Suppression of Progression and Metastasis of Established Colon Tumors in Mice by Intravenous Delivery of Short Interfering RNA Targeting KITENIN, a Metastasis-Enhancing Protein

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Abstract

KITENIN promotes invasion of mouse colon adenocarcinoma (CT-26) cells *in vivo*. Here, we studied the effects of *in vivo* KITENIN ablation on established tumors by using pSUPER vectors (pSUPER-KITENIN) producing short interfering RNA (siRNA). When pSUPER-KITENIN was given weekly or semiweekly for 1 month into tail vein of syngeneic mice that have established colon tumors, tumor size regressed markedly and metastases were inhibited. In mice injected with pSUPER-KITENIN, serum interleukin-2 (IL-2) and IFN-γ increased and $CD4^+$ and $CD8^+$ T cells infiltrated in the regressed tumor tissues. These effects, observed beginning 2 days after i.v. injection, imply that immune response is involved in the antitumor action of pSUPER-KITENIN. Using a yeast two-hybrid assay, we identified two KITENIN-interacting proteins for the possible mediators of these actions: 90K protein, a known immune modulatory glycoprotein, and protein kinase C inhibitor (PKCI). 90K was increased in the culture medium from CT-26/antisense KITENIN/90K cells. Double culture of accessory cells with CT-26/antisense KITENIN/90K cells revealed increased secretion of IL-1 and IL-6. Overexpression of 90K in CT-26/antisense KITENIN cells further delayed tumor growth compared with that of CT-26/antisense KITENIN cells. Actin arrangement was distorted in CT-26/antisense KITENIN and CT-26/antisense PKCI cells, whereas overexpression of PKCI resulted in increased invasiveness to fibronectin. Thus, antitumor effects of KITENIN siRNA derives from both the generation of a tumor-specific immune response *in vivo* through increased 90K secretion from tumor cells and the suppression of tumor invasion in which PKCI is related to increased invasiveness. Moreover, siRNA targeting of KITENIN can function as a chemotherapeutic strategy against colon cancer. (Cancer Res 2005; 65(19): 8993-9003)

Introduction

Most tumor cells are poorly immunogenic and do not elicit an immune response sufficient for elimination of the tumor. Alternatively, the failure of the immune system to recognize and react to tumor antigens may be due to improper antigen presentation or insufficient costimulation (1). Certain cytokines, when administered systemically or locally, can lead to successful immune recognition of the tumor and mobilization of an effective immune response. Thus, cytokine-engineered nonimmunogenic tumor cells can elicit a strong tumor-specific immune response. In addition, tumor cells produce and secrete proteins that can directly affect the host immune response to the tumor, either positively or negatively (2, 3).

90K, a tumor-associated protein with possible immunoregulatory properties in CG-5 human breast cancer cells, is detected in the sera of normal as well as tumor-bearing patients (4, 5). The 90K protein belongs to the scavenger receptor cysteine-rich domain superfamily of proteins implicated in immune defense and immunoregulation (6). This protein has been shown to enhance the *in vitro* generation of cytotoxic effector cells from peripheral blood mononuclear cells (PBMC) and to indirectly enhance interleukin-2 (IL-2) production by PBMC and suggested to activate an early step in the immune response to tumors, resulting in a more effective antitumor response (7–10).

Recently, we cloned KITENIN (KAI1 COOH-terminal interacting tetraspanin), a member of the tetraspanin protein family, which interacted specifically with the COOH-terminal cytoplasmic domain of KAI1 (11). We found that, compared with parental cells, KITENIN-overexpressing CT-26 mouse colon cancer cells showed greater tumorigenicity and early hepatic metastasis *in vivo* as well as greater invasiveness and adhesion to fibronectin *in vitro*. There was a positive correlation between the expression of KITENIN and the presence of distant metastasis, indicating that KITENIN can function as a metastasis-promoting gene. Our previous results suggest that an antisense KITENIN strategy may be able to inhibit metastasis in colon cancers (11).

