A humanized single-chain antibody against beta 3 integrin inhibits pulmonary metastasis by preferentially fragmenting activated platelets in the tumor microenvironment

Abbreviated Title: Human Anti-GPIIIa Ab inhibits pulmonary metastasis

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Abstract

Platelets play a supportive role in tumor metastasis. Impairment of platelet function within the tumor microenvironment may provide a clinically useful approach to inhibit metastasis. We developed a novel humanized single-chain antibody (scFv Ab) against integrin GPIIIa49-66 (named A11) capable of lysing activated platelets. In this study, we investigate the effect of A11 on the development of pulmonary metastases. In the Lewis lung carcinoma (LLC) metastatic model, A11 decreases the mean number of surface nodules and mean volume of pulmonary nodules. It protects against lung metastases in a time window that extended 4 hours prior to and 4 hours after the i.v. injection of LLCs. Co-injection of GPIIIa49-66 albumin reverses the anti-metastatic activity of A11 in B16 melanoma model, consistent with the pathophysiological relevance of the platelet GPIIIa-49-66 epitope. Significantly A11 had no effect on angiogenesis using both in vitro and in vivo assays. The underlying molecular mechanisms are a combination of inhibition of each of the following interactions: between activated platelets and tumor cells, platelets and endothelial cells, and platelets and monocytes, as well as disaggregation of an existing platelet/tumor thrombus. Our observations may provide a novel anti-metastatic strategy through lysing activated platelets in the tumor microenvironment using humanized anti-GPIIIa49-66 scFv Ab.
Introduction

Tumor metastasis is the main cause of death from cancer and a major challenge for improving cancer management. Hematogenous tumor cell spreading is a highly complex process, including detachment of cancer cells from the primary site, migration into and transport along the bloodstream, and finally tumor cell arrest and proliferation within distant tissue. Thus survival of tumor cells within the bloodstream and adhesion in the vasculature at the metastatic site are crucial for tumor cell dissemination. Extensive evidence indicates that the interaction of tumor cells with platelets within the bloodstream plays an important role during the early phase of metastasis.1, 2

The involvement of platelets and coagulation factors in hematogenous tumor metastasis has long been recognized. Cancer patients frequently present with signs of thrombosis, and these are most severe if the disease has progressed to a metastatic stage.1–7 Furthermore, thrombocytopenia or the inhibition of platelet function can markedly suppress tumor metastasis.8–11 Subsequent animal models in which specific platelet functions were altered through drug treatment or controlled genetic ablation have led to a model of platelet-supported tumor metastasis in which tumour cells enter the bloodstream (intravasation), and bind and activate platelets (cohesion) and leukocytes.12,13 These host cells then assist tumour cell arrest at the vessel wall (adhesion) and survival within the vasculature (immune evasion), which enables exit from the circulation (extravasation), and tumour cell survival and proliferation within target tissues of metastasis.14–16 These contributions of platelets to tumour cell survival and spread suggest that agents directed against these processes may give rise to new therapies for patients with a high risk of metastasis or for minimizing the risk of cancer cell dissemination during tumor surgery.

Integrin αIIbβ3 (platelet glycoprotein GPIIb/IIIa) is a heterodimeric receptor of the integrin family expressed at high density (50,000–80,000 copies/cell) on the platelet membrane.17 In the circulation it is normally in a resting state but is activated during platelet aggregation and adhesion, which in binding to fibrinogen and von Willebrand factor allows formation of a platelet aggregate or a mural thrombus on damaged vessel walls.
GPIIIa49-66 (CAPESIEFPVSEAREVLED) is a linear epitope of integrin subunit β3 (GPIIIa) on the surface of platelets. We have previously described a unique antiplatelet autoantibody in patients with HIV- or hepatitis C-related thrombocytopenia that recognizes the sequence GPIIIa49-66 and induces complement-independent platelet lysis by generation of reactive oxygen species and peroxide after platelet-reduced nicotinamide adenine dinucleotide phosphate oxidase activation.18-22 By screening a human single-chain fragment variable region (scFv) library with the GPIIIa49-66 peptide as bait, we identified a human monoclonal scFv Ab that recognized GPIIIa49-66 (named A11), with similar functional properties to the patient autoantibody in that it preferentially binds to activated platelets and can also lyse platelet thrombus in vitro.23-25 We therefore sought to determine whether A11 would be associated with any significant anti-metastatic effect by clearance of functional, activated platelets in the tumor environment.

Materials and methods

Cell lines

Lewis lung carcinoma cells (LLCs), Human umbilical vein endothelial cells (HUVECs) and B16 melanoma cells (B16) were all purchased from American Type Culture Collection (Rockville, MD). LLC and B16 cells were maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% newborn calf serum (Gibco). HUVECs were cultured in iscove’s modified Dulbecco’s medium (Gibco) containing 10% (v/v) fetal bovine serum (Gibco) and supplemented with 90 μg/mL heparin sodium (Sigma,St.Louis,MO), 2 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN). All culture systems were incubated at 37°C in a humidified, 5% CO2 atmosphere.

