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Expression of Aggrus/podoplanin in bladder cancer and its role in pulmonary metastasis

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Platelet aggregation-inducing factor Aggrus, also known as podoplanin, is associated with tumor malignancy by promoting hematogenous metastasis. Aggrus overexpression has been reported in some tumor tissues including lung, esophagus, head and neck and brain. We here found the frequent upregulation of *aggrus* mRNA in urinary bladder cancers using cancer tissue panels from various organs. Immunohistochemical analysis confirmed Aggrus protein expression in urinary bladder cancers and suggested a positive correlation between Aggrus expression and metastatic tendency in bladder cancers. Endogenous expression of Aggrus protein on the cell surface was found in the mouse bladder cancer MBT-2 cell line and human bladder cancer SCaBER cell lines. Knockdown of Aggrus expression in MBT-2 cells decreased their ability to induce platelet aggregation and form pulmonary metastasis in syngeneic mouse models. Knockdown of Aggrus expression in the human bladder cancer SCaBER cells also attenuated their ability to induce platelet aggregation and form pulmonary metastasis in mice. Moreover, pulmonary metastasis of SCaBER cells was prevented by prior administration of our generated anti-Aggrus neutralizing monoclonal antibodies by attenuating their retention in lung. These results indicate that Aggrus plays an important role in bladder cancer metastasis. Thus, anti-Aggrus neutralizing antibodies would be useful for the prevention of hematogenous metastasis of Aggrus-positive bladder cancer.

Key words: Aggrus/podoplanin, platelet aggregation, bladder cancer

Abbreviations: ADC: adenocarcinomas; ADCC: antibody-dependent cellular cytotoxic; ATCC: American Type Culture Collection; CDC: complement-dependent cytotoxic; CLEC-2: C-type lectin-like receptor 2; ET-1: endothelin-1; FBS: fetal bovine serum; HBSS: Hanks' Balanced Salt Solutions; IHC: immunohistochemical; qRT-PCR: quantitative reverse transcription polymerase chain reaction; SCC: squamous cell carcinomas; TCC: transitional cell carcinomas. Additional Supporting Information may be found in the online version of this article.

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Bladder cancer is the sixth most common cancer in the western world; with an estimated 73,510 new cases of urinary bladder cancer being diagnosed in 2012 in the United States.[1,2] Bladder cancer is known to be a smoking-related disease that most frequently occurs in males living in industrialized countries.[3] Approximately 90% of bladder cancers are transitional cell carcinomas (TCC), 5% are squamous cell carcinomas (SCC), and less than 2% are adenocarcinomas (ADC). Although 70% of patients with bladder cancer present with superficial tumors that are generally not life-threatening, 30% present with muscle-invasive disease associated with a high risk of death from distant metastasis.[4] Despite of good prognosis for patients with non-muscle-invasive disease, recurrence is common and associated with the development of muscle invasion disease in up to 30% of the cases.[5] The incidence of distant metastasis is 50% in recurrent TCC and 8–10% in SCC; distant metastasis is uncommon in ADC.[6,7] Lung and liver are two common sites of dissemination for bladder cancer. Some genes, including endothelin-1 (ET-1), CD24 and LAMC-2, are reportedly associated with lung and liver metastasis of bladder cancer.[8–10]

Aggrus, also known as podoplanin, T1-alpha, OTS-8, or D2–40 antigen, was previously identified as a platelet aggregation-inducing factor expressed in highly metastatic tumor cells.[11] Because Aggrus mutants lacking platelet aggregation-inducing abilities were unable to form hematogenous metastasis, Aggrus-induced platelet aggregation is directly associated with metastasis formation.[11] The metastasis-promoting effects of platelets have been revealed in several reports. *For example*, platelets have been reported to facilitate tumor cell survival in blood circulation by enhancing the formation of tumor cell clusters that increase

What's new?

Expression of the platelet-aggregation-inducing factor Aggrus (podoplanin) by tumor cells is associated with metastasis via the bloodstream. In this study, the authors found that Aggrus is over-expressed in highly metastatic human bladder cancers, and that knockdown of this factor decreased metastasis of both mouse and human bladder-cancer cells in mice. Anti-Aggrus antibodies were also able to prevent this metastasis. This type of antibody may therefore be a promising adjunct to bladder-cancer therapy, and Aggrus over-expression may be a useful biomarker for predicting metastasis.

embolization and epithelial–mesenchymal transition in the microvasculature and protecting the tumor cells from shear stress or immunological assault.[12] In contrast, the risk of hematogenous metastasis is low in the patients with thrombocytopenia.[13,14] In addition, antiplatelet agents have been reported to reduce the number of experimental tumor metastasis.[15] Therefore, Aggrus with platelet aggregation-inducing ability would increase the risk of hematogenous metastasis with assistance from host platelets.

Aggrus is a type-I transmembrane sialoglycoprotein that is frequently upregulated in several cancers, including SCC, mesothelioma, Kaposi's sarcoma, testicular germ cell tumors and brain tumors.[16–19] Aggrus upregulation in cancer-associated fibroblasts has also been reported. Its expression in these cells is associated with poor prognosis in patients with lung ADC.[20] Recently, Aggrus expression was found in tumor-initiating cells, suggesting a role of Aggrus in cancer progression.[21] The C-type lectin-like receptor 2 (CLEC-2) expressed on platelets was recently identified as one of the counter receptors of Aggrus.[16,22,23] Aggrus binding to CLEC-2 transmits platelet activation signals through Src family kinases, Syk and phospholipase Cγ2 in platelets.[24,25] CLEC-2 was previously identified as a platelet receptor for the snake venom toxin rhodocytin.[26] Because of the similarities in platelet-aggregation phenotypes induced by rhodocytin and Aggrus, CLEC-2 was identified as a platelet receptor of Aggrus.[22] In addition, similarities were observed in the phenotype of mice deficient in CLEC-2 to that of Aggrus/podoplanin knockout mice,[25,26] confirming that CLEC-2 functions as a platelet receptor of Aggrus *in vivo*.