Synthetic short interfering RNA (siRNA), known as RNA interference, is a powerful tool to induce strong and specific suppression of gene expression (12). However, this reduction in gene expression is transient, which severely restricts its applications. Recently, a new expression vector system, pSUPER (suppression of endogenous RNA) was developed that directs the synthesis of siRNA-like transcripts in mammalian cells (13). The siRNA expression mediated by this vector causes efficient and specific down-regulation of gene expression, resulting in functional inactivation of targeted genes.

Thus, in this study, we used the pSUPER vector system to deliver KITENIN siRNA for the efficient and stable suppression of KITENIN expression in the established tumors of syngeneic mice. Surprisingly, we observed marked inhibition of the growth of established tumors as well as suppression of distant metastases of colon cancer following four weekly or semiweekly i.v. injections of pSUPER-KITENIN. In addition, we characterized the effect of antisense KITENIN and two of its interacting proteins, 90K and

Note: J.H. Lee and E.S. Cho contributed equally to this work.

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protein kinase C inhibitor (PKCI) proteins, both *in vitro* and *in vivo*. Here, we provide *in vitro* and *in vivo* evidences showing that antitumor effects of KITENIN siRNA on established colon tumors derives from both the generation of a tumor-specific immune response *in vivo* through increased 90K protein secretion from nonimmune tumor cells and the suppression of tumor invasion in which PKCI is related to increased invasiveness as well as helping the KAI1's suppressive action. The present study suggests that siRNA targeting the KITENIN could be useful in the treatment of colon cancer, together with other chemotherapeutic agents.

Materials and Methods

Yeast two-hybrid assay. To identify the protein interacting with KITENIN, full-length KITENIN cDNA was used as bait. The yeast reporter strain AH109 was sequentially transformed with pGBKT7-KITENIN and pGADT7-mouse testis cDNA library. Positive clones were selected and prepared as previously described (11). The positive clone was identified by sequencing.

Constructs of KITENIN short interfering RNA and cDNA. For silencing the expression of KITENIN, five regions of human and mouse KITENIN were targeted for siRNA. Two of the five regions were shown to specifically reduce the expression of human KITENIN (KIT-siRNA 2, 5′-ACGCTACCTGGGCCTCACC-3′; KIT-siRNA 5, 5′-AGAAGCGGAAAGCAAGGCT-3′). In mice, the two identical regions (KIT-siRNA 2, 5′-GCGCTATCTGGGCCTTACC-3′; KIT-siRNA 5, 5′-AGAAGCGGAGAGCAAGACT-3′) were selected and constructed in the pSUPER vector (OligoEngine, Seattle, WA). To generate siRNAs, equimolar amounts of complementary sense and antisense strands were mixed and annealed each in a 50-μL reaction at 90°C for 4 minutes, 70°C for 10 minutes, 60°C for 30 minutes, 37°C for 20 minutes, and 25°C for 10 minutes. The pSUPER vector was digested with *Bgl*II and *Hin*dIII, and the annealed oligos were ligated into the vector. The NH_2-terminal half of full-length KITENIN cDNA (antisense KITENIN cDNA) was inserted inversely into the mammalian expression vector pREP4 (Invitrogen, Carlsbad, CA).

Cell culture and transfection. CT-26 cells, human colon cancer (KM1214) cells, human embryonic kidney (293) cells, and mouse accessory (RAW 264.7) cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Transfection was done using FuGENE 6 (Roche, Indianapolis, IN) as described (14). The KITENIN cDNA, antisense KITENIN cDNA, antisense PKCI cDNA, or 90K cDNA was transfected into CT-26/parent cells (CT-26/KITENIN, CT-26/antisense KITENIN, CT-26/antisense PKCI, and CT-26/90K cells). Antibiotic-resistant cells were selected and maintained by culture with G418 (500 μg/mL), zeocin (200 μg/mL, Invitrogen), and/or hygromycin (100 μg/mL, Clontech, Palo Alto, CA). Two weeks later, surviving clones were analyzed by Western blot for expression of the respective protein.