Antibodies

Human monoclonal scFv Ab against GPIIIa49-66 (A11) and control scFv Ab (13CG2) were prepared as described.24, 25
Mice
C57BL/6J background mice used in the following experiments were raised in our laboratory under specific pathogen-free conditions. All procedures in animal experiments were approved by the Institutional Animal Care and Use Committee of East China Normal University.

Monocyte isolation and assay of platelet adhesion to monocytes\(^{26,27}\)
See “Supplemental data”.

Fluorescence labeling of tumor cells
Tumor cells were incubated with fluorescent dye DIO (Beyotime Institute of Biotechnology, China) (5 \(\mu\)g/mL) for 30 minutes at 37\(^\circ\)C. After washing three times with PBS, the pellet was resuspended in RPMI 1640 medium for later use.

Establishment of hypoxia-reoxygenation model
The cells were cultured in a 37\(^\circ\)C, 95% air and 5% CO\(_2\) incubator to 90% confluence, and then the culture flasks or plates were transferred into an air-tight container infused with a mixture air of 95% N\(_2\) and 5% CO\(_2\) for 24 hours at 37\(^\circ\)C followed by culturing at 37\(^\circ\)C, 95% air and 5% CO\(_2\) incubator for 2 hours.

Disaggregation and destruction of ex vivo tumor-platelet aggregates
Disaggregation and destruction of ex vivo tumor-platelet aggregates were determined as previously published.\(^{23}\)
To create tumor-platelet aggregates, thrombin (0.25 U/mL) was incubated with a tumor cell - platelet mixture (platelet: tumor = 3,000 : 1) for 1 hour at 37\(^\circ\)C with intermittent shaking, followed by gravity sedimentation at room temperature for 30 minutes. Excess reagents were removed by washing in PBS. A total of 0.5 \(\mu\)M A11 or
control scFv (13CG2) was added at different time intervals, and the remaining tumor-platelet/aggregate was enumerated as previously described.23

**Assay of tumor cells adhesion to activated platelets**

The adhesion of tumor cells to activated platelets was measured as previously described with slight modifications.28 Briefly, thrombin (0.25 U/mL) activated platelets were incubated in 96-well flat bottomed microtitre plates at a density of $3 \times 10^7$ cells/well, and then treated with various concentrations of A11 for 4 hours at 37°C. Plates were then blocked with 0.01M PBS plus 1% BSA for 1 hour at 37°C, and washed three times with PBS-BSA plus 0.9 mM CaCl$_2$, 0.9 mM MgCl$_2$ before the addition of $1 \times 10^4$ DIO-labeled tumor cells for a 4 hours co-incubation. The non-adherent tumor cells were discarded. The fluorescent intensity of adherent tumor cells was observed with a fluorescence plate reader (Molecular Devices Corporation, USA).

**Assay of activated platelets adhesion to endothelial cells**

For the platelet-endothelial adhesion assay, HUVECs were incubated in 96-well flat bottomed microtitre plates at a density of $1 \times 10^4$ cells/well until the cellular confluence reached 90%. The treatment of hypoxia-reoxygenation was applied as described above. After labeling platelets with sulfo-NHS-LC-Biotin (PIERCE, USA) as described previously,23 labeled platelets were activated by thrombin, and then added into the HUVEC culture plate, followed by co-incubation at 37°C for 4 hours in the presence of various concentrations of A11 or control scFv (13CG2) (platelet: HUVECs = 1,000 : 1). The non-adherent platelets were aspirated off before the addition of horseradish peroxidase-conjugated streptavidin to be developed with the TMB substrate. The extent of adhesion at each concentration of A11 was detected by comparison with the standard curve and expressed as the percentage of the control where the platelets were not preincubated with A11.

**Assay of tumor cells adhesion to endothelial cells**
HUVECs were incubated in 96-well flat bottomed microtitre plates at a density of $1 \times 10^4$ cells/well until the cellular confluence reached 90%. The treatment of hypoxia-reoxygenation was applied as described above. Platelets were activated by thrombin (0.25 U/mL), and then co-incubated with the HUVEC culture plate at 37°C for 4 hours in the presence of A11 or control scFv 13CG2 (platelet: HUVECs = 1,000 : 1). The non-adherent platelets were aspirated off before the addition of Dio- labeled B16 melanoma cells. The adhesion status of B16 melanoma cells with HUVECs was detected under a fluorescence microscope.

**MTT, transwell migration and tube formation assay**

See “Supplemental data”.

**Animal experiments**

**Detection of the effect of platelets on metastasis of B16 tumor cells in vivo**

Platelets were isolated from healthy C57BL/6J mice as described previously. Then B16 cells were co-incubated with isolated platelets for 30 minutes (platelet: tumor cell ratio=1000:1). The C57BL/6J mice were randomly divided into two groups, and were injected with B16 cell ($2 \times 10^5$/mouse) or platelet-incubated B16 cells via the tail vein, respectively. After 3 weeks, the mice were sacrificed and the surface metastatic nodules on the lungs were counted.