In this study, we screened the *aggrus* mRNA expression in various cancers and found that some bladder cancers showed high *aggrus* mRNA levels. Tissue microarray analysis confirmed that Aggrus expression is frequently upregulated in metastatic bladder TCC and SCC. Because Aggrus knockdown in Aggrus-positive bladder cancer cell lines decreased the number of pulmonary metastatic foci, Aggrus expression was directly linked to the lung metastasis of bladder cancers. Moreover, we found that our generated anti-Aggrus neutralizing antibodies attenuated the pulmonary metastasis of bladder cancers suggesting the usefulness of the neutralizing antibodies as metastasis-inhibitory drugs.

Material and Methods

Quantitative and semi-quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a LightCycler 480 Probes Master (Roch, Basel, Switzerland) and the LightCycler 480 Real-time PCR System (Roch). TissueScan Cancer Survey Panel 4× 96-III (OriGene Technologies, Rockville, MD) was screened by qRT-PCR using primers for human *aggrus* and *β-actin*. Standard curves were generated from a dilution series of cDNA prepared from HT1080 cells that were reported to express endogenous Aggrus.[27] The expression level of each *aggrus* mRNA was normalized by that of *β-actin*. Primer pairs used in qRT-PCR were as follows: human *aggrus* forward, 5′-AAATGTCGGGAAGGTACTCG-3′; human *aggrus* reverse, 5′-GCCAGGCAAGTGTTCCAC-3′; human *β-actin* forward, 5′-CCAACCGCGAGAAGATGA-3′; and human *β-actin* reverse, 5′-CCAGAGGCGTACAGGGA TAG-3′. Semi-quantitative RT-PCR was performed using KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) and the GeneAmp PCR System 9700. Complementary DNAs were prepared with SuperScript III RT according to the manufacturer's protocols. Primer pairs used in semi-quantitative RT-PCR were as follows: human *aggrus* forward, 5′-ATG TGGAAGGTGTCAGCTCTGC-3′; human *aggrus* reverse, 3′-GTGTGTCTCCATCCACTTTCTC-3′; human *β-actin* forward, 5′-ATCTGGCACCACACCTTCTACAATG-3′; human *β-actin* reverse, 3′-CGTCATACTCCTGCTTGCTGATCCA-3′; mouse *aggrus* forward, 5′-TGTTTTTCATCTTTTCACAA CCC-3′; mouse *aggrus* reverse, 3′-AGCTCTTTAGGGCGAG AACCTTC-3′; mouse *β-actin* forward, 5′-GATATCGCTGCG CTGGTCGTCGAC-3′; and mouse *β-actin* reverse, 3′-CAA GAAGGAAGGCTGGAAAAGAGC-3′.

Immunohistochemistry

Four human bladder cancer tissue arrays (BL801, BL804, BL806 and BL208) were obtained from US Biomax (Rockville, MD). Overlapped samples among the four arrays were omitted, and the remaining 135 samples were assessed. Tissue array sections were deparaffinized, rehydrated and treated with peroxidase-blocking solution (DAKO, Glostrup, Denmark). Anti-human Aggrus/podoplanin mAb (clone: D2–40, DAKO) was treated for 30 min at room temperature, then incubated with EnVision+ System-HRP labeled polymer anti-mouse (DAKO). Color was developed with ImmPACT DAB (Vector Laboratories, Burlingame, CA). Mayer's hematoxylin solution (Wako, Osaka, Japan) was used for nuclei counter staining. Evaluation of the stain score (defined as the sum of the proportion score and intensity score) was entrusted to Kyodo Byori (Hyogo, Japan). The proportion score (the percentage of positive staining) was defined as follows: 0: 0%, 1: <10%, 2: 11–49%, 3: 50–79%, 4: >80%. The

intensity score (the average staining intensity) was defined as follows: 0: negative, 1: weakly positive, 2: moderately positive, 3: strongly positive. Scoring of immunohistochemical (IHC) analyzed slides was performed by two independent pathologists who were blind to diagnosis.

Plasmid construction

Human *aggrus* cDNA was cloned as described previously.[15] MISSION shRNA targeting mouse *aggrus* (TRCN0000176005: shAgg), human *aggrus* (TRCN0000061924: shAgg1h and TRC N0000061926: shAgg2h) and empty vector (SHC001: shCont) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines

CHO cells were purchased from the American Type Culture Collection (ATCC) and MBT-2 cells were obtained from the RIKEN Cell Bank (Yokohama, Japan). Both cell lines were cultured in RPMI 1640 media containing 10% fetal bovine serum (FBS). HT1080 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium containing 10% FBS. UM-UC-3 (ATCC) and T24 (RIKEN Cell Bank) cells were cultured in minimum essential medium (MEM) containing 10% FBS. UM-UC-5 cells (Health Protection Agency, Salisbury, UK) were cultured in MEM containing 1 mM nonessential amino acids (NEAA, Sigma-Aldrich) and 10% FBS. SCaBER (ATCC) and J82 (ATCC) cells were cultured in MEM containing 1 mM sodium pyruvate, 1 mM NEAA and 10% FBS. RT-4 cells (ATCC) were cultured in McCoy's 5A media containing 10% FBS. CHO cells that had stably transfected with human *aggrus* gene (CHO/Aggrus) and NL-17 cells (a highly metastatic variant of colon 26 ADC) were established in our laboratory and cultured in RPMI 1640 media containing 10% FBS.[11,28]

Immunoblot analysis

Sample preparation was performed as described previously.[29] In brief, cells were lysed in lysis buffer [25 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0.2% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 2% NP-40, 66 mU/ml of aprotinin and 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes and immunoblotted with anti-human Aggrus/podoplanin mAb (clone: E-1, Santa Cruz Biotechnology), anti-mouse Aggrus mAb (clone: 8F11, established in our laboratory),[30] or anti-β-actin mAb (clone: AC-15, Santa Cruz). Enhanced chemiluminescence reagent (GE Healthcare) and luminescence image analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan) were used in detection.

Flow cytometric analysis

Cells were harvested and treated with or without recombinant $(His)_{10}$-tagged human CLEC-2 (10 μg/ml) following incubation with the Alexa Fluor 488-conjugated anti-$(His)_5$ antibody (QIAGEN, Hilden, Germany). Flow cytometric analysis was performed using a Cytomics FC500 flow cytometry system (Beckman Coulter).