Staining of filamentous actin. CT-26/antisense KITENIN or CT-26/antisense PKCI cells were seeded onto an eight-well Lab-Tek Chamber Slide Glass (Nunc, Scotts Valley, CA) and were grown in DMEM supplemented with 10% FBS. Cells were washed thrice with PBS (pH 7.4) and fixed in 3.7% formaldehyde solution in PBS for 10 minutes at room temperature. After washing with PBS and placing with 0.1% Triton X-100 in PBS for 5 minutes, the cells were incubated with Alexa fluor 488 phalloidin (A12379, Molecular Probes, Eugene, OR) for 30 minutes at room temperature and then washed thrice with PBS buffer. The cells were mounted using Prolong Gold (P36939, Molecular Probes) and examined with a Laser Scanning Confocal Microscope (Leica Microscope Systems, Wetzlar, Germany).

***In vivo* tumor growth.** Prior approval of the experimental protocol was obtained from the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Maintenance of animals and all *in vivo* experiments were done according to The Guiding Principles in the Care and Use of Animals (DHEW publication, NIH 80-23). CT-26 cells (1×10^6 cells per mouse) were injected s.c. into BALB/c syngeneic mice. The mice received tail vein injections of vector or KITENIN siRNA with FuGENE 6 at 1 or 2 weeks after CT-26 injection, and the injection interval was 1 week. The pSUPER-KITENIN was the mixture of KITENIN S2 and S5 administered at 100 μg doses in ~100 μL per injection in each mouse as described (15). Tumor size and volume were measured daily from the first to the fifth week as previously described (14). At the fifth week after injection, the presence of metastasis in the liver and lung tissues of each group was evaluated by gross and microscopic examination.

Production of anti-protein kinase C inhibitor antibody. We prepared the GST-PKCI fusion construct by subcloning the full-length of PKCI into the unique *Eco*RI and *Xho*I sites of pGEX-4T as described (14). Rabbit polyclonal antiserum recognizing PKCI was prepared using the GST-PKCI fusion protein. The serum was filtered through a column of GST-PKCI fusion protein, and the column was eluted with a low-pH buffer. It was then filtered through a column of glutathione *S*-transferase (GST) protein to remove the anti-GST component.

***In vitro* translation.** To verify the interactions with KITENIN identified in the yeast two-hybrid screen, *in vitro* transcription and translation was done using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI). Translations were synthesized *in vitro* by T7 RNA polymerase promoter and rabbit reticulocyte lysate. Circular plasmid DNA (1 μg) is added directly to TNT lysate, TNT T7 RNA polymerase, amino acid mixture (−Leu/−Met), and RNasin, and incubated in a 50-μL reaction for 90 minutes at 30°C.

Immunoprecipitation. Proteins of indicated cells were prepared as described (11), incubated with anti-KITENIN or anti-V5 antibody and protein A/G-agarose beads (Pierce, Rockford, IL), and then were analyzed by blotting with anti-PKCI or anti-KITENIN polyclonal antibody.

Western blot analysis. Transfer of proteins and immunoblotting were done as described (14). The blot was reprobed with anti-actin antibody (I-19, Santa Cruz Biotechnology, Santa Cruz, CA) to control for loading.

Cell invasion assay. Cell migration was measured using the Transwell migration apparatus (Costar, Inc., Cambridge, United Kingdom) as described (14).

Purifying 90K protein from culture supernatant. Culture medium was harvested from CT-26/90K/antisense KITENIN cells between 24 and 48 hours after seeding, and cell culture supernatant (150 mL) was applied onto Ni-NTA spin columns (Qiagen, Valencia, CA) equilibrated with buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L $CaCl_2$, 20 mmol/L imidazole]. The absorbed proteins were eluted with 2×750 μL buffer containing 200 mmol/L imidazole. Eluants were combined and dialyzed against buffer [5 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaCl, 0.2 mmol/L $CaCl_2$] overnight. Aliquots (15 μL) were separated by SDS-PAGE under reducing conditions and confirmed by immunoblotting.

ELISA. ELISA was used to quantify cytokines in mouse serum and cell lines after KITENIN siRNA treatment. For collecting mouse serum, vector or KITENIN siRNA was injected at 1 week after the injection of CT-26 cell in BALB/C mice. The blood of each group ($n = 6$) was collected everyday for 6 days. IL-1β, IL-2, IL-4, IL-5, IL-6, and IFN-γ were measured as recommended (R&D Systems, Minneapolis, MN).