**Determination of Mouse Platelet Count**

Platelet counts were done as described previously. Briefly a total of 20 μL of blood was drawn into Unopettes (No. 365855, Becton Dickson) that contained an optimal anticoagulant concentration and diluent for quantitating platelet counts by phase-contrast microscopy.

**Bleeding Time**

To determine the bleeding time, the mouse tail vein was severed 2 mm from its tip and blotted every 30 seconds on a circular sheet of filter paper to obtain an objective measurement. Termination of the bleeding time was
recorded after absence of blood on the filter paper. Bleeding time differences were recorded by an unbiased observer and confirmed by two other observers, blinded to the experimental status of the mice.

**Spontaneous lung metastasis assay**

For spontaneous metastasis (which measures metastasis from a primary tumor), 6-week-old C57BL/6J female mice were subcutaneously injected with $2.5 \times 10^5$ viable LLC tumor cells. The primary tumor was resected 10 days later, and mice were treated with A11 or control scFv Ab (13CG2), and three times per week thereafter. After 3 weeks, the mice were sacrificed, and the surface metastatic nodules on the lungs were counted. The volume of each nodule was calculated from its diameter ($d$), by assuming the nodules are spheres. Nodule volumes were calculated by the following formula: $\text{volume (mm}^3) = \pi/6 \times d^3$. The mean nodule volume for each mouse was calculated by dividing the total pulmonary nodule volume for each group of mice by the number of mice in each group.

**Experimental lung metastasis assay**

Experimental metastasis refers to the later steps of the metastatic migration process (extravasation from the blood stream and then growth into pulmonary tumor). In the LLC model, 6-week-old C57BL/6J female mice were randomly divided into five groups: Group 1: mice intravenously pretreated with A11 (25 $\mu$g/mice) 12 hours prior to i.v. injection of LLC tumor cells ($2.5 \times 10^5$/mouse); Group 2: mice were pretreated with A11 4 hours prior to injection of tumor cells; Group 3: mice were first given injections of tumor cells and then simultaneously treated with A11; Group 4: mice were first given injections of tumor cells and then treated with A11 4 hours later; Group5: mice were treated with A11 12 hours after injection of tumor cells. These mice were then given injections of A11 at 24 hours, and three times per week thereafter. Suitable controls were given injections of control scFv 13CG2. In the B16 melanoma model, mice first received an i.v. injection of B16 tumor cells ($2 \times 10^5$/mouse) and then were treated with A11 at 0 and 24 hours post tumor inoculation, followed by three treatments per week thereafter. In a control group, mice received both A11 and an equal molar quantity
of GPIIIa49-66 albumin conjugate. After 3 weeks, the mice were sacrificed to harvest the lungs. The mean number of surface nodules per lung and mean volume of nodules per lung were determined as described above.

Assay of tumor growth

Six-week-old C57BL/6J mice were subcutaneously (s.c.) implanted with $5 \times 10^5$ LLC tumor cells. When tumors became easily palpable, mice were randomly divided into two groups. A11 (25 μg/mice) was injected i.v. into mice thrice per week for 2 successive weeks, whereas the controls received injection of saline. Tumor dimensions were measured with calipers for calculation of tumor volume.

In vivo Matrigel plug assay

See “Supplemental data”.

Assay of early invasion of B16 melanoma in lung tissue

The C57BL/6J mice were randomly divided into two groups, and then treated with A11 or control scFv Ab (13CG2) after i.v. injection of DIO labeled B16 cells. 6 hours later, the mice were sacrificed. The lung tissue was collected at the maximal longitudinal cut surface across the hilus pulmonis and prepared into frozen slices. The adhesion of B16 to vessels was observed under a fluorescence microscope.

Statistical analysis

$P$ values were determined through the two-tailed Student’s $t$ test. Differences were considered statistically significant when $p<0.05$.

Results

Platelets promote pulmonary metastasis in the B16 tumor model

Extensive experimental evidence and clinical studies suggested platelets play an important role during the early phase of tumor metastasis. 10-13 Figure1A show the events occurring following tumor intravasation, tumor-
platelet-endothelial cell binding, platelet-tumor thrombus formation, tumor-platelet embolization, angiogenesis, and extravasation. We further confirmed platelet promotion of the experimental metastasis of B16 melanoma in vivo. As noted in Figure 1B, B16 melanoma cells co-incubated with platelets had a markedly increased number of surface pulmonary nodules compared to B16 melanoma cells alone (16 ± 3.2 vs 5 ± 1.3, **p<0.001). In lung tissue H-E slides, the number of metastatic tumors also increased markedly in comparison to the platelet-free control group (data not shown). We have previously demonstrated that humanized anti-platelet GPIIIα49-66 scFv Ab (A11) preferentially fragments activated versus resting platelets. We therefore hypothesized that A11 may inhibit tumor metastasis by preferentially fragmenting activated platelets in the tumor microenvironment (Figure 1C).