Platelet-aggregation assay

Murine whole blood was drawn by cardiac puncture from Jcl:ICR mice terminally anesthetized with chloroform and taken with heparin solution. Platelet aggregation was measured using the screen filtration pressure method and a WBA Carna aggregometer (IMI, Saitama, Japan). Whole blood samples (200 μl) were stirred in the reaction tubes at 1,000 rpm at 37°C and preincubated for 2 min, followed by cell addition (MBT-2 and its transfectants: 1.6×10^5 cells, SCaBER: 1.3×10^5 cells). Using a 3.7-mm diameter syringe containing screen microsieves made of nickel with 300 openings per 30×30 μm^2 in an area 1 mm in diameter, whole blood were sucked to detect aggregation pressure at 1–12 min later. The final platelet-aggregation pressure in each reaction tube was determined depending on the pressure rate (%) detected by the pressure sensor. In some experiments, SCaBER cells were preincubated for 30 min on ice with 300 μg/ml of anti-human Aggrus neutralizing mAbs, previously established in our laboratory (clone: P2-0 and MS-1),[31,32] or control mouse IgG (Sigma-Aldrich).

Animals

Male C3H/HeNCrlCrlj and female CB17/Icr-*Prkdc*^{scid}/CrlCrlj mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Jcl:ICR mice were purchased from Clea Japan. (Tokyo, Japan). All animal procedures were performed using protocols approved by the Japanese Foundation for Cancer Research Animal Care and Use Committee.

Experimental pulmonary metastasis

MBT-2 cells were suspended in Hanks' Balanced Salt Solutions (HBSS) and intravenously injected (6.0×10^5 cells/mouse) into the lateral tail vein of 8-week-old male C3H/HeNCrlCrlj mice. Control mouse IgG or anti-human Aggrus mAb (30 μg/mouse) was intravenously injected into the lateral tail vein of CB17/Icr-*Prkdc*^{scid}/CrlCrlj mice the day before intravenous injection of SCaBER cells suspended in HBSS (5.0×10^5 cells/mouse). After 17–20 days (MBT-2 cells) and 32–35 days (SCaBER cells), mice were euthanized; their lungs were stained with saturated picric acid solution. Lung surface metastatic foci were then counted.

In vitro and *in vivo* proliferation assays

To assess *in vitro* cell proliferation, the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI) was used after 24, 48 or 72 h in cell culture. For the *in vivo* proliferation assay, SCaBER cells were harvested, washed and resuspended in HBSS. In total, 3.0×10^6 cells suspended in 50 μl HBSS were subcutaneously injected into the backs of 8-week-old male C3H/HeNCrlCrlj mice. Tumor volume was calculated using the following formula: volume $= W^2 \times L/2$, where W is width (the shortest dimension) and L is length (the longest dimension).

Statistical analysis

The Mann–Whitney *U* test was performed to determine the statistical significance of the results of the metastasis assays. Some results were compared using Student *t*-test. Significant *p* values are shown as $^{*}p < 0.05$, $^{**}p < 0.01$. All statistical tests were two-sided.

Results

Aggrus expression in bladder cancers

To identify new tumors expressing Aggrus, qRT-PCR was initially performed using the TissueScan Cancer Survey Panel containing 381 human tissues from patients with 22 different forms of cancer. Upregulation of *aggrus* mRNA was detected in cancer tissues from esophagus, lung, testis and urinary bladder, which were then compared with normal tissues (Fig. 1). Because several groups have already reported Aggrus overexpression in esophageal, lung and testicular tumors,[18,33–35] we focused on *aggrus* expression in urinary bladder cancers in this study. Although we compared the *aggrus* mRNA level among histological types or differentiation degrees of urinary bladder cancers, no correlation could be found (Supporting Information Table S1).

Correlation of Aggrus expression with metastatic tendency in bladder cancers

We next examined Aggrus protein expression in 135 different cases of human bladder cancer by IHC staining of tissue arrays with anti-human Aggrus/podoplanin mAb D2–40. Although most G1, G2 and G3 TCCs received scores of 0–3, approximately half of the metastatic TCC tissues received scores ≥ 4 (Fig. 2*a* and Supporting Information Table S2). Moreover, clear staining of SCC (but not ADC) of bladder was observed (Fig. 2*b*). Because distant metastasis is more common in TCC and SCC than in ADC,[6,7] these results suggest that Aggrus would be useful as a biomarker for prediction of metastatic TCC and SCC of bladder.

Aggrus expression in mouse bladder cancer cell line MBT-2

Aggrus is known to enhance hematogenous metastasis of various cancers by interacting with and activating platelet receptor CLEC-2.[16] To investigate the effects of Aggrus expression in bladder cancer metastasis, the chemically induced mouse bladder TCC cell line MBT-2 was initially utilized because of its applicability to syngeneic tumor models. Expression of mouse Aggrus protein has been reported in NL-17 cells, a highly metastatic subclone of the mouse colon ADC 26 cell line.[28] Therefore, NL-17 cells were used as a positive control in this study. Aggrus protein and mRNA expression was detected in MBT-2 cells (Figs. 3*a* and 3*b*). Aggrus-knockdown MBT-2 cells were then established and designated as MBT-2/shAgg cells (Figs. 3*c* and 3*d*). These cells were examined for their binding capacity against the recombinant CLEC-2 protein. Although cell surface binding

