

Fig. 1 Evaluation of MSP expression in human SCLC cells. **a** The expression levels of MSP mRNA in SBC-5 cells and their subclones were determined by RT-PCR, as described in Materials and methods. **b** The expression levels of MSP protein in SBC-5 cells and their subclones were determined by Western blotting, as described in Materials and methods. **c** The expression levels of MSP protein in H1048 cells and their subclones were determined by Western blotting

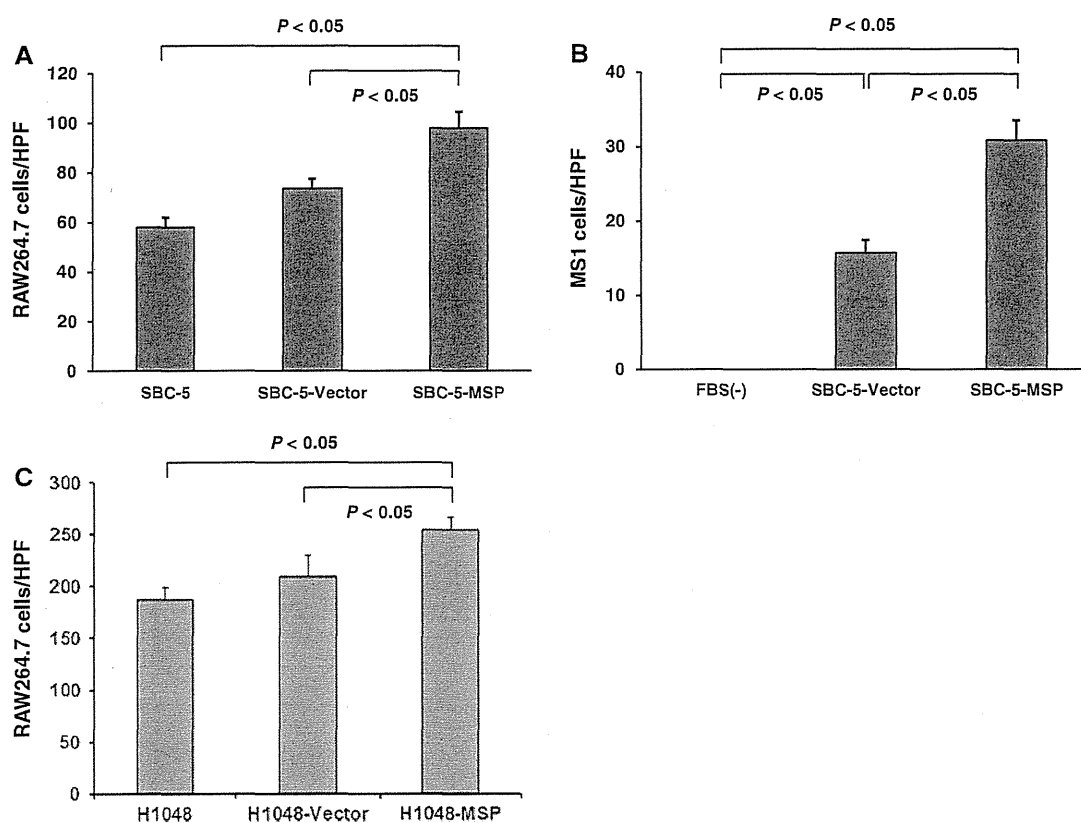
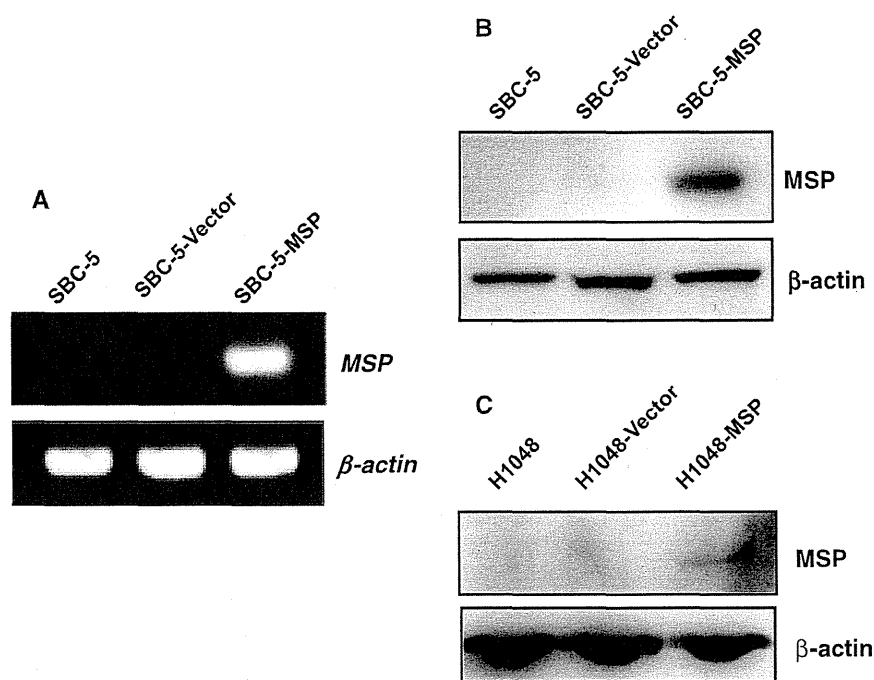


Fig. 2 Analysis of the bioactivity of MSP secreted by the MSP transfectant. The bioactivity of MSP, produced by SBC-5-MSP cells, was measured by a two-chamber migration assay. **a** The culture supernatant of SBC-5-MSP cells, but not that of parental cells or the vector control clone, induced migration of RAW 264.7 cells. **b** The

culture supernatant of SBC-5-MSP cells significantly enhanced the migration of MS1 cells compared with that of SBC-5-Vector cells ($P < 0.05$). **c** The culture supernatant of H1048-MSP cells, but not that of parental cells or the vector control clone, induced migration of RAW 264.7 cells

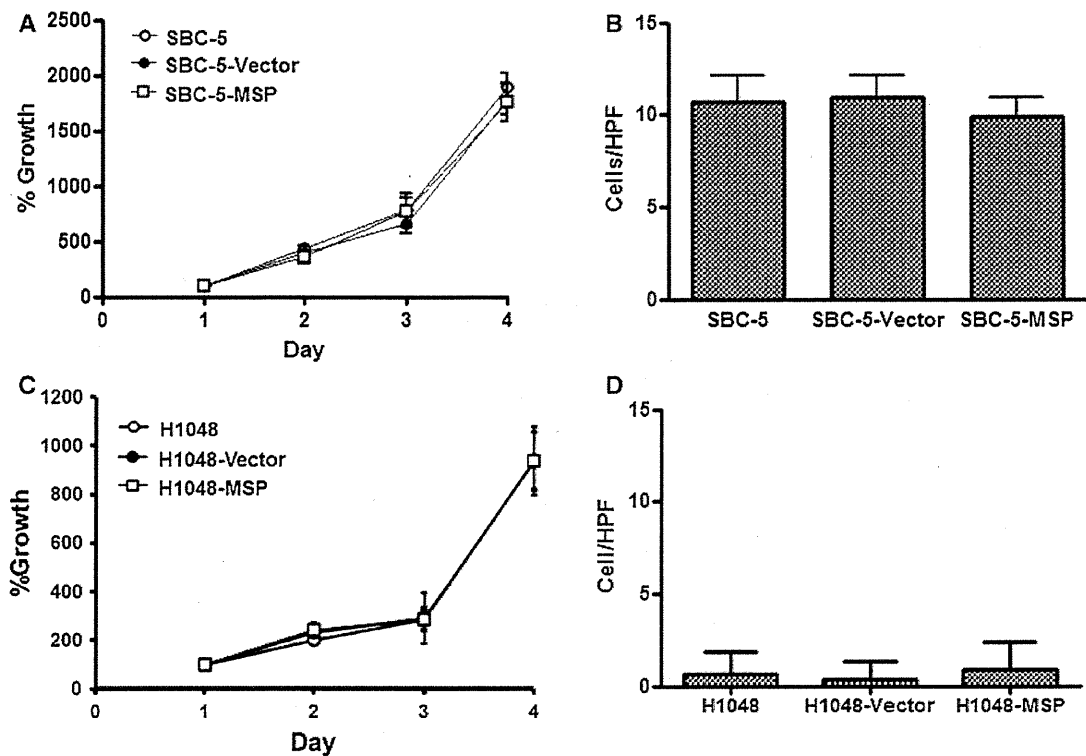


Fig. 3 The effect of MSP gene transduction on in vitro tumor cell behavior. **a** The anchorage-dependent cell growth of SBC-5 cells and their subclones was determined by a MTT assay, as described in Materials and methods. **b** Cell motility of SBC-5 cells and their subclones was determined by a two-chamber migration assay, as

described in Materials and methods. **c** The anchorage-dependent cell growth of H1048 cells and their subclones was determined by a MTT assay. **d** Cell motility of H1048 cells and their subclones was determined by a two-chamber migration assay

several human SCLC cell lines, but neither SBC-5 cells nor H1048 cells expressed human RON (Fig. 4a). Since our purpose is to study the effect of MSP to mouse organ microenvironment, we transfected mouse MSP to human cancer cell lines not expressing human RON. Hence, overexpressed mouse MSP was not able to affect cancer cell themselves.

MSP gene transduction resulted in the promotion of experimental liver metastasis in vivo

To evaluate whether MSP signaling could affect cancer metastasis in the presence of the murine microenvironment, we performed in vivo experiments using a previously established model of multiple-organ metastasis in NK-cell depleted SCID mice. In this model, SBC-5 cells produce metastatic colonies predominantly into the liver, lung, and bone [21]. The number of liver metastases was significantly higher in SBC-5-MSP cells than those of parental SBC-5 cells or SBC-5-Vector cells (Table 1; Fig. 5a). There was no statistically significant difference between the numbers of bone and lung metastases among these three cell lines. The expression of MSP mRNA in liver metastatic nodules

produced by SBC-5-MSP cells was confirmed to be maintained (Fig. 5c).

To certify that these effects were not cell-specific, we generated another MSP-overexpressing transfectant, H1048-MSP cells, which were constructed in the same way as SBC-5-MSP (Fig. 1c, 2c). MSP gene transduction did not affect the behavior of H1048-MSP cells in vitro (Fig. 3c, d). H1048 cells also produced metastatic colonies predominantly in the liver, kidney, and bone in NK-cell depleted SCID mice. As with SBC-5-MSP, the number of liver metastases, but not those of kidney and bone metastases, was significantly increased in H1048-MSP cells than those of parental cells or the vector control clone (Table 2; Fig. 5b). These results indicated that MSP has the potential to enhance liver metastases produced by human SCLC cells.