**Effect of A11 on spontaneous metastasis of Lewis Lung Carcinoma (LLC)**

We first tested the effect of A11 on spontaneous lung metastases of LLCs. Figure 2A shows the treatment protocol. Figure 2B demonstrates that the mean number of surface nodules per lung was significantly decreased in the A11 group compared to the control scFv 13CG2 (6.5 ± 2.8 vs 18 ± 4.3, *p<0.01). The mean volume of nodules per lung was reduced in the A11 group compared to the control (1.47 ± 0.35 vs 3.45 ± 0.64, *p<0.01) (Figure 2C). Microscopic evaluation of lung tissues gave results that correlated with the macroscopic findings, i.e. the number of microscopic metastatic focuses was lower in the A11 group than in the control (Figure 2D).

**Effect of A11 on experimental metastasis of Lewis Lung Carcinoma (LLC)**

We next evaluated the effect of A11 on experimental lung metastases of LLC cells inoculated via the tail vein. Figure 3A shows five different pre-treated or treated protocols. Figure 3B demonstrates that A11 immediate injection (0 h) after i.v. injection of LLC provided the best anti-metastatic effect. The mean number of surface nodules per lung was significantly decreased in the A11 group compared to the control scFv 13CG2 (5.9 ± 1.7 vs 18.3 ± 2.9, **p<0.001). A11 4 hours prior- or post- injection of LLCs also inhibits the mean number of surface nodules per lung [A11 (Pre-4 h) vs Ctrl, 12.1 ± 2.3 vs 18.3 ± 2.9; A11 (Post-4 h) vs Ctrl, 7.4 ± 1.8 vs 18.3 ± 2.9; *p<0.01]. No effect was noted on the inhibitory of the mean number of surface nodules per lung if...
A11 was given 12 hours prior- or post-injection of LLCs [A11 (Pre-12 h) vs Ctrl, 20.3 ± 2.7 vs 18.3 ± 2.9, $p=0.17$; A11 (Post-12 h) vs Ctrl, 15.8 ± 1.9 vs 18.3 ± 2.9; $p=0.06$]. A similar effect was noted in the mean volume of nodules per lung [A11 (0h) vs Ctrl, 0.9 ± 0.2 vs 3.1 ± 0.9, **$p<0.001$; A11 (Pre-4 h) vs Ctrl, 1.3 ± 0.5 vs 3.1 ± 0.9; A11 (Post-4 h) vs Ctrl, 1.5 ± 0.4 vs 3.1 ± 0.9; *$p<0.01$; A11 (Pre-12 h) vs Ctrl, 3.1 ± 0.8 vs 3.1 ± 0.9, $p=0.88$; A11 (Post-12 h) vs Ctrl, 2.5 ± 1.9 vs 3.1 ± 0.7; $p=0.15$] (Figure 3B).

**Pathophysiologic relevance of platelet GPIIIa49-66 epitope (GP49) on lung metastases**

To prove the pathophysiologic relevance of the platelet GPIIIa49-66 epitope (GP49) on lung metastases, we examined the effect of a GPIIIa49-66 albumin conjugate on blocking the A11 triggered anti-metastatic function in experimental metastasis of B16 melanoma. We postulated that a GPIIIa49-66 albumin conjugate would prevent the protective effect of A11 on pulmonary metastasis. This proved to be the case. Figure 4A clearly demonstrated that A11 significantly decreased the mean number of surface nodules per lung in comparison with control scFv (13CG2) (A11 vs 13CG2, 4.0 ± 1.5 vs 13.6 ± 4.5, *$p<0.001$); however, injection of A11 with the simultaneous injection of equal molar quantities of GPIIIa49-66 albumin conjugate prevents the protective effect of A11 on pulmonary metastasis (A11+GP49 vs 13CG2, 9.8 ± 2.7 vs 13.6 ± 4.5, $p>0.05$). The same effect was noted on the mean volume of nodules per lung (A11 vs 13CG2, 0.4 ± 0.15 vs 1.6 ± 0.5, *$p<0.001$; A11+GP49 vs 13CG2, 1.1 ± 0.4 vs 1.6 ± 0.5, $p>0.05$) (Figure 4B). Figure 4C demonstrates the prevention of a platelet count drop induced by A11 in animals simultaneously treated with GPIIIa49-66 albumin conjugate.

**Effect of A11 on early invasion of tumor cells in vivo**

Since A11 is more effective in the time window from 4 hours prior- to 4 hours post-injection of tumor cells, we reasoned that A11 may block the early invasion of tumor cells *in vivo*. Mice were treated with 13CG2 or A11 after i.v. injection of Dio- labeled B16 melanoma cells. Six hours later, the lungs were perfused and then used for frozen sections. The accumulation of B16 melanoma cells in lung tissue was observed. Figure 5A demonstrates treatment with A11 results in a significant decrease of lung fluorescent loci in comparison with
the 13CG2 control. The number of fluorescent loci associated with A11 was lower than 13CG2 control (49.8 ± 8.6 vs 13.6 ± 4.0, p<0.01) (Figure 5B).