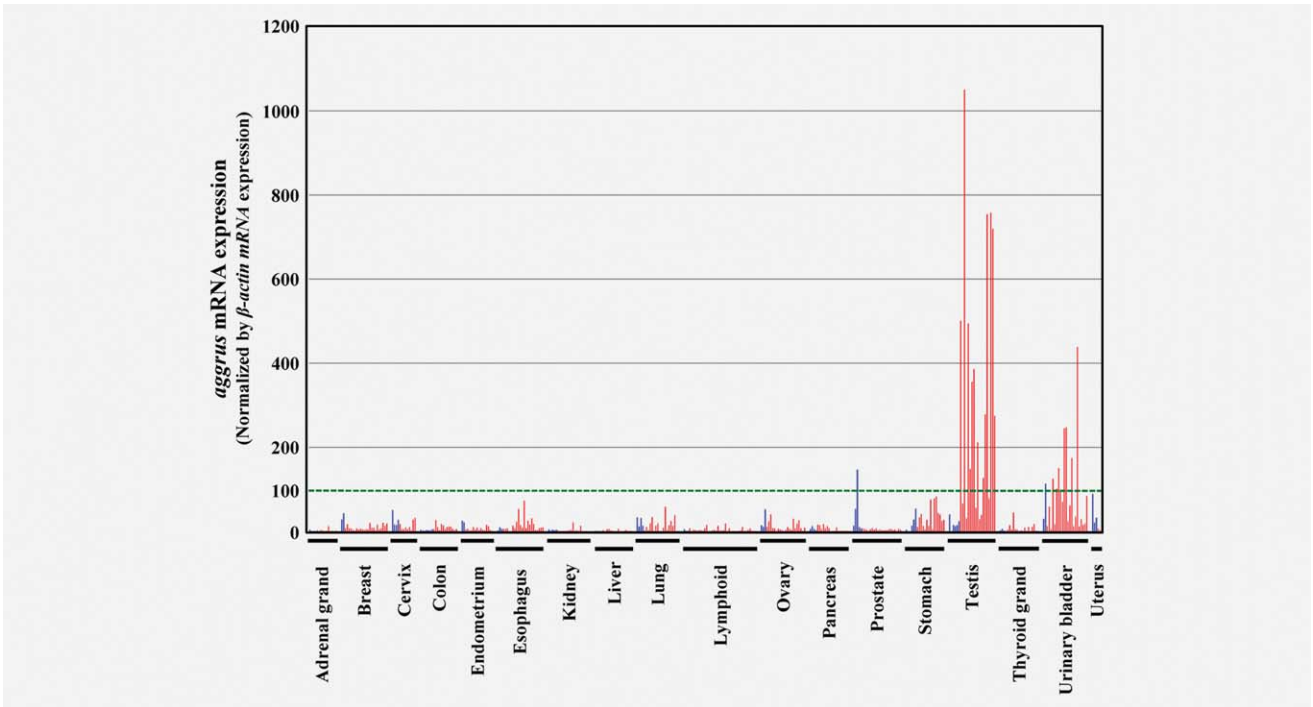

Figure 1. Overexpression of *aggrus* mRNA in bladder cancers. Expression of *aggrus* mRNA were screened using TissueScan Cancer Survey Panel 4 × 96-III (OriGene Technologies, Rockville, MD) containing 381 human tissues covering 22 different cancers. The expression level of each *aggrus* mRNA was normalized by that of *β-actin*. The blue bar represents the expression level of normal tissue, red bar represents that of cancer tissue.

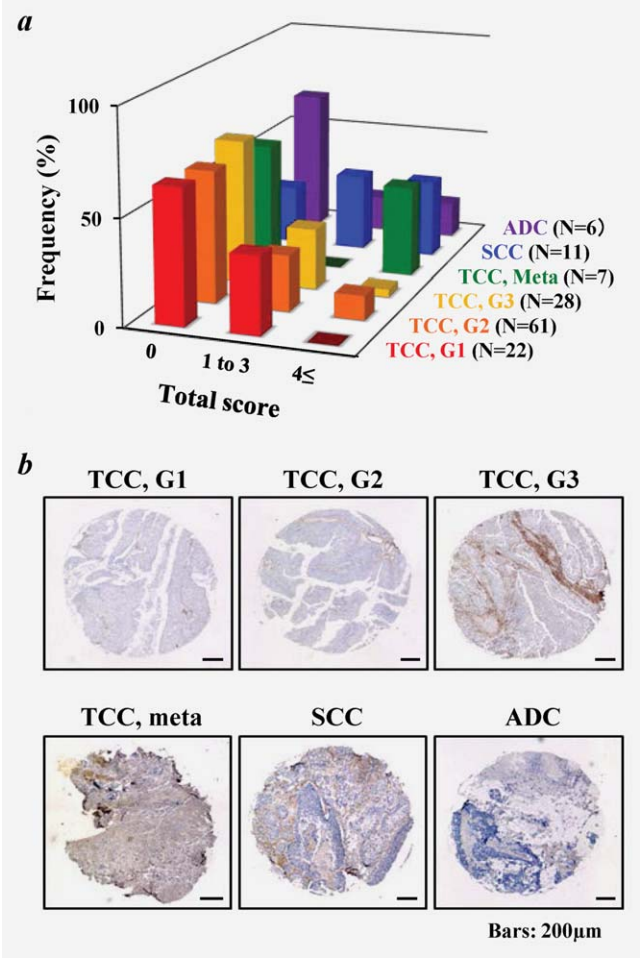

Figure 2. Correlation between Aggrus expression and metastatic tendency in bladder cancers. (*a*) and (*b*) Human bladder cancer tissue arrays containing 135 different cases were stained with anti-human Aggrus/podoplanin mAb (clone: D2–40) and Mayer's hematoxylin solution. Stain score was the sum of proportion score and intensity score as described in Materials and Methods (*a*). Typical microscopic photographs of the human bladder cancer sections were shown (*b*).

