antitumor treatments (27, 32). In the tumor inhibition experiment, a delay in growth was observed in the group treated with mAb TP41.2 compared with the control-treated group (Fig. 6A). Tumor growth was
significantly \( (P < 0.0001) \) inhibited by mAb TP41.2 within 4 weeks, as compared with 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 with IgG control-treated animals (Fig. 6C).

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 preincubated 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 cell 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. \(* * * , P < 0.0005.\)

Figure 5. CSPG4-specific mAb TP41.2 induces apoptosis of MM cells. CSPG4-positive PPM-Mill and CSPG4-negative REN MM cells were preincubated 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. \(* * * , P < 0.0001.\)
Figure 6. CSPG4-specific mAb TP41.2 inhibits human MM cell growth in xenografts. CSPG4-positive PPM-Miiluc cells (1 x 10^6/mouse) were either preincubated with mAb TP41.2 or IgG control for 1 hour and injected intraperitoneally in SCID mice. (A, treatment started at day 0) or without preincubation injected intraperitoneally in SCID mice (D, treatment started at D5). mAb TP41.2 was administered intraperitoneally twice a week for 4 weeks. Tumor size was monitored weekly by IVIS with luciferin injection. The luminescence signals are expressed as total flux of photons/sec (A, inhibition of MM growth, and D, therapy for 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 200 μL per 20 g mouse (B, inhibition of MM growth, and E, therapy for MM). Kaplan–Meier survival plots of mice with MM xenografts. Survival curves of xenograft-bearing mice treated with CSPG4-specific mAb TP41.2 or isotype IgG control. (C, inhibition of MM growth, and F, therapy for MM).
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 with 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 with 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 Institutional Animal Care and Use Committee (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 alive till 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 conducted 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 5 paired animal groups tested (data not shown).

Together, these data indicate that mAb TP41.2 is a well tolerated and promising antitumor agent that is capable of inhibiting MM and prolonging survival in an animal model.

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, and that 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 histologic subtype that is completely resistant to present therapies (6), suggesting that CSPG4 could be a potential target also for therapy for 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 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 of 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 through studies of integrin-mediated adhesion and spreading, and the subsequent signaling involving CDC42, ACK1, and p130CAS, which in turn activate FAK, Src, and ERK1/2 (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 overexpression allows MM tumor survival in the absence of adhesion.

CSPG4-specific mAbs inhibition of tumor growth, metastasis, and tumor recurrence was shown 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 2 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 heterogeneous 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 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 shown in breast cancer (39). Similarly, it is conceivable that mAb TP41.2 may also enhance the sensitivity toward chemotherapeutic drugs such as cisplatin and pemetrexed, currently used in MM as first-line treatment (7).

Overall, our results provide preclinical data for the translation of an antibody-based therapeutic regimen to clinical
trials for MM. On the basis of 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 histologic variant comprising about 30% of MM that are associated with 6 to 9 months survival, because it is completely resistant to current therapies. We do not expect significant side effects of mAb TP41.2 due to the lack of expression of CSPG4 in normal tissues, as shown by our thorough screening of a large FDA Standard Frozen Tissue Array.

Disclosure of Potential Conflict of interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Z. Rivera, S. Ferrone, X. Wang, H.I. Pass, G. Gaudino, M. Carbone
Development of methodology: Z. Rivera, X. Wang, H. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Jube, M. Carbone
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Rivera, X. Wang, H. Yang, H.I. Pass, S. Kanodia, G. Gaudino, M. Carbone
Writing, review, and/or revision of the manuscript: Z. Rivera, S. Ferrone, X. Wang, H. Yang, H.I. Pass, S. Kanodia, G. Gaudino, M. Carbone
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Rivera, X. Wang, S. Kanodia
Study supervision: Z. Rivera, H. Yang, G. Gaudino, M. Carbone

Grant Support
These studies were supported by the P01 CA114047 grant (M. Carbone), R01 CA160715 (H. Yang), R01 CA105500 and R01 CA138188 (S. Ferrone), awarded by the National Cancer Institute, by the Hawaii Community Foundation (H. Yang and G. Gaudino), and by the Butitta Mesothelioma Foundation (M. Carbone)

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 21, 2012; revised July 20, 2012; accepted July 23, 2012; published OnlineFirst August 14, 2012.

References


CSPG4 as a Target of Antibody-Based Immunotherapy for Malignant Mesothelioma

Zeyana Rivera, Soldano Ferrone, Xinhui Wang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0628

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/08/14/1078-0432.CCR-12-0628.DC1

Cited articles
This article cites 38 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/19/5352.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/18/19/5352.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.