Molecular mechanisms by which A11 inhibits tumor metastasis

We next investigated the molecular mechanisms by which A11 inhibits tumor metastasis. We first investigated the effect of A11 on the interaction between activated platelets and tumor cells. Figure 6A demonstrates the LLCs adhesion to activated platelet was significantly decreased when activated platelets were pretreated with A11. Note the approximately 82% fluorescence intensity reduction at the maximum A11 concentration, corresponding to reduced LLCs adhesion to activated platelets (Figure 6B). A similar result was obtained using B16 melanoma cells (data not shown).

Since platelets mediated tumor cells adhesion to endothelial cells, we reasoned that the adherence between platelets and endothelial cells will also be impaired by A11. Figure 6C demonstrates that A11 reduced activated platelets adhesion to hypoxic-treated HUVECs in a dose-dependent manner with a maximum 71% inhibition. As expected the irrelevant control, scFv 13CG2, had no effect.

We further examined the effect of A11 on platelets mediated tumor cells adhesion to endothelial cells in vitro. Hypoxic-treated HUVECs were coated on wells, and then incubated with 13CG2- or A11-treated activated platelets, respectively, followed by addition of Dio-labeled B16 melanoma cells. The adhesion status of B16 melanoma cells with HUVECs was detected using a fluorescence microscope (Figure 6D). Figure 6E demonstrates that the fluorescence intensity, corresponding to B16 melanoma cell adhesion to HUVECs, significantly decreased when activated platelets were treated with A11 (291.25 ± 31.98 vs 95 ± 17.79, p<0.05).

We then investigated the effect of A11 on the destruction of already formed tumor-platelet aggregates. Figure 6F clearly demonstrates that A11 disaggregate thrombin-induced tumor-platelet clumps with a nadir at 4 hours, whereas control scFv (13CG2) had no effect.

Since the adhesion of platelets to monocytes is also vital for tumor cell recruitment. We finally evaluated the effect of A11 on the adherence between platelets and monocytes (the protocols are illustrated in supplemental Figure S1). As shown in Table 1, normally about 66% of monocytes are saturated with more than 10 activated
platelets and about 31% contained between 5 to 10 attached platelets, with the remaining 3% containing only 5 or less attached platelets. However treatment of activated platelets with various concentration of A11 changed this distribution. The number of monocytes with less than 5 attached platelets increased from 3% to 29%, between 5 to 10 attached platelets increased from 31% to 57%, and the number of monocytes with more than 10 attached platelets dropped from 66% to 14%, after platelets were treated with A11. This suggests that A11 decreases the attachment of platelets to monocytes.

Effect of A11 on the biological traits of tumor cells and endothelial cells

To rule out the possibility that A11 may be tumoricidal or cross-reactive with tumor cell antigens (for example human melanoma cells containing $\alpha_v\beta_3$), A11 was incubated with B16 melanoma cell and LLC for 7 days in vitro and cell death was evaluated using the MTT assay. No loss of viability could be detected for B16 (Figure 7A) or LLC (Figure 7B) at various concentrations of A11.

Since endothelial integrin $\alpha_v\beta_3$ also shares the A11 epitope-bearing $\beta_3$ chain, we then evaluated the effect of A11 on endothelial cell (HUVEC). In vitro, HUVECs grown in the presence of various concentrations of A11 for 7 days had no impairment (Figure 7C). Figure 7D shows that A11 had no effect on the chemotaxis of both tumor cells and HUVECs. We also demonstrated A11 had no effect on s.c. LLC tumor growth compared to a saline control (Figure 7E). Immunohistochemistry staining for CD31 demonstrated that the blood vessel density in the tumors of A11-treated mice had no significance difference compared to saline control (data not shown). These results indicate that A11 had no effect on the biological traits of tumor cells and endothelial cells, precluding possible side-effects of A11 such as damaging endothelial cells.

Effect of A11 on the angiogenesis process

We also tested the effect of A11 on the angiogenesis process. In vitro, HUVECs form tube and capillary-like structures on the surface of basement membrane extract (Matrigel) in the presence of 50 ng/mL FGF-2 (Trevigen), through a process involving attachment, alignment, and migration. Treatment with different dose of A11 (0.25-2.5 $\mu$M) had no significantly effect on this process (supplemental Figure S2). To determine whether
A11 had an effect on VEGF-induced angiogenesis *in vivo* we used an established *in vivo* angiogenesis model, the mouse Matrigel plug assay. Plugs with VEGF alone or mixed with 50 μg A11 appeared a similar red color, contrasting to plugs with Matrigel alone, which were pale in color, indicating no or reduced blood vessel formation (supplemental Figure S3A). The vessels were abundantly filled with intact RBCs, which indicate the formation of a functional vasculature inside the Matrigel and blood circulation in newly formed vessels by the angiogenesis induced with VEGF. The Hb content inside the Matrigel plug was measured to quantify the effect of A11 on angiogenesis. It demonstrated that A11 had no effect on VEGF-induced neovessel formation *in vivo* (supplemental Figure S3B).