The expression of the factors involved in MSP/RON signaling pathway in normal organs of mice

To elucidate the mechanisms of obvious liver tropism in metastasis formation of MSP-expressing SCLC cells, we examined the expression of the factors involved in MSP/

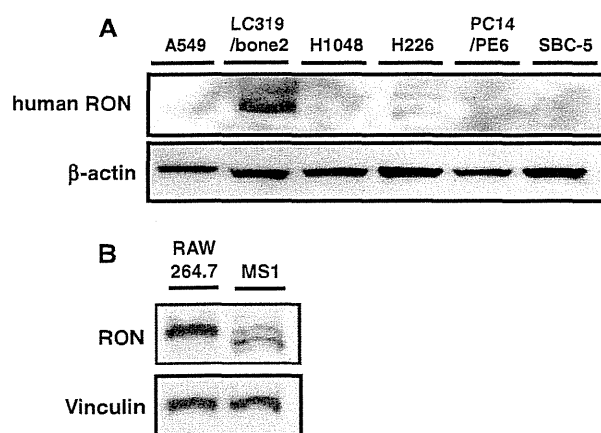


Fig. 4 The expression of RON in several human lung cancer cells, RAW264.7 and MS1 cells. **a** The expression levels of human RON protein in several lung cancer cells were determined by Western blotting, as described in Materials and methods. **b** The expression levels of mouse RON protein in RAW264.7 and MS1 cells were determined by Western blotting, as described in Materials and methods

RON signaling pathway in normal organs of mice. Then, we found that MSP and HGFA are highly expressed in the liver compared to other organs (Fig. 6a). HGFA is trypsin-like serine proteases, and is known to activate pro-MSP to mature MSP [23], leading to increase mature MSP derived from pro-MSP secreted by tumor cells themselves and/or tumor stroma. Thus, the microenvironment in the liver is favorable for pro-MSP to be translated to activated form. Moreover, RON was phosphorylated in the liver but not in the kidney and the lung, while RON was expressed in all three organs (Fig. 6b), suggesting that the MSP/RON axis was activated in the liver. Taken together, the abundant MSP can affect liver microenvironment and activate MSP/RON axis, which leads to promote liver metastases of MSP-producing SCLC cells.

Microvessel density and tumor-infiltrating macrophages were significantly increased in liver metastatic lesions produced by MSP transfectants

To further explore the mechanism by which MSP enhanced experimental liver metastases, histological analysis was performed using metastatic lesions from the liver by SBC-5, SBC-5-Vector, and SBC-5-MSP cells. For the detection of tumor-associated microvessels and tumor-infiltrating macrophages, we used an anti-mouse CD31 and an anti-mouse CD68 antibody, respectively. Immunohistochemical staining showed that tumor-associated microvessel densities and tumor-infiltrating macrophages were significantly higher in lesions produced by SBC-5-MSP cells, than those produced by parental SBC-5 cells or SBC-5-Vector cells (Fig. 7). These results suggest that MSP secreted from

tumor cells elicited the activation of tumor-infiltrating macrophages and that activated macrophages induced angiogenesis, which may promote liver metastases.

Discussion

Molecular interactions between cancer cells and their microenvironments play crucial roles throughout the multiple steps of metastasis [24, 25]. In this study, we evaluated the interaction of MSP-positive tumor cells with the murine microenvironment using a multiple-organ metastasis model in NK-cell depleted SCID mice. While there was no increase in cell growth or migration in MSP-overexpressing human SCLC cells, MSP overexpression enhanced liver metastases produced by these cells. These findings indicated that MSP may affect organ microenvironments, rather than the tumor cells themselves, to promote metastases.

Tumor-associated macrophage (TAM) infiltration is associated with increased microvessel density, which results in poor prognosis in lung cancer [26]. Moreover, the angiogenic factors secreted by activated macrophages in response to various stimuli in the tumor microenvironment contribute to tumor growth, angiogenesis, and metastasis. In this study, we found that tumor-infiltrating macrophages and microvessel densities were significantly increased in liver metastases by MSP-overexpressing human SCLC cells. These results suggested that MSP secreted from tumor cells elicited chemotaxis and activation of TAMs and that activated TAMs produced angiogenic factors, which could promote angiogenesis and liver metastases. We also demonstrated that the culture supernatant of SBC-5-Vector cells induced migration of murine endothelial cells, and this affects were enhanced by MSP-overexpressing (Fig. 2c). These results indicated that MSP could enhance angiogenesis via a direct effect on endothelial cells in addition to an indirect effect through macrophage activation. Taken together, these data indicate that MSP may be one of the major determinants that affect the properties of tumor stroma and that produce a permissive microenvironment to promote cancer metastasis.

Interestingly, MSP-overexpressing SCLC cells showed liver tropism in metastasis formation. While the number of liver metastases was significantly higher in MSP-overexpressing SCLC cells than those of parental cells or the vector control clone, there were no discernible differences in the numbers of bone and lung metastases among these three cell lines. The reason for these phenomena remains to be elucidated. One possible explanation is that the expression of MSP and HGFA are prominent in the liver compared with other organs (Fig. 6a). MSP and HGFA are primarily produced by hepatocytes and secreted mainly

Table 1 Production of metastasis by SBC-5 cells with or without MSP in NK-cell depleted SCID mice

Cell line	Incidence of metastasis	Liver weight (g) ^a	Number of metastatic colonies ^a		
			Liver	Lung	Bone
SBC-5	6/6	1.3 (1.0–2.2)	70 (50–136)	29 (15–48)	6 (4–8)
SBC-5-Vector	8/8	1.5 (1.2–2.4)	66 (32–133)	26 (8–54)	4 (3–6)
SBC-5-MSP	7/7	1.5 (1.3–1.9)	107 (59–139) ^b	24 (10–47)	5 (3–7)

SBC-5 cells (1×10^6) with or without MSP gene were inoculated intravenously into NK-cell depleted SCID mice on day 0

Mice were killed on day 28 and the formation of metastases was evaluated

Data are representative of five independent experiments with similar results

^a Values are the means (range) for the indicated numbers of mice

^b Significantly different from SBC-5 and SBC-5-Vector cells ($P < 0.05$)

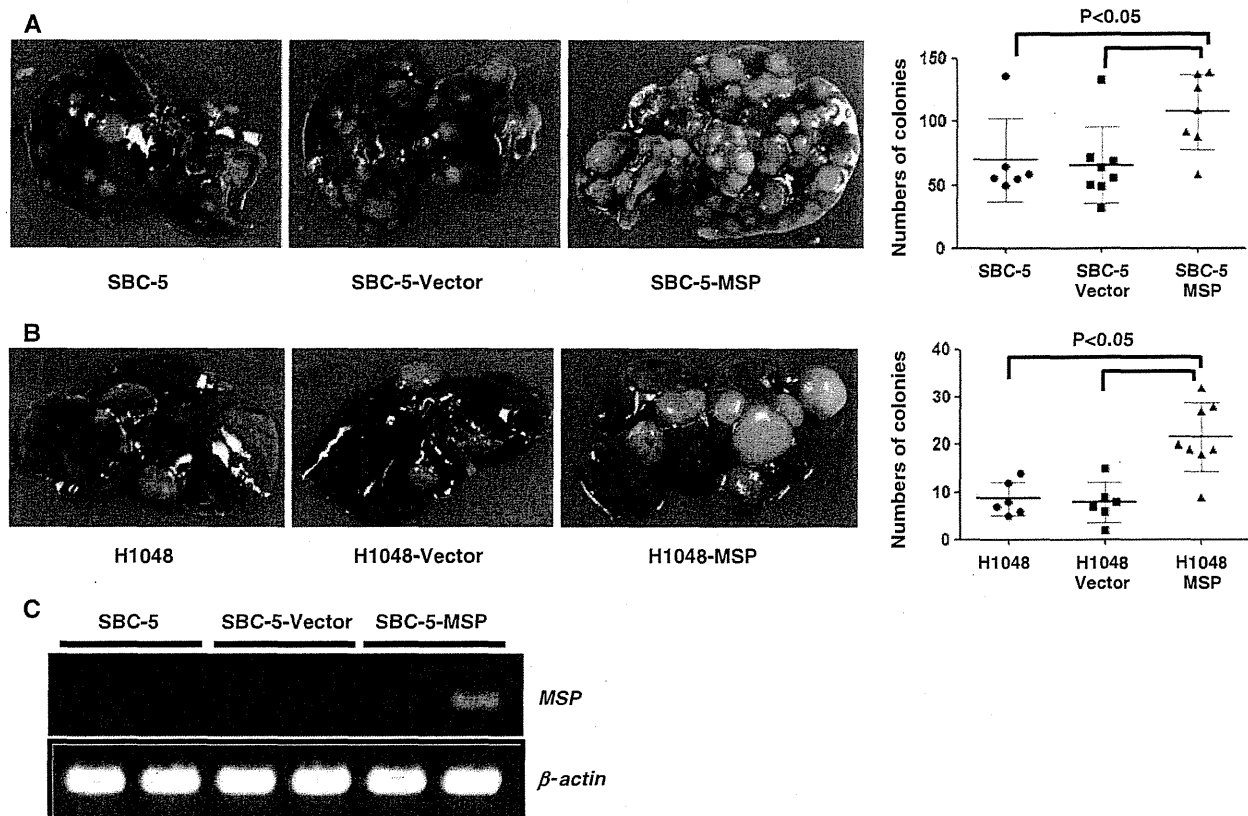


Fig. 5 The effect of MSP gene transduction on experimental liver metastasis formation produced by human SCLC cells. **a** The experimental liver metastases by SBC-5-MSP cells significantly increased compared with SBC-5 and SBC-5-Vector cells. Representative photographs of the liver (left panel) and the numbers of liver metastatic colonies in each experimental group (right panel) are shown. **b** The experimental liver metastases by H1048-MSP cells

significantly increased compared with H1048 and H1048-Vector cells. Representative photographs of the liver (left panel) and the numbers of liver metastatic colonies in each experimental group (right panel) are shown. **c** The expression levels of MSP mRNA in metastatic liver lesions by SBC-5 cells and their subclones were determined by RT-PCR, as described in Materials and methods

from the liver [27, 28]. RON mRNA and protein are also expressed in the normal liver, and its expression is localized to hepatocytes and Kupffer cells, the resident macrophage population of the liver [27]. These facts means that liver have the most advanced environment used to convert pro-