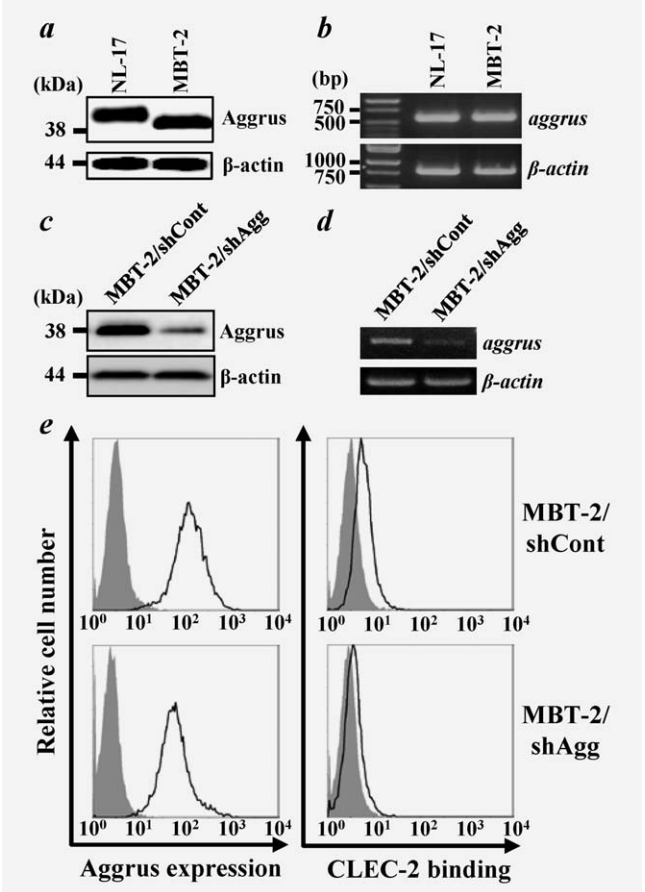

Figure 3. Aggrus expression in mouse bladder cancer MBT-2 cells. (*a*) Aliquots of cell lysates were immunoblotted with the anti-mouse Aggrus mAb (clone: 8F11, upper panel) or anti-*β*-actin mAb (lower panel). (*b*) The mouse *aggrus* (upper panel) and mouse *β-actin* (lower panel) mRNAs were amplified by semi-quantitative RT-PCR from cDNA of MBT-2 and NL-17 cells. NL-17 cell line was used as a positive control of Aggrus-expressing mouse cancer cells. (*c*) MBT-2/shAgg or MBT-2/shCont cells were lysed and immunoblotted with anti-mouse Aggrus mAb (upper panel) or anti-*β*-actin mAb (lower panel). (*d*) The mouse *aggrus* (upper panel) and mouse *β-actin* (lower panel) mRNAs were amplified by semi-quantitative RT-PCR from cDNA of MBT-2/shCont and MBT-2/shAgg cells. (*e*) Left panels, MBT-2 cells were treated with control rat IgG (gray area) or anti-mouse Aggrus mAb (bold lines). Aggrus expression was detected by flow cytometry (left panels). (*e*) Right panels, MBT-2 cells were treated with or without His-tagged recombinant human CLEC-2 protein (10 μg/ml). CLEC-2 binding was detected by flow cytometry (right panels).

of CLEC-2 was detected by flow cytometry in a control cell line (MBT-2/shCont); the CLEC-2 binding was dramatically attenuated in MBT-2/shAgg cells (Fig. 3*e*). These results indicated an interaction of MBT-2 cells with the CLEC-2 protein through Aggrus expression on the cell surface.

Involvement of Aggrus in pulmonary metastasis of MBT-2 cells

Aggrus–CLEC-2 interaction is critical for Aggrus-induced platelet aggregation and hematogenous metastasis.[16] Thus, platelet aggregation-inducing ability and lung metastasizing ability of Aggrus-knockdown cells were measured. It was observed that platelet-aggregation rate and the number of lung surface metastatic foci were attenuated in MBT-2/shAgg cells, which is consistent with the results shown in Figure 3*e*, but the attenuation was not observed in MBT-2/shCont cells (Figs. 4*a*–4*c*). The *in vivo* growth rate was similar among the established MBT-2 clones (Supporting Information Fig. S1). These results indicated that Aggrus plays an important role in pulmonary metastasis of MBT-2 cells.

Aggrus expression in human bladder cancer cells and its role in pulmonary metastasis

By collecting several human bladder cancer cell lines, we screened the expression of human Aggrus protein in these cells. Our previously established Aggrus-transfected CHO

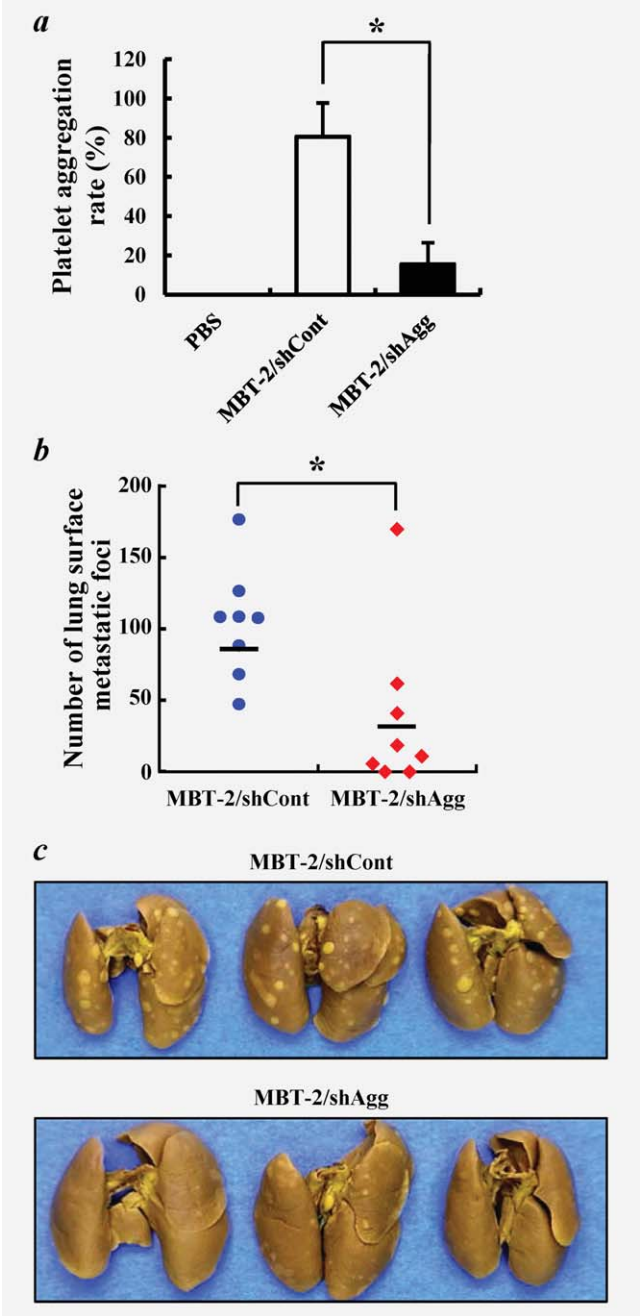

Figure 4. Attenuation of pulmonary metastasis of mouse bladder cancer cells by Aggrus knockdown. (*a*) Whole-blood samples (200 μl) in the reaction tubes were stirred at 1,000 rpm at 37°C and preincubated for 2 min, followed by MBT-2 cells addition (1.6×10^5 cells). The final platelet-aggregation pressure of each reaction tube was determined at the pressure rate (%) of a pressure sensor using WBA Carna aggregometer. NS: not significant. *$p < 0.05$ by the Mann–Whitney *U* test. (*b*) and (*c*) the MBT-2/shCont or MBT-2/shAgg cells were intravenously inoculated into syngeneic C3H/HeNCrlCrlj mice (6.0×10^5 cells/mouse). After 19 days of tumor inoculation, lung surface metastatic foci were counted. Numbers of metastatic foci in each mouse were shown (*b*). Bars, mean. $N = 8$. NS: not significant. *$p < 0.05$ by the Mann–Whitney *U* test. Representative pictures of the lungs and lung surface metastatic foci were shown (*c*).