**The biological safety of A11 in vivo**

We finally evaluated the biological safety of A11 on C57BL/6J mice. Previous studies had revealed that the greatest platelet drop induced by an optimal dose of A11 (25 μg/mouse) in BALB/c mice was at 4 hours (~18% platelet drop) with recovery to normal levels at 24 hours, we therefore speculated that it should have a similar effect on C57BL/6J mice. This proved to be the case. Supplemental Figure 4A demonstrates that injection of the same dose of A11 (25 μg/mouse) resulted in an average 21% drop in the platelet count at the 4 hours post-injection time point (*p*<0.001 t=4 hours vs t=0). Injection of the same dose of control scFv Ab (13CG2) did not significantly affect the platelet count (*p*=0.8 t=4 hours vs t=0). Supplemental Figure 4B demonstrated that A11 had no significant effect on the mouse vein tail bleeding time 4 hours (nadir time point) compared to 13CG2 control (205 ± 86 vs 179 ± 58 ; *p*=0.23).

In addition, tumor-free C57BL/6J mice were given same dose of A11 at the same frequency as above and monitored daily for clinical signs of complications. A11-treated mice had no noticeable changes in fur, body weight, appetite, spontaneous bleeding, or life span. No significant pathologic changes were found in the heart, lung, liver, spleen, kidney, or brain by histologic examination (data not shown). This suggests that the treatment was apparently harmless to mice.
Discussion

Cancer cells have been shown to aggregate platelets and this ability correlates with the metastatic potential of cancer cells. The ability of malignant tumor cells to aggregate platelets is called tumor cell-induced platelet aggregation (TCIPA). There is strong evidence suggesting that platelet receptors, including GPIb-IX-V, GPIIb/IIIa and P-selectin, are crucial for TCIPA. Indeed antagonists of GPIIb/IIIa receptor are the most effective known inhibitors of TCIPA. Karpatkin et al. first demonstrated that the drugs belonging to this group hold a potential to reduce TCIPA. A study using an oral GPIIb/IIIa antagonist, XV454, in a mouse model of experimental metastasis showed that this agent is able to inhibit lung metastases formation. Currently, a number of agents directed against human GPIIb/IIIa including abciximab, eptifibatide, and tirofiban have been evaluated in murine tumor models. However, the most challenging problem for the clinical use of antithrombotic approaches in cancer is their lack of selectivity. They affect both haemostasis and cancer-induced thrombosis, which results in severe bleeding complications. Furthermore, currently available antiplatelet drugs target prevention rather than the more clinically relevant issue of resolution of an existing platelet-tumor thrombus. We describe a novel antimetastatic strategy through preferential dissolution of activated platelets in the tumor microenvironment using a humanized anti-GPIIIa49-66 scFv Ab, which has significant translational value since it avoids interfering with hemostasis and minimizes bleeding side effects.

In this report, we demonstrate the following: (1) In the spontaneous LLC metastatic model, A11 treatment decreased the mean number of surface nodules (A11 vs control scFv 13CG2, 6.5 ± 2.8 vs 18 ± 4.3, *p<0.01), and reduced the mean nodule volume per lung (A11 vs control scFv 13CG2, 1.47 ± 0.35 vs 3.45 ± 0.64, *p<0.01). (2) In an experimental LLC metastatic model, A11 provided protection against lung metastases in a time window from 4 hours before to 4 hours after i.v injection of tumor cells. (3) Similar protective effects were observed in experimental metastasis using B16 melanoma. Simultaneously injection of a GPIIIa49-66 albumin conjugate prevents the anti-tumor activity induced by A11. (4) *In vitro*, the number of tumor cells adhering to platelets, platelets adhesion to HUVECs, platelet mediated tumor cells adhesion to HUVECs, and platelets...
adhesion to monocytes is significantly decreased in the presence of A11. A11 disaggregates thrombin-induced platelet-tumor clumps, whereas control scFv (13CG2) has no effect. (5) A11 had no effect on the angiogenesis process using \textit{in vitro} and \textit{in vivo} Matrigel assays. Thus, a new antimetastatic strategy is proposed through lysing activated platelets in the tumor microenvironment with humanized anti-GPIIIa49-66 scFv Ab.