MSP to mature form, and are susceptible organs by pro-MSP secreted from cancer cells. Therefore, in liver metastatic lesions, MSP which was secreted by tumor cells may induce RON activation in hepatocytes and Kupffer cells, which may produce permissive organ microenvironments for the

Table 2 Production of metastasis by H1048 cells with or without MSP in NK-cell depleted SCID mice

Cell line	Incidence of metastasis	Liver weight (g) ^a	Number of metastatic colonies ^a		
			Liver	Lung	Bone
H1048	6/6	0.9 (0.6–1.1)	9 (5–14)	24 (11–30)	3 (1–5)
H1048-Vector	6/6	1.0 (0.7–1.5)	8 (2–15)	31 (19–36)	5 (3–6)
H1048-MSP	8/8	1.2 (0.8–1.5)	22 (9–32) ^b	29 (15–43)	3 (1–6)

H1048 cells (1 × 106) with or without MSP gene were inoculated intravenously into NK-cell depleted SCID mice on day 0
Mice were killed on day 56 and the formation of metastases was evaluated

Data are representative of five independent experiments with similar results

^a Values are the means (range) for the indicated numbers of mice
^b Significantly different from H1048 and H1048-Vector cells (*P* < 0.05)

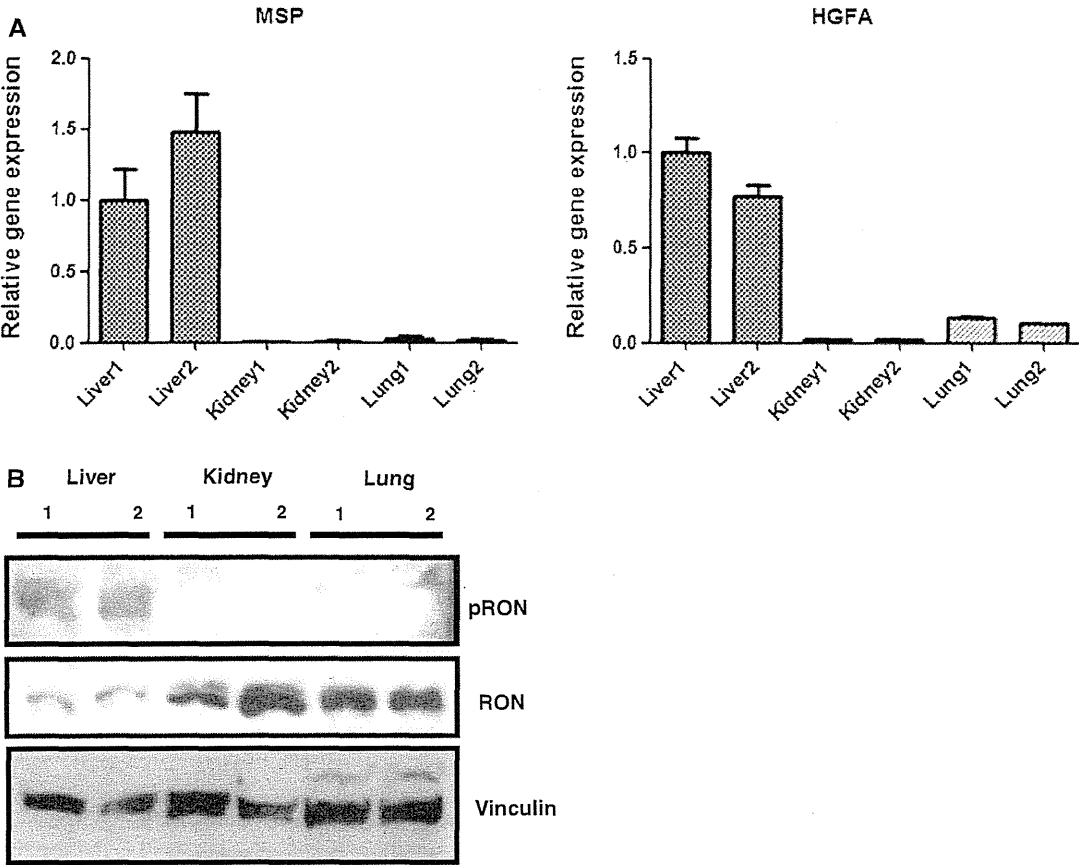


Fig. 6 The expression of the factors involved in MSP/RON signaling pathway in normal organs of mice. **a** The expression levels of MSP and HGFA mRNA in the liver, the kidney and the lung of mice were determined by quantitative PCR, as described in Materials and

methods. **b** The expression levels of RON and pRON protein in the liver, the kidney and the lung of mice were determined by Western blotting, as described in Materials and methods

promotion of tumor growth. Another explanation is that angiogenesis is more important for metastasis formation in the liver than other organs. Indeed, disease-free and overall survival of metastatic liver cancer was reported to be closely associated with the number of microvessels in the tumor [29], and the liver parenchyma adjacent to synchronous liver

metastases provides an angiogenic prosperous environment for metastatic tumor growth in the presence of the primary tumor [30]. Moreover, we recently reported that angiogenesis is hardly involved in experimental bone metastasis formation by SBC-5 cells [31]. Further studies are warranted to ascertain the precise mechanisms for liver tropism in

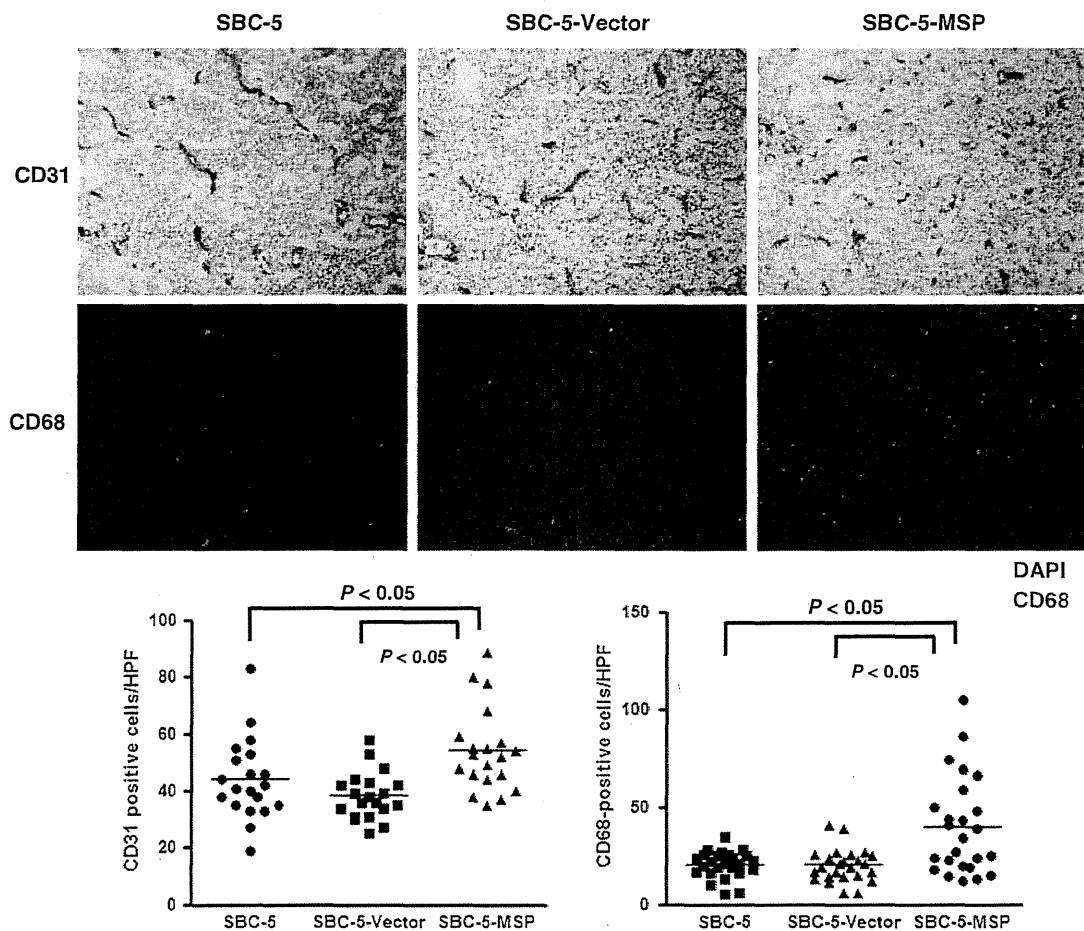


Fig. 7 Analyses of the microvessel density and tumor-infiltrating macrophages in liver metastatic lesions produced by SBC-5 cells transfected with or without the MSP gene. Immunohistochemical staining of the liver was performed as described in Materials and

methods. The amount of vascularization determined by CD31 staining and macrophages by CD68 staining were significantly increased in SBC-5-MSP tumors than those of SBC-5 and SBC-5-Vector tumors. * $P < 0.05$ in comparison to SBC-5 tumors and SBC-5-Vector tumors

metastasis formation observed in MSP-overexpressing SCLC cells.

Accumulating evidence indicates that activation of the MSP/RON axis affected the ability of tumor cells themselves to migrate, invade, and/or grow in distant tissues [11–13]. For example, the overexpression of MSP facilitated metastases to various organs, especially to bone, produced by mammary tumors in transgenic mice [12]. Constitutive expression of RON was shown to induce epithelial mesenchymal transition, marked by phenotypic changes and alterations in cell motility [32], and was correlated with reduced disease-free survival of breast cancer patients [33]. In lung cancer, MSP and its receptor, RON, were also overexpressed in 25–44 % and 62–72 % of tumors, respectively [22, 34]. In vitro tests of RON activity in certain lung cancer cell lines demonstrated that MSP stimulation induced RON phosphorylation and increased cell motility in an autocrine and/or paracrine manner [22].

Furthermore, MSP expression was up-regulated in bone metastatic lesions by SCLC cells [13]. These observations suggested that MSP may affect not only tumor microenvironments but also the tumor cells themselves to promote the progression and metastases of lung cancer. While there are no clinical reports about MSP expression in SCLC, recent evidence has demonstrated that the frequency and intensity of RON expression was much higher in lung tumors of neuroendocrine origin such as SCLC and in secondary tumors which metastasized to the brain [34]. These findings also suggest the importance of MSP/RON axis to interact with organ microenvironment and promote distant metastases of SCLC cells.