(CHO/Aggrus) cells were used as a positive controls.[15] Aggrus expression was detected in SCC cell lines such as UM-UC-5 and SCaBER, and a transitional cell papilloma cell line, RT4 (Figs. 5*a* and 5*b*). No Aggrus expression was detected in the collected TCC cell lines (J82, UM-UC-3 and T24). To investigate the role of Aggrus expression in hematogenous metastasis in human bladder cancer cells, Aggrus-knockdown SCaBER cells were established (SCaBER/shAgg1h and SCaBER/shAgg2h; Figs. 5*c* and 5*d*) and their role in pulmonary metastasis was examined. Metastatic ability was attenuated in SCaBER/shAgg1h and SCaBER/shAgg2h cells consistent with Aggrus expression levels (Figs. 5*e* and 5*f*). These results indicate that human Aggrus also plays an important role in pulmonary metastasis of human bladder cancer cells.

Efficacy and mechanism of anti-Aggrus neutralizing antibodies on pulmonary metastasis of human bladder cancer cells

We have previously established anti-human Aggrus neutralizing antibodies designated as P2-0 and MS-1, and clarified their efficacy as hematogenous metastasis inhibitors using Aggrus-overexpressing cell lines.[31,32] Thus, we attempted to examine the efficacy of anti-human Aggrus neutralizing antibodies on SCaBER cell-mediated platelet aggregation. Platelet aggregation induced by SCaBER cells was significantly suppressed by the addition of P2-0 or MS-1 mAb (Fig. 6*a*). The effects of these antibodies on pulmonary metastasis of SCaBER cells were then investigated. Consistent with the earlier results, pulmonary metastasis of SCaBER cells was prevented by prior administration of P2-0 or MS-1 mAb into mice (Figs. 6*b* and 6*c*). To clarify how these mAbs suppress lung metastasis, calcein-AM-labeled SCaBER cells were intravenously injected into nude mice and fluorescent cells in lung microvessels were counted after 30 min. Fluorescent cells were detected in lungs of control IgG-administrated mice, indicating that several SCaBER cells were trapped in lung (Figs. 6*d* and 6*e*). In contrast, prior administration of P2-0 or MS-1 mAbs dramatically decreased the number of cells trapped in lung. These results indicated that P2-0 and MS-1 mAbs suppressed pulmonary metastasis of SCaBER cells by inhibiting cell retention in lung. Therefore, anti-Aggrus neutralizing antibodies would be useful for the prevention of pulmonary metastasis of Aggrus-positive bladder cancer cells.

Discussion

Although superficial tumors in patients with bladder cancer are generally not life-threatening, occult distant metastasis in recurrent cancer is associated with poor 5-year survival rates.[5] Molecular markers to identify localized tumors with high metastatic potential are currently lacking.[36] Thus, identification and inhibition of key molecules that induce distant metastasis is important for improving the 5-year survival rate of patients with bladder cancer.