Compared to traditional anti-platelet drugs, which prevent thrombosis by inhibiting normal platelet function, including platelet adhesion, aggregation and activation, A11 has a different mechanism of action and has distinct properties. It has no effect on normal platelet function.\textsuperscript{23} However, it can induce oxidative platelet fragmentation in the absence of complement activation via Ab activation of platelet nicotinamide adenine dinucleotide phosphate oxidase and 12-lipoxygenase releasing reactive oxygen species.\textsuperscript{23-25} More importantly, it preferentially binds to activated versus resting platelets since activated platelets display more GPIIb-IIIa reactive receptors on the surface,\textsuperscript{23-25} making it more likely to be clinically useful by avoiding an increased bleeding risk. In addition, our data clearly demonstrates that A11 can dissolve an already formed platelet-tumor thrombus, making it more clinically relevant for cancer treatment. A11 impair the adhesion of tumor cells to activated platelets, weakens platelet mediated tumor-endothelium adhesion and decreases the activated platelet numbers bound to monocytes. Each of these properties can additively help inhibit platelet-tumor thrombus formation.

In this study, we focused on the effect of A11 on activated platelet-mediated adhesion between tumor cells and endothelial cells \textit{in vitro} and \textit{in vivo}. To demonstrate the anti-metastasis effect is due to activated platelet lysis rather than disruption of endothelial cell biological function, we designed several experiments. We demonstrated, in HUVECs, that A11 had no effect on endothelial cell proliferation, chemotaxis, and tube formation. In the plug assay model of angiogenesis \textit{in vivo}, A11 had no inhibitory effect on neovascularization. However, the effects of A11 on activated platelet-mediated tumor angiogenesis will be further investigated in our future studies.

This novel strategy confers a number of advantages for the prevention of tumor cell metastases including: 1. Tumor cells lacking an intact coat of platelets will lose their ability to evade the body’s immune system. 2. Fragmented platelets will lose their ability to shield cancerous cells from the high shear forces seen in flowing
blood that could potentially damage tumor cells. 3. Lysing activated platelets could disaggregate already-formed large tumor-platelet aggregates, which could embolize in the microvasculature to a new extravasation site. 4. Lysed platelets will lose their ability to facilitate the adhesion of tumor cells to the vascular endothelium, as well as, decreasing the release of a number of growth factors that can stimulate tumor cell growth.

Of particular interest was our observation that injection of A11 4 hours before or after inoculation of tumor cells provided a protective effect against tumor metastases, whereas injection of A11 given 12 hours prior or post-inoculation of tumor cells did not decrease metastases. It is likely that platelets play a crucial role within the first 12 hours of tumor inoculation. Indeed, tumor cells have been found to spread within pulmonary microvessels 2-6 hours post-injection, where they were associated with platelets and fibrin clots. Our data confirms this observation. We show that mice treated with A11 after inoculation of tumor cells have reduced early invasion of the lung.

Our previous studies have revealed that the platelet drop nadir induced by patient anti-GPIIIa49-66 Ab or its mimic Ab occurs at 4 hours with a recovery to normal at 24 hours. We found A11 had similar pharmacokinetic properties as their parental Ab. In this study, injection of an optimal dose of A11 (25 μg/mouse) resulted in a modest drop of the non-activated circulating platelet count (~21%) at the nadir time point, which resulted in no significant change in the C57BL/6J mouse vein bleeding time. We found no evidence of complications due to A11 injections in mice at the same frequency as used in our tumor treatments. However prior to any clinical use, further studies on the safety of A11 scFv Ab regarding hemostasis following repeated administration of various doses of A11 are warranted since hemostasis and hemorrhage are a complex processes. Nevertheless, the current data have established the concept of developing a novel approach to combat tumor metastasis by preferentially fragmenting activated platelets in the tumor microenvironment via A11 or compounds with similar properties.

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Authorship
Contribution: W.Z., S.D., T.H., J.T., J.F., DW. B., and YJ. S.. performed research and analyzed data; W.Z., ZG. W., and T.W. designed the research and analyzed data; and W.Z. and T.W. wrote the paper. Conflict-of-interest disclosure: The authors declare no competing financial interests.

References


Table 1 Effects of A11 on the adhesive properties of thrombin activated platelets to human monocytes

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Attached plts (&lt;5)</th>
<th>Attached plts (5-10)</th>
<th>Attached plts (&gt;10)</th>
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<tr>
<td>A11</td>
<td>3%</td>
<td>31%</td>
<td>66%</td>
</tr>
<tr>
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<td>48%</td>
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</tr>
<tr>
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<td>51%</td>
<td>27%</td>
</tr>
<tr>
<td>1000</td>
<td>29%</td>
<td>57%</td>
<td>14%</td>
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Platelets (2 × 10^8 cells/mL) were first activated by thrombin, and then incubated with various concentrations of A11 for 4 hours at 37°C. Twenty microliter of A11-treated activated platelets were added to an equal volume of human monocytes (2 × 10^6 cells/mL) and incubated at room temperature for 30 minutes followed by inspection and scoring of the monocytes with various numbers of attached platelets: monocytes with less than 5, between 5 to 10 and more than 10 attached platelets.
LEGENDS TO FIGURES