Recently, a humanized monoclonal antibody, IMC-41A10, that blocks the interaction of RON with MSP has been developed. IMC-41A10 not only inhibits the binding of MSP to RON, but also diminishes RON phosphorylation and its downstream signaling. In addition, IMC-41A10 also

significantly decreased tumor growth of murine xenografts from various subcutaneously injected cancer cell lines, including lung cancer [35]. These results highlight the potential therapeutic usefulness of MSP/RON axis inhibition in human cancers.

In conclusion, we showed that MSP gene transduction resulted in the facilitation of experimental liver metastases via the promotion of angiogenesis in NK-cell depleted SCID mice by human SCLC cells. The capability of MSP to modify organ microenvironments may uncover a unique and effective strategy to inhibit liver metastases of SCLC in humans.

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Conflict of interest The authors declare that we have no conflict of interest.

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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Surfactant Protein A Suppresses Lung Cancer Progression by Regulating the Polarization of Tumor-Associated Macrophages

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Surfactant protein A (SP-A) is a large multimeric protein found in the lungs. In addition to its immunoregulatory function in infectious respiratory diseases, SP-A is also used as a marker of lung adenocarcinoma. Despite the finding that SP-A expression levels in cancer cells has a relationship with patient prognosis, the function of SP-A in lung cancer progression is unknown. We investigated the role of SP-A in lung cancer progression by introducing the SP-A gene into human lung adenocarcinoma cell lines. SP-A gene transduction suppressed the progression of tumor in subcutaneous xenograft or lung metastasis mouse models. Immunohistochemical analysis showed that the number of M1 antitumor tumor-associated macrophages (TAMs) was increased and the number of M2 tumor-promoting TAMs was not changed in the tumor tissue produced by SP-A-expressing cells. In addition, natural killer (NK) cells were also increased and activated in the SP-A-expressing tumor. Moreover, SP-A did not inhibit tumor progression in mice depleted of NK cells. Taking into account that SP-A did not directly activate NK cells, these results suggest that SP-A inhibited lung cancer progression by recruiting and activating NK cells via controlling the polarization of TAMs. (*Am J Pathol* 2013, 182: 1843–1853; <http://dx.doi.org/10.1016/j.ajpath.2013.01.030>)

Lung cancer is the major cause of malignancy-related death worldwide. Mortality is 80% to 90%, which makes this disease the leading cause of cancer-related deaths.¹ The high mortality rate of this disease is primarily due to the difficulty of early diagnosis, the high metastatic potential, and the poor responses to chemical therapy and radiotherapy. Because there is no established curative therapy for advanced lung cancer to date, clinical management is palliative in many cases. Therefore, it is crucial to investigate and understand the underlying biological and molecular mechanisms of lung cancer progression.

Surfactant protein A (SP-A) is a large multimeric protein found in the airways and alveoli of the lungs. SP-A is a member of the collectin family of proteins, characterized by NH₂-terminal collagen-like regions and COOH-terminal lectin domains. Although other SPs, such as SP-B, function

to reduce surface tension in the lungs, SP-A (and SP-D) regulates the pulmonary immune response.² Previous *in vivo* studies have shown that SP-A regulates responses involved in initiation and potentiation of inflammation by regulating the production of proinflammatory cytokines, such as tumor necrosis factor α (TNF- α), in response to lipopolysaccharide³ or by accelerating the clearance of a variety of pathogens.^{4–8} Because SP-A has the ability to opsonize and enhance pathogen uptake by phagocytes, the immunoregulatory roles of SP-A have been studied mainly in the field of infectious diseases. Recently, we reported that

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SP-A has a role in regulating bleomycin-induced acute noninfectious lung injury by inhibiting lung epithelial cell apoptosis.⁹ Pastva et al¹⁰ reported that SP-A regulates T_H2 cytokine production in a mouse asthma model. These results suggest that SP-A has diverse functions to control various lung diseases. Considering that SP-A contributes to multiple aspects of pulmonary host defense, we hypothesized that SP-A might have a role in lung cancer progression.

In a lung cancer study, SP-A was expressed in approximately 49% of primary non-small cell lung carcinomas¹¹ and is used as a specific marker of carcinoma that originates in type II pneumocytes. In addition, a previous study demonstrated that deletion of the *SFTPA1* (alias, *SPA*) gene in non-small cell lung cancer cells was associated with tumor progression.¹² Tsutsumida et al¹³ found that patients with lung adenocarcinoma with relatively high MUC1 mucin expression and low SP-A expression in cancer cells had a poor outcome. These clinical studies demonstrate that in addition to use as a diagnostic marker, SP-A expression in lung cancer cells could be a useful biomarker of good prognosis. Although these studies suggested that SP-A might have a role in suppressing lung cancer progression, the role of SP-A in lung cancer has not been extensively studied, and the mechanisms by which SP-A controls lung cancer progression remain unknown.

In this study, we generated SP-A-overexpressing human lung adenocarcinoma cells and evaluated the role of SP-A in lung cancer progression using experimental mouse models.

Materials and Methods

Cell Lines

The human lung adenocarcinoma cell line PC14PE6 was a gift from Dr. Isaiah J. Fidler (The University of Texas MD Anderson Cancer Center, Houston TX). The human lung adenocarcinoma cell line A549 was purchased from ATCC (Manassas, VA). These cell lines were authenticated by BEX Co. Ltd. (Tokyo, Japan) using a multiplex short tandem repeat assay. Both cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin, and 50 µg/mL of streptomycin and were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air.

Reagents

An anti-mouse IL-2 receptor β-chain monoclonal antibody, TM-β1 (IgG2b), was a gift from Drs. Masayuki Miyasaka and Toshio Tanaka (Osaka University, Osaka, Japan).

SP-A Purification

SP-A was purified from the lung lavage fluid of patients with alveolar proteinosis as previously described¹⁴ and was routinely tested to reduce endotoxin contamination.¹⁴ Briefly, SP-A was suspended in 100 mmol/L octylglucoside and 5 mmol/L Tris, pH 7.4, after butanol extraction.

Table 1 Primer Sequences Used in Quantitative PCR

	Forward	Reverse	Product size (bp)
Gene (mouse)			
<i>IL-1β</i>	5'-TGACGTTCCCATTAGACAAC-3'	5'-ATTTTGTCTGCTTGCTTGGTTC-3'	171
<i>IL-6</i>	5'-GTACCATAGCTACCTGGAGT-3'	5'-GGAAATTGGGGTAGGAAGGA-3'	154
<i>TNF-α</i>	5'-CCTATGTCTCAGCCTCTTCT-3'	5'-TTGGGAAGTCTCATCCCTT-3'	107
<i>IL-12</i>	5'-CACACTGGACCAAGGGACT-3'	5'-TGGTTTGATGATGTCCCTGA-3'	169
<i>IFN-γ</i>	5'-TAGCTCTGAGACAATGAACG-3'	5'-CACATCTATGCCACTTGAGT-3'	145
<i>CCL2</i>	5'-TTCACAGTTGCCGGCTGG-3'	5'-TGAATGAGTAGCAGCAGGTGAGTG-3'	81
<i>CCL5</i>	5'-CAGCAGCAAGTGCTCCAATCTT-3'	5'-TTCTTGAACCCACTTCTCTCTGG-3'	91
<i>IL-10</i>	5'-AAGGACCAGCTGGACAACAT-3'	5'-TCTCAGCCAGGGAATTCAAA-3'	172
<i>MRC-1</i>	5'-TGCAAGGATCATACTTCCCT-3'	5'-TGATGTTCTCCAGTAGCCAT-3'	240
<i>Arg1</i>	5'-GAATGGAAGAGTCAGTGTGG-3'	5'-AATGACACATAGGTCAGGGT-3'	97
<i>CD163</i>	5'-GACGACAGATTGAGCGACTT-3'	5'-CCGAGGATTTCAGCAAGTCCA-3'	114
<i>Pf1</i>	5'-GACACAGTAGAGTGTCGCAT-3'	5'-TTTGAAGTCAAGGTGGAGTG-3'	70
<i>GzmB</i>	5'-AGAGAGCAAGGACAACACTC-3'	5'-ATCGAAAGTAAGGCCATGTAG-3'	176
<i>B2M (β₂M)</i>	5'-GGAAGCCGAACATACTGAAGT-3'	5'-TTTCCCGTTCTTCAGCATTTGG-3'	80
Gene (human)			
<i>IL-1β</i>	5'-GACAGGATATGGAGCAACAA-3'	5'-GCTGTAGAGTGGGCTTATCA-3'	147
<i>IL-6</i>	5'-CCTCTTCAGAACGAATTGAC-3'	5'-AGTCTCCTCATTGAATCCAG-3'	186
<i>TNF-α</i>	5'-GGCAGTCAGATCATCTTCTCG-3'	5'-CAGCTGGTTATCTCTCAGCTC-3'	148
<i>CCL2</i>	5'-CTCATAGCAGCCACCTTCATT-3'	5'-ACAGATCTCCTTGGCCACAA-3'	192
<i>CCL3</i>	5'-GGCAGTCAGATCATCTTCTCG-3'	5'-CAGCTGGTTATCTCTCAGCTC-3'	81
<i>CCL5</i>	5'-CTGTCATCCTCATTGCTACTG-3'	5'-GCCACTGGTGTAGAAATACTC-3'	140
<i>MRC-1</i>	5'-CCATCGAGGAATTGGACTTT-3'	5'-TGTCATTTAAGCCGATCCAC-3'	78
<i>RPL27</i>	5'-ATCGCCAAGAGATCAAAGATAA-3'	5'-TCTGAAGACATCCTTATTGACG-3'	123

Polymyxin-agarose (Sigma-Aldrich, St. Louis, MO) was added 1:5 (v/v) and allowed to incubate at room temperature for 30 minutes. The mixture was then dialyzed (14,000 molecular weight cutoff value) for four changes ≥ 4 hours each against autoclaved 5 mmol/L Tris, pH 7.4. The mixture was then centrifuged, and the supernatant, containing SP-A, was removed by gentle aspiration. SP-A preparations had final endotoxin concentrations of <0.1 pg/ μ g of SP-A as determined by the Limulus amoebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD).

SPA Gene Transduction

The human *SPA* gene—expressed region [SFTPA1 (NM_005411)] (OriGene Technologies, Rockville, MD) was introduced into the pMIG vector (a gift from Dr. Alana L. Welm, University of Utah, Salt Lake City). The Platinum-E packaging cell line (a gift from Dr. Toshio Kitamura, Tokyo University, Tokyo, Japan)¹⁵ was transfected with pMIG or derivative vector DNA by using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). PC14PE6 or A549 cells were infected using the viral supernatant as described previously.¹⁶ The proportion of green fluorescent protein—positive cells was $>90\%$ in the entire population.