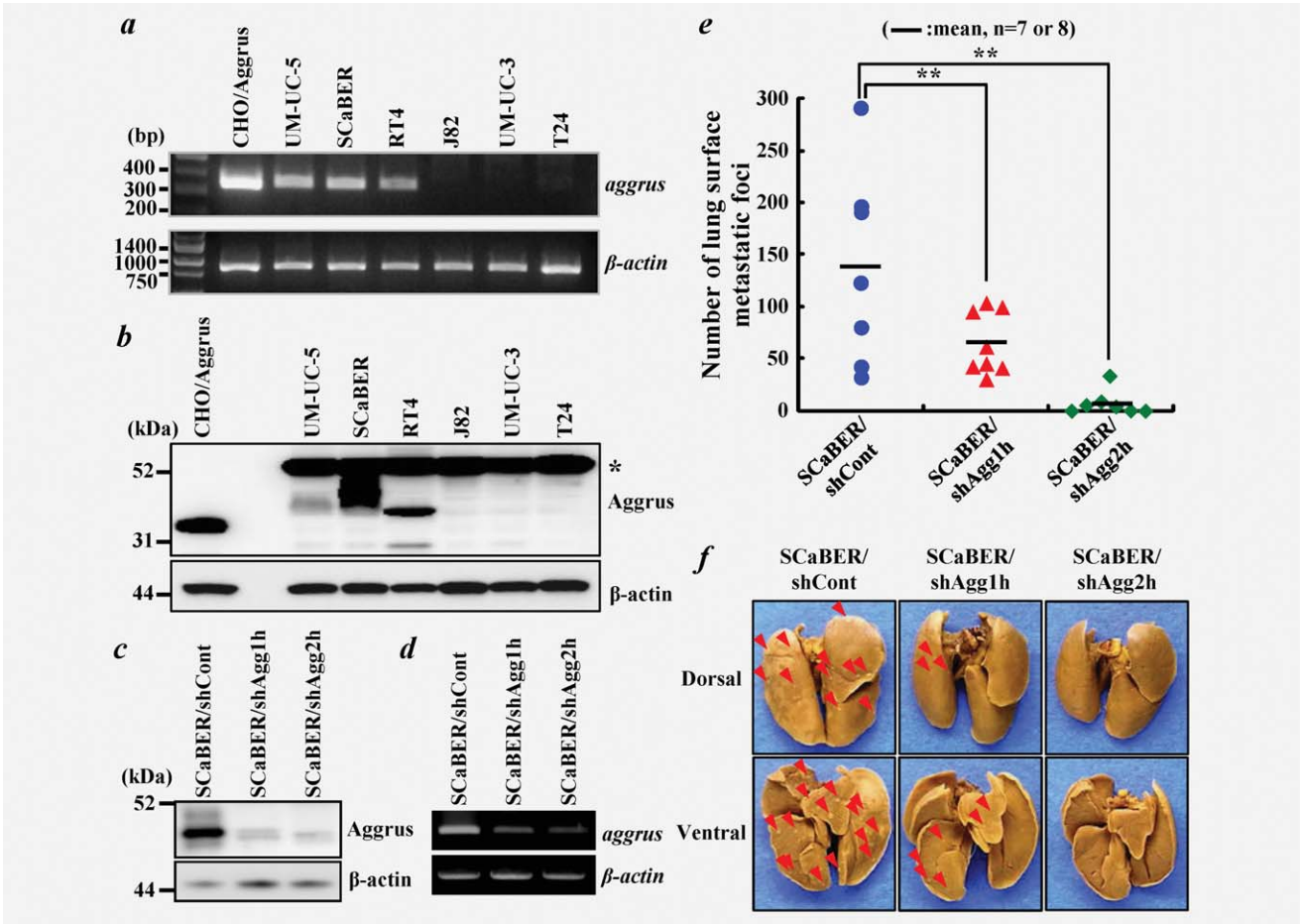

Figure 5. Involvement of Aggrus in pulmonary metastasis of human bladder cancer cells. (*a*) The human *aggrus* (upper panel) and human *β-actin* (lower panel) mRNAs were amplified by semi-quantitative RT-PCR from cDNAs of the indicated cell lines. CHO/Aggrus cell line was used as a positive control of human Aggrus-expressing cell line. (*b*) Aliquots of cell lysates were immunoblotted with the anti-human Aggrus mAb (clone: E-1, upper panel) or anti-*β*-actin mAb (lower panel). The asterisk (*) indicates non-specific band. (*c*) SCaBER cells that had been stably transfected with shRNA targeting human *aggrus* (SCaBER/shAgg1h and SCaBER/shAgg2h) or empty vector (SCaBER/shCont) were lysed and immunoblotted with anti-human Aggrus mAb (clone: E-1, upper panel) or anti-β-actin mAb (lower panel). (*d*) The human *aggrus* (upper panel) and human *β-actin* (lower panel) mRNAs were amplified by semi-quantitative RT-PCR from cDNA of SCaBER/shCont, SCaBER/shAgg1h and SCaBER/shAgg2h cells. (*e*) and (*f*) SCaBER/shCont, SCaBER/shAgg1h, or SCaBER/shAgg2h cells were intravenously inoculated into ICR/scid mice (5.0×10^5 cells/mouse). After 33 days of tumor inoculation, lung surface metastatic foci were counted. Numbers of metastatic foci in each mouse were shown (*e*). Bars, mean. $n = 7$ or 8. NS, not significant. $^{**}p < 0.01$ by the Mann–Whitney *U* test. Representative pictures of the lungs and lung surface metastatic foci were shown (*f*).

In this study, upregulation of the platelet aggregation-inducing factor Aggrus was found in bladder TCC tissues from patients with distant metastasis (Fig. 2). Although Aggrus reportedly enhances hematogenous metastasis in experimental pulmonary metastasis models,[11,15] the correlation between Aggrus expression and distant metastasis in cancer patients is not so clear. This is the first study to report the Aggrus upregulation in bladder cancer patients with distant metastasis and to indicate the possibility of Aggrus as a biomarker for predicting distant metastasis of bladder cancer. Previous reports have examined ET-1, ET-1 receptor, and CD24 as possible diagnostic markers of distant metastasis in bladder cancer.[8,9] ET-1 is known to promote tumor colonization through ET-1 receptor activation without affecting primary tumor growth.[8] CD24 is reported to be involved in the progression of tumorigenesis and metastasis of male bladder cancer under androgen regulation.[9] Using existing diagnostic markers such as ET-1 or CD24 in combination with Aggrus would improve accuracy in identification of distant metastasis in bladder cancer patients.

Moreover, Aggrus expression was found in clinical specimens of bladder SCC by IHC analysis (Fig. 2) and in bladder SCC cell lines (SCaBER and UM-UC-5, Fig. 5). Several groups have reported Aggrus upregulation in SCC of lung, esophagus and head and neck.[18,34,35,37] Thus, our finding is consistent with those of the above mentioned reports.

An association of Aggrus with pulmonary metastasis of bladder cancer cell lines was also found in this study (Figs. 4 and 5). The results of this study indicate that pulmonary