Figure 1
Schema of anti-platelet GPIIIa49-66 scFv Ab (A11) properties for the inhibition of tumor metastasis. (A) Summary figure: Events occurring following tumor intravasation, tumor-platelet-endothelial cell binding and platelet-tumor thrombus formation, tumor-platelet embolization, angiogenesis, and extravasation. T, refers to tumor cells; AP, activated platelets. (B) Platelets promoting metastases an in experimental pulmonary metastasis B16 tumor model. The upper row denotes lung specimens with metastastic colonies. Plt, refers to platelet. The bottom row denotes numeric results of surface pulmonary nodules (n=8, **p<0.01). (C) Hypothesized mechanisms of A11 on the inhibition of tumor metastasis by lysing activated platelets within the tumor microenvironment.

Figure 2
Effect of A11 on spontaneous metastasis of Lewis Lung Carcinoma (LLC). (A) It illustrates the treatment protocols in the spontaneous lung metastasis model. (B) Mean number of surface nodules per lung. (C) Mean volume of nodules per lung. (D) Representative histologic evidence from tumor sections of the different groups. (a-b) 13CG2 (× 40) (a), and (× 200) (b); (c-d) A11 (× 40) (c), and (× 200) (d), 13CG2 refers to control scFv Ab, n=8, *p<0.01.

Figure 3
Effect of A11 on experimental metastasis of Lewis Lung Carcinoma (LLC). (A) The preventive (a, b) and therapeutic (c, d, e) protocols in lung metastasis model are shown. i.v. represents intravenous injection. (B) Mean number of surface nodules per lung in the different treatments. (C) Mean volume of nodules per lung in the different treatment groups. n=8/group, **P<0.001, *P<0.01, Ctrl. refers to control scFv Ab 13CG2.

Figure 4
The pathophysiologic relevance of the platelet GPIIIa49-66 epitope (GP49) on experimental lung metastases of B16 melanoma. Mice were treated as described in methods. At day 21, mice were sacrificed. (A) Mean number of surface nodules per lung. (B) Mean volume of nodules per lung. (C) Induction of platelet count drop was determined at different time points. n=8, * refers to values with significant differences, n.s. refers to no significant difference.

Figure 5

Effect of A11 on early invasion of B16 melanoma cells in lung tissue (A) Frozen slide of lung tissue (20 ×) observed under a fluorescence microscope. (B) Quantitative result of adhesion of B16 in lung tissue as measurements of fluorescent intensity under a fluorescence plate reader. n=5, *p<0.01.

Figure 6

Molecular mechanisms by which A11 inhibits tumor metastasis. (A-B) Effect of A11 on the adhesion of tumor cells to platelets in vitro. (A) The adhesion of LLCs to activated platelets as observed under fluorescence microscope. (B) The quantitative analysis of adhesion of LLC with activated platelet in the presence of various concentration of A11, as measurements of fluorescent intensity under a fluorescence plate reader. The experiment was repeated three times and each concentration had 4 wells. (C) Effect of A11 on the adhesion of platelets to HUVECs in vitro. The extent of adhesion was expressed as the percentage of control platelets adhering without preincubation with A11 or control scFv (13CG2). The experiment was repeated three times and each concentration had 4 wells. (D-E) Effect of A11 on platelet mediated tumor cell adhesion to endothelial cell in vitro. B16 melanoma cells adhesion to HUVECs was performed as described in the methods. The adhesion efficiency of B16 tumor cell was observed under a fluorescence microscope (D). Quantitative result of adhesion of B16 melanoma cells with HUVECs (E). (F) Effect of A11 on the destruction of already formed tumor-platelet aggregates. Data and SD are given for 3 separate experiments at 0.5 μM reagent in which each time point represents 5 measurements.
Figure 7
Effect of A11 on the viability of Tumor Cells and Endothelial cells in vitro and in vivo. (A-C) Effect of A11 on the viability of B16 (A) and LLC (B) and HUVEC (C) as evaluated by the MTT assay. Data are mean ± SD of three different determinations. (D) Effect of A11 on the chemotaxis of tumor cells and HUVECs. Chemotaxis was performed in Transwell plates as described in the methods. Data and SD are given for 3 independent experiment results in which each concentration represent 4 measurements. (E) Effect of A11 on the s.c. tumor growth. Tumor dimensions were measured with calipers for calculation of tumor volume. n=5, error bars indicate SD.
Figure 3

A

A11(i.v.)

LLC i.v.

12h

24h

1w

2w

3w

A11(i.v. 3 times/w)

B

Mean number of surface nodules per lung

P<0.05

Ctrl  Pre-12h  Pre-4h  0h  Post-4  Post-12

C

Mean volume of nodules per lung (mm³)

P<0.05

Ctrl  Pre-12h  Pre-4h  0h  Post-4  Post-12
A humanized single-chain antibody against beta 3 integrin inhibits pulmonary metastasis by preferentially fragmenting activated platelets in the tumor microenvironment

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