Animals

Male athymic BALB/c nude mice and SCID mice were obtained from Charles River Laboratories Japan (Yokohama) and CLEA Japan (Tokyo), respectively, and were maintained under specific pathogen-free conditions throughout the study. All the experiments were performed in accordance with the guidelines established by The University of Tokushima Committee on Animal Care and Use, Tokushima, Japan. At the end of each *in vivo* experiment, the mice were anesthetized with isoflurane and euthanized humanely by cutting the subclavian artery. All the experiment protocols were reviewed and approved by the Animal Research Committee of The University of Tokushima.

In Vivo Subcutaneous Xenograft Model

PC14PE6 cells (1.0×10^6 per mouse) or A549 cells (3.0×10^6 per mouse) suspended in 0.1 mL of PBS were subcutaneously inoculated into the right flank of nude mice. Tumor size was measured using a vernier caliper three times a week (volume = $ab^2/2$, where a indicates long diameter; b , short diameter). The mice were euthanized humanely on day 21, and the tumors were resected for further analyses.

In Vivo Lung Metastasis Model

To establish lung metastasis, nude mice were intravenously inoculated via the tail vein with 1.0×10^6 tumor cells per mouse.¹⁷ The mice were euthanized humanely on either day 28 (PC14PE6) or day 42 (A549). The lungs were weighed,

and the number of metastatic colonies on the surface of the lungs was determined by visual examination. Because PC14PE6 cells produce large amounts of pleural effusion,¹⁸ the volume of the effusion was also evaluated. In some experiments, natural killer (NK) cells were depleted by treating nude mice with TM- β 1 5 days after inoculation of PC14PE6 cells.¹⁹

Immunofluorescence

The excised tumor tissue was placed into OCT compound (Sakura Finetechnical Co., Tokyo, Japan) and snap frozen. Eight-micrometer-thick frozen tissue sections were fixed with 4% paraformaldehyde solution in PBS and were used for identification of macrophages using 1:150 rat anti-mouse CD68 monoclonal antibody (Serotec, Oxford, UK) and of NK cells using 1:100 goat anti-mouse Nkp46/NCR1 monoclonal antibody (R&D Systems, Minneapolis, MN). Alexa Fluor 488-labeled secondary antibodies (dilution 1:250; Invitrogen, Carlsbad, CA) were used for immunofluorescence (IF) detection. To identify M1 or M2 macrophages, the sections were stained with 1:150 fluorescein isothiocyanate—conjugated rat anti-mouse TNF- α antibody (BD Pharmingen, Franklin Lakes, NJ) or 1:150 fluorescein isothiocyanate—conjugated rat anti-mouse CD206 [mannose

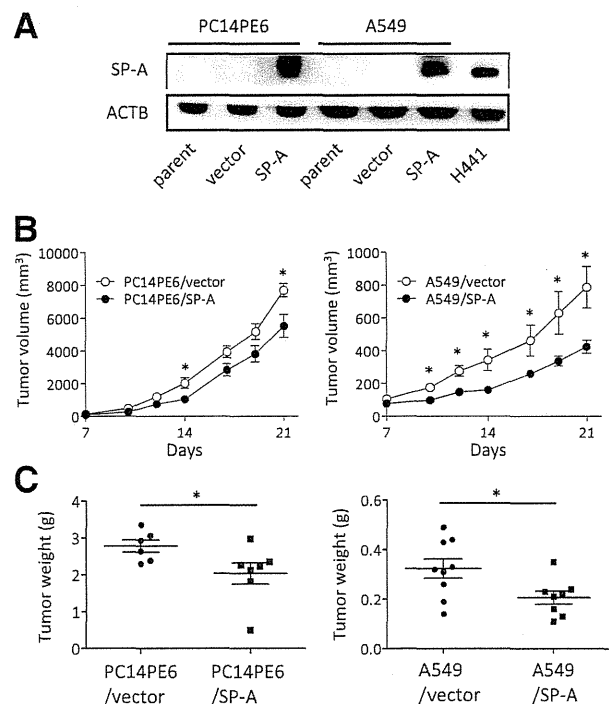


Figure 1 The effect of SP-A on lung cancer subcutaneous xenografts *in vivo*. **A**: SP-A expression of human SP-A stable transfectants was confirmed by Western blot analysis. H441 cells were used as a positive control. ACTB, β -actin. Tumor growth (**B**) and weight (**C**) of xenografts produced by PC14PE6 (left panels; $n = 6$ per group) and A549 (right panels; $n = 8$ per group) cells transduced with SP-A or vector. Data are presented as means \pm SEM (horizontal lines in **C**). * $P < 0.05$.

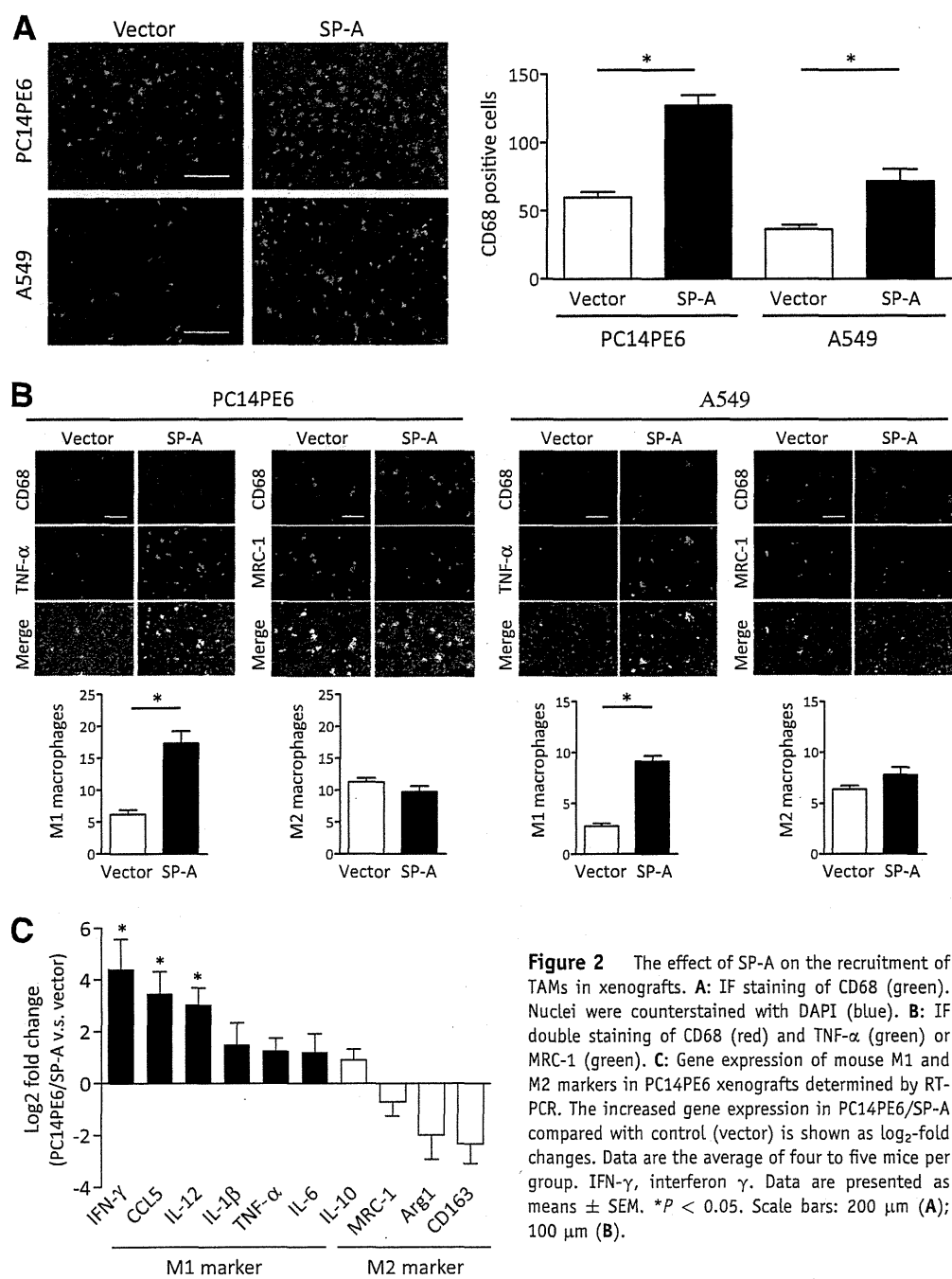


Figure 2 The effect of SP-A on the recruitment of TAMs in xenografts. **A:** IF staining of CD68 (green). Nuclei were counterstained with DAPI (blue). **B:** IF double staining of CD68 (red) and TNF- α (green) or MRC-1 (green). **C:** Gene expression of mouse M1 and M2 markers in PC14PE6 xenografts determined by RT-PCR. The increased gene expression in PC14PE6/SP-A compared with control (vector) is shown as log₂-fold changes. Data are the average of four to five mice per group. IFN- γ , interferon γ . Data are presented as means \pm SEM. * P < 0.05. Scale bars: 200 μ m (**A**); 100 μ m (**B**).

receptor C type 1 (MRC-1)] antibody (BioLegend, San Diego, CA) after CD68 staining. Alexa Fluor 594-labeled anti-rat secondary antibodies (dilution 1:250; Invitrogen) were used for CD68 IF detection. M1 or M2 macrophages were identified as CD68-positive/TNF- α -positive or CD68-positive/MRC-1-positive cells, respectively. Nuclei were counterstained with DAPI (blue). In each slide, the number of positive cells was counted in five areas under fluorescent microscopy at $\times 100$ (single staining) or $\times 200$ (double staining) magnification.

RT-qPCR

Total RNA was extracted from the tumors using the RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed using the CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA) using SYBR Premix Ex Taq (Takara, Kyoto, Japan). Human RPL27²⁰ and mouse β 2m mRNA were used

as housekeeping genes, and quantification was determined by using the $\Delta\Delta C_T$ method. Specific PCR primer pairs for each studied gene are shown in Table 1.