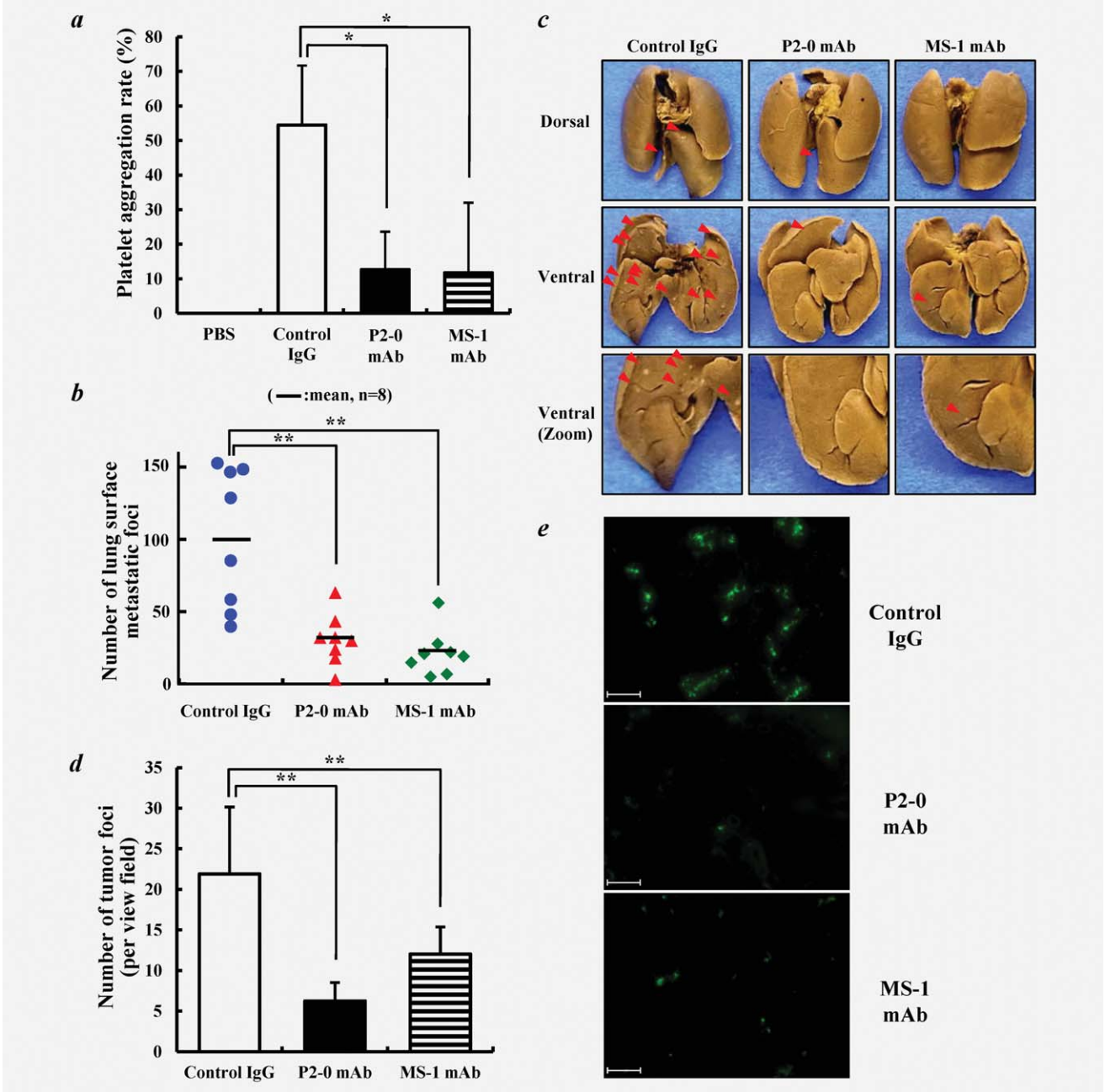

Figure 6. Efficacy and mechanism of anti-Aggrus neutralizing mAbs on pulmonary metastasis of human bladder cancer cells. (*a*) SCaBER cells were incubated with 300 μg/ml of anti-human Aggrus mAbs (clone: P2-0 and MS-1) or control mouse IgG for 30 min on ice, followed by incubation with whole-blood samples. The platelet-aggregation rate was determined using WBA Carna aggregometer. $*p < 0.05$ by the Mann–Whitney *U* test. (*b*) to (*e*) CB17/Icr-*Prkdc*^{scid}/CrlCrlj mice were intravenously injected with control mouse IgG, P2-0 mAb, or MS-1 mAb (30 μg/mouse). After 24 hr of mAb administration, SCaBER cells were intravenously inoculated into the mice. After 35 days of tumor inoculation, lung surface metastatic foci were counted. Bars, mean. $n = 8$. $**p < 0.01$ by the Mann–Whitney *U* test (*b*). Representative pictures of the lung surface metastatic foci were shown (*c*). After 24 hr of mAb injection, calcein-AM-labeled SCaBER cells were intravenously inoculated into the mice (*d* and *e*). After 30 min of tumor inoculation, frozen section of lung was prepared and number of micro metastatic foci labeled by calcein-AM was counted (*d*). Representative pictures of the frozen section of lung are shown (*e*). Scale bar, 0.5 mm. Bars, mean. $n = 3$. $**p < 0.01$ by the Mann–Whitney *U* test (*d*).

metastasis may be prevented by prior administration of anti-Aggrus neutralizing mAbs (Fig. 6). The two anti-Aggrus neutralizing mAbs that we established, P2-0 (mouse IgG1) and MS-1 (mouse IgG2a), possess similar affinity, epitope and platelet-neutralizing ability. However, only MS-1 exhibits Antibody-dependent cellular cytotoxic (ADCC) activity and complement-dependent cytotoxic (CDC) activity.[32] Because P2-0 and MS-1 mAbs suppressed lung retention of bladder

cancer cells, the anti-metastatic effects of these anti-Aggrus neutralizing antibodies would be platelet-aggregation inhibitory activity rather than effector activity. Although inhibitory effects of P2-0 and MS-1 mAbs are similar in the platelet-aggregation assay (Fig. 6*a*), P2-0 mAb exhibited inhibitory activity higher than MS-1 mAb in the lung retention assay (Fig. 6*d*). We performed platelet-aggregation assay after mixing cancer cells with antibodies, while in the lung retention assay the cancer cells were intravenously inoculated on the day following administration of antibodies into mice. Because P2-0 and MS-1 mAbs are similar in the affinity (9.3 and 9.0 nM, respectively) but slightly different in the dissociation rate constant (8.5×10^{-3} and 1×10^{-2} sec^{-1}, respectively),[31,32] it might be appeared as a difference of the capability to block Aggurs–CLEC-2 interaction in a short period until the retention of cancer cells in lung microvessels. We have also confirmed that the administration of either P2-0 or MS-1 mAb on the day following intravenous inoculation of tumor cells significantly suppressed the number of lung metastatic foci (data not shown). Thus, anti-Aggrus neutralizing antibodies may be appropriate as both neo-adjuvant and adjuvant therapy in patients with highly metastatic bladder cancer. However, further studies are required to evaluate the function of anti-Aggrus neutralizing mAbs on Aggrus-positive cancer cells in the bloodstream.

Because systemic chemotherapy regimens used to treat bladder TCC are generally ineffective for treatment of pure bladder SCC,[38] effective molecular target drugs against bladder SCC are urgently needed. ADCC and CDC activities are effective modes of action for therapeutic antibodies. Drugs based on these activities have been approved for clinical usage (*i.e.*, trastuzumab and rituximab). Abe *et al.* reported the efficacy of co-administration therapy of anti-Aggrus antibodies exhibiting ADCC activity with natural killer cells against Aggrus-positive malignant pleural mesothelioma,[39] suggesting that anti-Aggrus antibody-based immunotherapy may be a promising strategy for the treatment of bladder SCC.

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