SP-A Stimulation of Monocytes, Macrophages, and NK Cells

Human monocytes were separated from the peripheral blood of healthy volunteers as described previously.²¹ The purity and viability of the monocytes was confirmed to be >98% by staining with Diff-Quik (Baxter Diagnostics, Deerfield, IL) and trypan blue, respectively. Mouse alveolar macrophages (AMs) were collected by using bronchoalveolar lavage as described previously.⁹ More than 95% of the cells were confirmed to be AMs. For eliciting mouse peritoneal macrophages (PMs), 2 mL of thioglycollate (BD Biosciences, San Jose, CA) was injected into the peritoneal cavity of SCID mice. After 3 days, peritoneal exudative cells were harvested by intraperitoneal lavage with ice-cold PBS. Approximately 80% of isolated cells were macrophages. NK cells from SCID mice were isolated as previously described.²² These immune cells were stimulated with 20 $\mu\text{g/mL}$ of human SP-A for 4 hours in RPMI 1640 medium containing 1% fetal bovine serum. Total RNA was extracted for quantitative RT-PCR.

Cell Migration Assay

The migration assay was performed using 8- μm pore size cell culture inserts (BD Biosciences). After 24 hours of serum starvation, PMs in serum-free media were added to the inner chamber in the presence or absence of 20 $\mu\text{g/mL}$ of SP-A. RPMI 1640 medium containing 10% fetal bovine serum was added to the lower chamber. After 17 hours of incubation, the cells that had migrated to the bottom surface of the filter were counted in six randomly selected fields on each filter under a microscope at $\times 200$ magnification.

Western Blot Analysis

Twenty micrograms of total protein extracted from tumor cell lines was resolved by SDS-PAGE (Invitrogen) and was

transferred to polyvinylidene difluoride membrane (Atto Corp., Tokyo, Japan), and Western blot was performed as described previously.⁹ Immunoreactive bands were visualized using SuperSignal west femto maximum sensitivity substrate (Thermo Scientific, Waltham, MA).

Statistical Analysis

Data are given as means \pm SEM. Statistical analysis was performed using the Student's *t*-test of unpaired samples or the *U*-test. Values of *P* < 0.05 were considered statistically significant.

Results

Effect of SP-A on Lung Cancer Xenografts *in Vivo*

Human lung adenocarcinoma cell lines (PC14PE6 and A549 cells) were transduced with vectors encoding human SP-A by the retroviral transduction system (termed PC14PE6/SP-A and A549/SP-A, respectively). The empty vector was transduced as a control (termed PC14PE6/vector and A549/vector, respectively) (Figure 1A). To investigate the effect of SP-A on *in vivo* tumor growth, we initially injected male nude mice subcutaneously with these cells. For both cell lines, the growth of xenografts was significantly inhibited when cells were overexpressing SP-A compared with the vector control cells (Figure 1, B and C).

Direct Effect of SP-A on Lung Cancer Cell Proliferation

To explore the underlying mechanism by which SP-A suppressed the growth of xenografts, we performed immunohistochemical staining of Ki-67, CD31, or TUNEL (Supplemental Figure S1A). The number of Ki-67-positive cells was significantly decreased in tumors formed by SP-A overexpressing PC14PE6 cells, whereas no difference was seen in A549 cells. The number of TUNEL-positive cells was increased in both cell lines expressing SP-A. No difference was seen in the number of CD31-positive cells. These results led us to consider that SP-A might have a direct effect on cancer cell proliferation or the cell

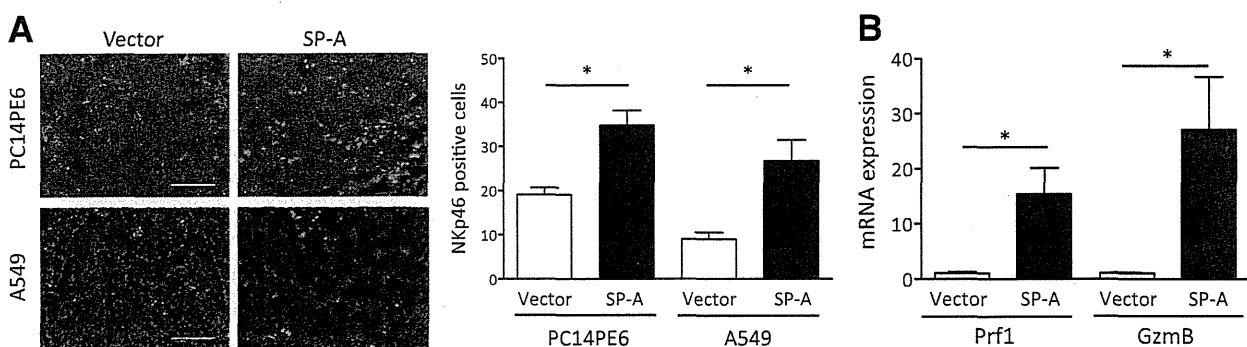


Figure 3 The effect of SP-A on NK cell recruitment in xenografts. **A:** IF staining of NKp46 (green). Nuclei were counterstained with DAPI (blue). Scale bars: 200 μm . **B:** Gene expression of mouse Prf1 and GzmB in PC14PE6 xenografts determined by RT-PCR. Data are the average of four to five mice per group. Data are presented as means \pm SEM. **P* < 0.05.

cycle. However, results of the *in vitro* MTT assay showed that SP-A–overexpressing cells had the same ability of cell proliferation with control cells in PC14PE6 and A549 cells (Supplemental Figure S1B). Propidium iodide staining revealed that the state of cell cycle and death was also similar by SP-A transduction in PC14PE6 cells (Supplemental Figure S1C). Moreover, the effect of SP-A on the cell cycle in PC14PE6 cells was also investigated by using the fluorescent ubiquitination-based cell-cycle indicator system, but no difference was observed (data not shown). Taken together, we considered that although SP-A inhibited tumor growth *in vivo*, its effect was not due to the direct effect on cell proliferation or the cell cycle or to the inhibition of tumor angiogenesis.

Effect of SP-A on the Recruitment of Tumor-Associated Macrophages

We next investigated whether cancer cell–produced SP-A might affect the tumor microenvironment. A variety of studies have shown that tumor-infiltrated tumor-associated macrophages (TAMs) play an important role in the progression of various types of cancers, including lung cancer.^{23,24} It is also known that activated macrophages are functionally polarized into either M1 (classically activated) or M2 (alternatively activated) macrophages. M1 macrophages produce large amounts of inflammatory cytokines, such as TNF- α and interferon- γ , and are essential for tumor suppression and host defense against bacteria.²⁵ In contrast, M2 macrophages play important roles in tumor progression, tissue remodeling, and angiogenesis. M2 macrophages are characterized by their high expression of several factors, such as arginase-1, MRC-1, and IL-10. To determine whether SP-A affects the recruitment of TAMs, sections from resected tumors were subjected to IF staining. As shown in Figure 2A, the number of CD68-positive macrophages was significantly increased in tumors formed by SP-A–transduced cells. We then assessed whether M1 and M2 macrophage polarizations were altered by SP-A transduction. We performed IF double staining of CD68 and TNF- α for M1 and MRC-1 for M2 and determined M1 and M2 macrophages in the xenografts. In both SP-A–transduced cell lines, the number of M1 macrophages was significantly increased versus vector controls, whereas the number of M2 macrophages was not changed (Figure 2B). To confirm that the number of M1 macrophages was increased in the SP-A–expressing tumors, mRNA was extracted from the resected tumor, and the expression of M1 and M2 markers was determined by RT-PCR using mouse-specific primers. Multiple M1 markers were up-regulated in the SP-A–expressing tumors, whereas M2 markers were not changed compared with the vector control tumors (Figure 2C).

Effect of SP-A on NK Cell Recruitment in Xenografts

We next focused on the other important immune cells, NK cells. Because cytokines/chemokines such as interferon- γ ,

CCL5, and IL-12 that were up-regulated in the SP-A–expressing tumors are known to be potent inducers of NK cells,^{26,27} we hypothesized that the *in vivo* tumor regression could be due to the recruitment of NK cells. Thus, we performed IF staining of NKp46 to determine the number of NK cells in the xenografts. As shown in Figure 3A, the number of

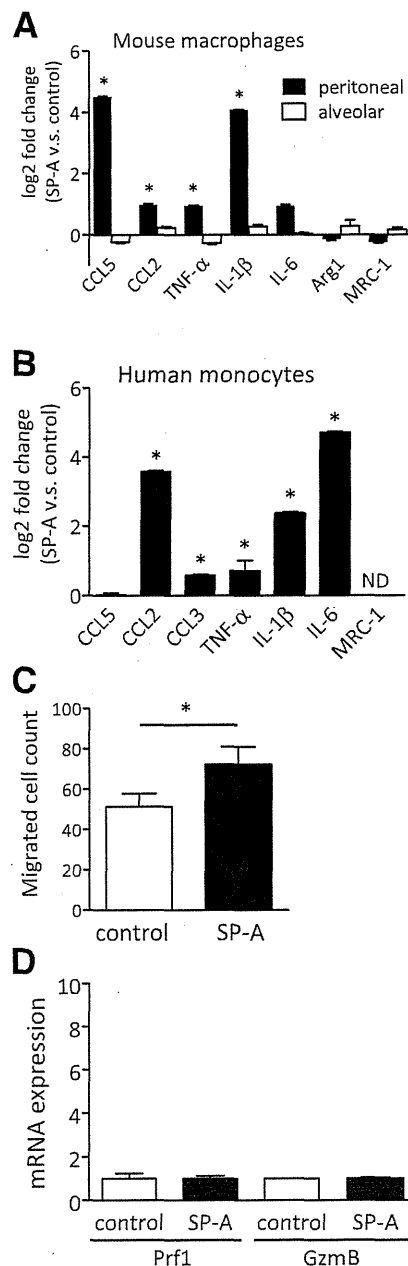


Figure 4 The effect of SP-A on various immune cells *in vitro*. After exogenous SP-A treatment, the expression of various genes was determined by RT-PCR in mouse PMs and AMs (A) and human peripheral monocytes (B). The increased gene expression in SP-A treatment compared with control (PBS) are shown as log₂-fold changes ($n = 3$ per group). ND, not detected. C: The effect of exogenous SP-A on the migration of mouse PMs ($n = 3$ per group). D: Gene expressions of Prf1 and GzmB in mouse NK cells treated with exogenous SP-A ($n = 3$ per group). Data are presented as means \pm SEM. * $P < 0.05$.

Table 2 Effect of SP-A on Lung Metastasis Produced by Lung Adenocarcinoma Cell Lines in Nude Mice

Cell line	Lung		Pleural effusion		
	Weight (g)	Metastasis Incidence	No.	Incidence	Volume (μ L)
PC14PE6					
Vector	0.36 (0.30–0.42)	6/6	117.7 (67–183)	5/6	176.7 (0–300)
SP-A	0.27 (0.22–0.31)*	6/6	58.8 (21–111)*	0/6	All 0*
A549					
Vector	0.48 (0.30–0.85)	9/9	All >200		
SP-A	0.26 (0.19–0.41) [†]	10/10	All >200		

PC14PE6 or A549 cells (1×10^6 per mouse) were i.v. injected into nude mice, and lung metastasis and pleural effusion were evaluated. Values are means (ranges).

*Statistically significant difference compared with PC14PE6/vector ($P < 0.05$).

[†]Statistically significant difference compared with A549/vector ($P < 0.05$).

recruited NK cells was significantly increased in the SP-A-expressing tumors. Moreover, the expression of mouse perforin 1 (Prf1) and granzyme B (GzmB), key factors produced by NK cells to exhibit cell killing, was strongly up-regulated in SP-A-expressing tumors (Figure 3B), suggesting that NK cell killing was activated by SP-A in the tumor microenvironment. Collectively, these results indicate that in the tumor microenvironment, SP-A led to increased numbers of activated M1 TAMs and NK cells, which, in turn, can inhibit tumor growth.

Effect of SP-A on Macrophages and NK Cells *in Vitro*

Given the observation that the numbers of M1 macrophages and NK cells were increased in SP-A-expressing tumors, we performed *in vitro* experiments using thioglycollate-elicited mouse PMs, AMs, NK cells, and human peripheral blood monocytes to further examine the effect of SP-A on these cells. As shown in Figure 4A, exogenous SP-A treatment increased the expression of M1-related genes, such as CCL5, CCL2, TNF- α , and IL-1 β , in mouse PMs. The expression of M2 markers did not change significantly. M1-related gene expression on human peripheral blood monocytes was also up-regulated by SP-A (Figure 4B). These results indicate that monocytes and macrophages can be directly targeted and activated toward the M1 phenotype by SP-A. Furthermore, the migration activity of PMs was increased by SP-A treatment (Figure 4C). Mouse AMs were not affected by exogenous SP-A treatment (Figure 4A), suggesting that AMs had developed to not overreact to SP-A exposure during their development and maturation in the pulmonary environment. The *in vivo* results also suggest that SP-A activated NK cells to demonstrate antitumor activity; however, exogenous treatment of SP-A did not directly affect Prf1 or GzmB gene expression in NK cells *in vitro* (Figure 4D). These results suggest that SP-A activated and attracted circulating monocytes/macrophages to obtain the M1 phenotype and that these increased M1 TAMs then recruited and activated NK cells to exhibit cell killing.

Effect of SP-A on the Lung Cancer Metastasis Model *in Vivo*

We next examined the effect of SP-A on lung metastasis induced by lung cancer cells. Intravenous injection of PC14PE6 or A549 cells into nude mice lead to the development of metastatic colonies in the lung. In addition, mice injected with PC14PE6 developed a large volume of pleural effusion.^{17,18} We compared the number of lung metastatic colonies, lung weight, and the amount of pleural effusion produced by vector control and SP-A-expressing cells. Mice injected with PC14PE6/SP-A cells produced significantly fewer lung metastatic colonies and a lower amount of pleural effusion than those injected with vector control cells (Table 2 and Figure 5A). Similarly, the lung metastasis formed by A549 cells was also suppressed by SP-A expression. Analogous to the results we obtained in the xenograft experiments, the numbers of CD68-positive macrophages, M1 macrophages, and NK cells were increased in the SP-A-expressing metastatic tumors compared with vector controls (Figure 5, B and C). The expression of multiple M1 markers, as well as Prf1 and GzmB, was up-regulated in the lung metastatic colonies formed by PC14PE6/SP-A, whereas the expression of M2 markers was not changed (Figure 5D).

Importance of NK Cells in the SP-A-Mediated Antitumor Effect in Lung Cancer Metastasis

To confirm that the activation of NK cells was essential in the antitumor effect of SP-A, we performed a lung metastasis experiment using nude mice depleted of NK cells.¹⁹ As shown in Table 3 and Figure 5E, PC14PE6/SP-A cells were confirmed to produce significantly fewer lung metastatic colonies and a lower volume of pleural effusion compared with control cells when injected into NK⁺ nude mice. However, when NK cells were depleted, no difference was observed in lung metastasis between PC14PE6/SP-A and control cells. These results indicate that the activation of NK cells was essential for SP-A-mediated suppression of lung cancer progression. Taken together, these findings suggest

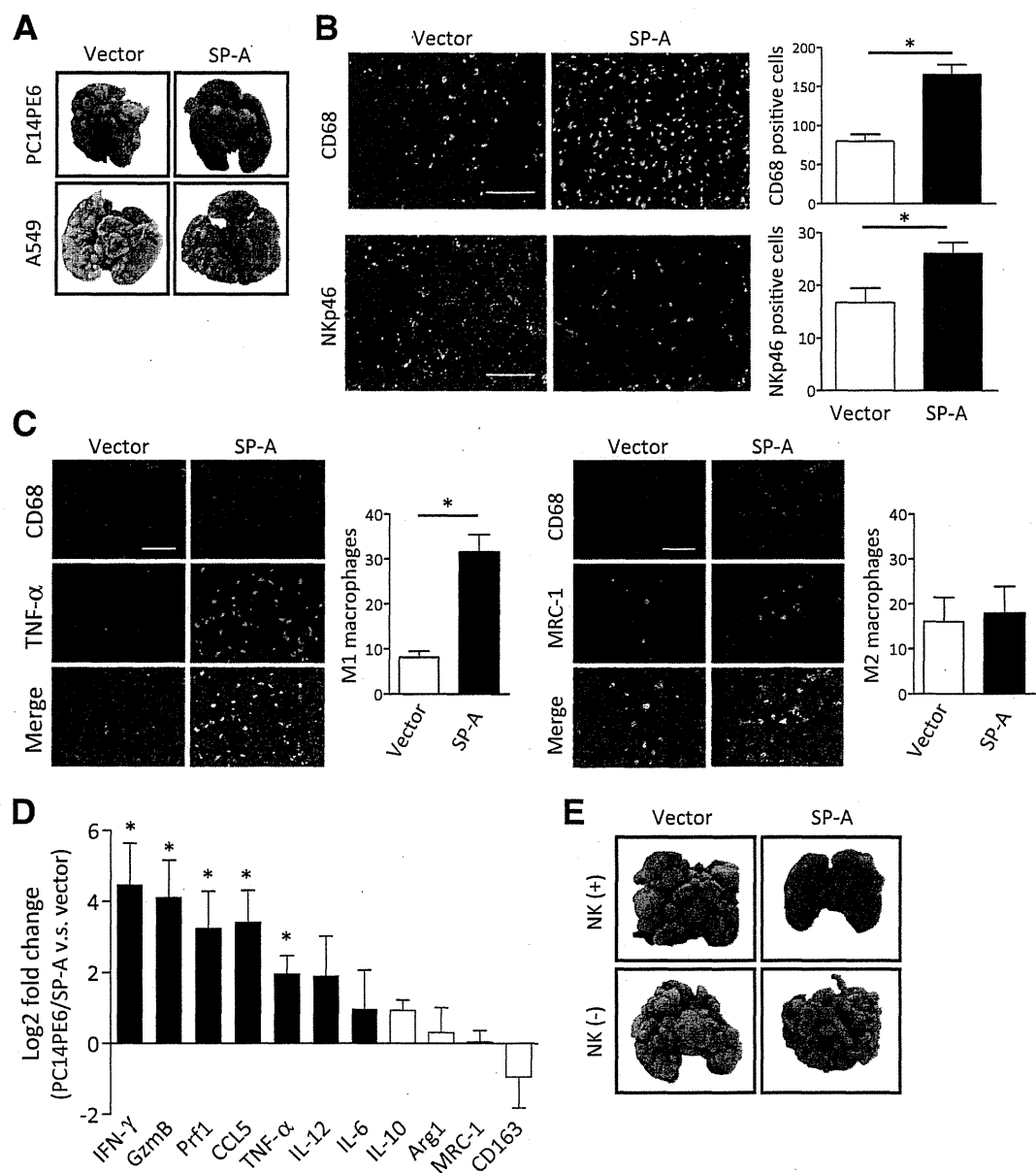


Figure 5 The effect of SP-A on lung cancer metastasis. **A:** Representative images of lung metastasis produced by SP-A- or vector-transduced lung cancer cell lines. **B:** IF staining of CD68 or Nkp46. Nuclei were counterstained with DAPI (blue). **C:** IF double staining of CD68 (red) and TNF- α (green) or MRC-1 (green) in PC14PE6 lung metastasis. **D:** Gene expression of mouse M1 and M2 markers in PC14PE6 metastatic lung nodules determined by RT-PCR. The increased gene expression in PC14PE6/SP-A compared with control (vector) is shown as log₂-fold changes. Data are the average of four to five mice per group. IFN- γ , interferon γ . **E:** Representative images of the lung metastasis formed by PC14PE6/SP-A or vector in nude mice (NK⁺) or NK cell-depleted nude mice (NK⁻). Data are presented as means \pm SEM. * P < 0.05. Scale bars: 200 μ m (**B**); 100 μ m (**C**).

that SP-A in the tumor microenvironment displays anti-tumor activity by mediating the polarization of TAMs toward an M1-dominant phenotype, which, in turn, activates NK cells that then limit tumor progression.

Discussion

In this study, we demonstrated that i) SP-A expression in cancer cells suppresses progression of lung adenocarcinoma in xenograft and lung metastasis models; ii) SP-A inhibits

lung cancer progression not by its direct effect on tumor cells but by regulating the host microenvironment, including macrophages and NK cells; and iii) SP-A increases the number of M1 TAMs in the tumor microenvironment, resulting in NK cell recruitment and activation in tumor tissue. These results suggest new immunoregulatory functions of SP-A, which is frequently expressed in pulmonary adenocarcinoma.

Tumors comprise not only malignant cells but also many other nonmalignant cell types, and they produce a unique

Table 3 Effect of SP-A on Lung Metastasis Produced by PC14PE6 Cells in Nude Mice Depleted of NK Cells

PC14PE6	Lung	Metastasis		Pleural effusion	
	Weight (g)	Incidence	No.	Incidence	Volume (μL)
Vector NK ⁺	0.41 (0.24–0.65)	6/6	113.0 (35–197)	6/6	313.3 (20–900)
SP-A NK ⁺	0.21 (0.15–0.26)*	5/5	25.6 (6–34)*	1/5	80.0 (0–400)
Vector NK [−]	0.50 (0.26–0.89)	7/7	148.6 (52–207)	7/7	438.6 (20–1000)
SP-A NK [−]	0.34 (0.21–0.48)	5/5	101.2 (41–181)	4/5	400.0 (0–1200)

PC14PE6 cells were i.v. injected into nude mice with or without NK cell depletion, and lung metastasis and pleural effusion were evaluated. Values are means (ranges).

*Statistically significant difference compared with vector NK⁺ ($P < 0.05$).

microenvironment that can modify the neoplastic properties of the tumor cells. Among the cells recruited in the tumor microenvironment, TAMs are one of the major players known to have pivotal roles in the progression and metastasis of tumors.^{23,24} Although partially contradictory, high numbers of TAMs often correlate with poor prognosis in various types of cancer.²⁸ Therefore, a better understanding of the role of TAMs seems crucial to control cancer progression.

Considering the character of TAMs, it is now generally accepted that TAMs usually polarize to M2 and represent protumoral functions.²³ Indeed, we have seen in this study that approximately 60% of TAMs had the M2 phenotype in PC14PE6 and A549 control (vector-transduced) tumors (Figures 2B and 5C). However, when tumor cells expressed SP-A, this M1/M2 balance was reversed, and M1 macrophages became dominant in both tumors. As far as we have investigated, the expression of multiple M1 markers was up-regulated in SP-A-expressing tumors, whereas the expression of M2 markers was not altered in xenograft and metastatic lung tumors. Together with the fact that the number of M1 TAMs was increased in SP-A-expressing tumors, these results indicate that SP-A aided in making the TAMs M1 dominant by increasing the number of recruited M1 macrophages rather than shifting the M2 TAMs into the M1 phenotype in the tumor microenvironment.

Numerous studies have shown that macrophages could be a target cell type that SP-A interacts with to regulate infectious inflammation, and, to date, diverse and contradictory functions of SP-A against monocytes/macrophages are reported.^{29–32} These studies all indicate that SP-A has various effects on inflammation induced by different agonists. Indeed, we also observed that the cytokine/chemokine expression profiles of PMs and human monocytes were different in response to SP-A (Figure 4), suggesting that SP-A may exert cell- and agonist-specific effects that contribute to the inflammation state of the host.

In addition to the observations investigating the role of SP-A during infection, we showed that SP-A activates and increases M1 macrophages in the tumor microenvironment and induces the production of inflammatory cytokines, suggesting that SP-A facilitates inflammation in the tumor to reduce tumor progression. The precise molecular mechanism by which SP-A activates M1 macrophages in the

tumor remains unclear with the current observation; however, several mechanisms should be considered. First, SP-A may enhance the binding of cytokines to their respective receptors. SP-A is reported to bind to several receptors, including Toll-like receptors 2 and 4, and to regulate inflammatory responses induced by pathogen-derived products, such as peptidoglycan and lipopolysaccharide via Toll-like receptors.^{33–35} In addition to its role in Toll-like receptor-mediated cellular responses induced by infectious challenges, it is very possible that SP-A regulates the function of TAMs in the tumor microenvironment through the interaction with Toll-like receptors. Second, signal transduction in the TAMs could be regulated by SP-A. SP-A has been shown to trigger rapid tyrosine, but not serine or threonine, phosphorylation³⁶ of macrophage proteins and could possibly enhance/accelerate the initial phosphorylation steps of the signal transduction pathway, which leads to the regulation of inflammation in the tumor. It was also possible that SP-A directly affects tumor cells and regulates cytokine expression. Thus, we compared the chemokine expression of SP-A-expressing PC14PE6 and control (vector-transduced) cells using the PCR array system. Of 92 chemokine and chemokine-related genes tested, none were altered by SP-A overexpression (data not shown), suggesting that SP-A did not directly affect chemokine expression of tumor cells.

Of note, we showed the effect of SP-A on M1 macrophage recruitment and tumor suppression in xenograft and lung metastasis models. This result indicated two important aspects of SP-A. First, the lung tumor-specific expression of SP-A is more important than the host SP-A in the lung to suppress lung cancer progression. Second, the recruited M1 TAMs by tumor-derived SP-A could be originated from circulating monocytes. This possibility was supported by the result that SP-A activated only circulating monocytes/macrophages (mouse PMs or human monocytes) and showed no effect on resident AMs in cytokine expression. In the lung, the sensitivity of resident AMs against SP-A is thought to be suppressed as they are continuously contacted by SP-A, which could be a plausible explanation because the host needs to be protected from the overzealous inflammation in the resting, normal, noninflamed lungs. The molecular mechanism of different SP-A sensitivities in different cells is

not still clearly understood. However, as stated previously herein, the expression of inflammatory signaling molecules that might be regulated by SP-A could be different between AMs and other type of monocytes/macrophages. In addition, there could be an unknown SP-A receptor(s) that might be critical in regulating inflammation in macrophages, as SP-A is reported to bind to multiple receptors.² Further studies are needed to understand the precise molecular mechanisms of the diverse and cell-specific function of SP-A against macrophages in the context of SP-A and lung cancer.

These results indicate that NK cell activation was the main mechanism by which SP-A lead to the reduction in tumor burden. The production of Prf1 and GzmB was strongly increased (15- to 30-fold), whereas the number of NK cells was increased only twofold to threefold in the SP-A-expressing tumor, suggesting the multiple pathways regulated by SP-A to recruit and activate NK cells. Because cytokines produced by M1 macrophages, such as interferon- γ and CCL2, are known to activate NK cells,^{37–39} it is likely that SP-A implicitly induces NK cell killing via activating M1 TAMs and increasing various inflammatory cytokines in the tumor microenvironment.

Two functional genes of SP-A were detected in a previous report⁴⁰: SP-A1 and SP-A2. These genes were differentially regulated by development⁴¹ and have a minor difference in carbohydrate-binding activity.⁴² However, SP-A protein derived from SP-A1 and SP-A2 genes are reported to be functional and to enhance TNF- α secretion by the monocytic cell line.⁴³ Thus, although we have transduced only the SP-A1 gene, we suspect that SP-A protein from the SP-A2 gene could also contribute to activation of the innate immune system and suppress tumor progression.

In conclusion, these findings demonstrate that SP-A regulates the tumor microenvironment by controlling the polarization of TAMs. SP-A expression by tumor cells leads to increased numbers and the activation of M1 TAMs. These activated M1 TAMs then recruit and activate NK cells that function in tumor suppression. These results indicate that SP-A plays an important protective role in the progression of lung cancer. Specifically targeting M1 TAMs (not bulk TAMs) to induce the activation of a proinflammatory program in the tumor, generating the pharmacologic modulators of SP-A for example, could be the therapeutic approach to improve the effect of anticancer therapy.

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Supplemental Data

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A Novel Targeting Therapy of Malignant Mesothelioma Using Anti-Podoplanin Antibody

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Podoplanin (Aggrus), which is a type I transmembrane sialomucin-like glycoprotein, is highly expressed in malignant pleural mesothelioma (MPM). We previously reported the generation of a rat anti-human podoplanin Ab, NZ-1, which inhibited podoplanin-induced platelet aggregation and hematogenous metastasis. In this study, we examined the antitumor effector functions of NZ-1 and NZ-8, a novel rat-human chimeric Ab generated from NZ-1 including Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity against MPM in vitro and in vivo. Immunostaining with NZ-1 showed the expression of podoplanin in 73% (11 out of 15) of MPM cell lines and 92% (33 out of 36) of malignant mesothelioma tissues. NZ-1 could induce potent ADCC against podoplanin-positive MPM cells mediated by rat NK (CD161a⁺) cells, but not murine splenocytes or human mononuclear cells. Treatment with NZ-1 significantly reduced the growth of s.c. established tumors of MPM cells (ACC-MESO-4 or podoplanin-transfected MSTO-211H) in SCID mice, only when NZ-1 was administered with rat NK cells. In vivo imaging, NZ-1 efficiently accumulated to xenograft of MPM, and its accumulation continued for 3 wk after systemic administration. Furthermore, NZ-8 preferentially recognized podoplanin expressing in MPM, but not in normal tissues. NZ-8 could induce higher ADCC mediated by human NK cells and complement-dependent cytotoxicity as compared with NZ-1. Treatment with NZ-8 and human NK cells significantly inhibited the growth of MPM cells in vivo. These results strongly suggest that targeting therapy to podoplanin with therapeutic Abs (i.e., NZ-8) derived from NZ-1 might be useful as a novel immunotherapy against MPM. *The Journal of Immunology*, 2013, 190: 6239–6249.

Malignant pleural mesothelioma (MPM) is an aggressive malignancy arising from the mesothelial cells lining the pleura (1). Previously, MPM was considered

to be rare; however, its incidence is increasing worldwide. According to various studies, the number of cases is estimated to reach a peak between 2010 and 2020 in Europe (2) and ~2030–2040 in Asia (3). Despite the combined therapies including surgery, radiotherapy, and chemotherapy, the prognosis of MPM is very poor: median survival from the time of diagnosis is 4–12 mo, and the 5-y survival rate is <5% (4–6). Therefore, novel therapeutic strategies for MPM are urgently necessary for improving the prognosis.

On the basis of the current status of MPM, immunotherapy has also been considered as one of the novel therapeutic approaches. Recently, tumor-specific immunotherapy using vaccination with antigenic peptides of tumor-associated Ags (TAAs) with or without dendritic cells or Abs has been conducted because several TAAs specific for MPM were reported, although their number is insufficient (7–9). Among them, immunotherapy with specific Abs seems to be the most promising approach, whereas clinical trials with humanized Abs for mesothelin as well as CD26 are still in phase I (10).

Podoplanin (Aggrus), which is a type I transmembrane sialomucin-like glycoprotein, induces platelet aggregation (11–14). Originally, podoplanin was detected on the surface of podocytes (15). Although podoplanin is reported to be expressed in limited normal tissues such as endothelium of lymphatic vessels and in type I alveolar epithelium (16, 17), various tumors such as squamous cell carcinomas, testicular seminomas, malignant brain tumors, fibrosarcomas, and malignant mesotheliomas (MMs) including MPM are known to show its overexpression (18–22). Furthermore, it has been reported that podoplanin is associated with cell migration (23, 24), epithelial–mesenchymal transition (25), and tumor metastasis (26, 27). Moreover, podoplanin serves through extra-

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Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CHO, Chinese hamster ovary; CRPMI 1640, RPMI 1640 medium supplemented with 10% FBS; MM, malignant mesothelioma; MNC, mononuclear cell; MPM, malignant pleural mesothelioma; MSFI, mean specific fluorescence intensity; PDPN, podoplanin; TAA, tumor-associated Ag.

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