In RAW 264.7 cells, the change of cytokine secretion by the effect of overexpressed 90K was measured. CT-26/parent, CT-26/90K, and CT-26/90K/antisense KITENIN cells (2×10^5) were cultured in the lower chamber of the Transwell, and RAW cells (1×10^5) were cultured in the upper chamber. After 2 days, the supernatant was centrifuged and was measured for IL-1β, IL-2, IL-4, IL-5, IL-6, and IFN-γ. In addition, the cultured medium was harvested from RAW cells at 24 hours after adding purified 90K protein derived from supernatant of CT-26/90K/antisense KITENIN cells, and IL-1 and IL-6 were measured by ELISA.

Histochemistry. KITENIN siRNA S2 or S5 (100 μg) was injected i.v. ($n = 4$), and internal organs (liver, lung, kidney, and spleen) were excised for histochemistry at 3 days after injection. The tissue sections were deparaffinized, rehydrated, and rinsed. Distribution of green fluorescent protein in mouse tissue, expressed by pSUPER KITENIN siRNA, was examined immediately by fluorescent microscope.

We prepared other groups of syngeneic mice, which were injected s.c. with CT-26/parent, CT-26/90K, or CT-26/antisense KITENIN/90K cells ($n = 12$ for each group). Two weeks after injection, liver and lung were excised weekly in each experimental group ($n = 3$) for histochemistry up to

5 weeks. The tissue sections were deparaffinized, rehydrated, and stained with H&E for examining metastatic cells with light microscope.

Reverse transcription-PCR. Reverse transcription was done as described (14). The reverse transcription-PCR (RT-PCR) exponential phase was determined to be 30 cycles to allow quantitative comparison of each cDNA from identical reactions. All reactions involved an initial denaturation at 94°C for 5 minutes followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and at 72°C for 2 minutes using PCR primers (for KITENIN, sense, 5′-GGAATTCCATTCGAAAAAATCTA-3′; antisense, 5′-CCGCTCGAGGCCCAGGTAGCGTTTGCA-3′; for 90K, sense, 5′-CTACCAGTCCTTCCAGACT-3′; antisense, 5′-AGCATCAGGGCTTTGTTTTCGTAG-3′).

Statistical analysis. Results are expressed as mean ± SE. Experimental differences were tested for statistical significance using ANOVA and Student's *t* test. All statistical tests were two sided, and *P*s < 0.05 were considered statistically significant.

Table 1. KITENIN-siRNA sequences

Species	Name	Sequence (19 mer)
Human	KIT-siRNA 1	5′-GCTGACATGCCACGGGTGT-3′
	KIT-siRNA 2	5′-ACGCTACCTGGGCCTCACC-3′
	KIT-siRNA 3	5′-GGAGGGATGAGCTGGAGCC-3′
	KIT-siRNA 4	5′-GGTTGCAGTGGAAGAGGCC-3′
	KIT-siRNA 5	5′-AGAAGCGGAAAGCAAGGCT-3′
Mouse	KIT-siRNA 1	5′-GCGGATGTGCCGCGGGTGT-3′
	KIT-siRNA 2	5′-GCGCTATCTGGGCCTTACC-3′
	KIT-siRNA 3	5′-GGAGGGAGGAGCTGAAACC-3′
	KIT-siRNA 4	5′-AGTGGCTGTGGAGGAAGCC-3′
	KIT-siRNA 5	5′-AGAAGCGGAGAGCAAGACT-3′

Results

Expression of KITENIN short interfering RNA using pSUPER vector induces KITENIN mRNA degradation. We designed five synthetic siRNA oligonucleotides targeted against different sequences of human KITENIN (Table 1), subcloned each of them into pSUPER vector, and compared their ability to inhibit KITENIN expression in a transient transfection experiment using 293 (Fig. 1*A, top*) and KM1214 (Fig. 1*A, bottom*) cells. Two KITENIN siRNAs (human pSUPER-KITENIN S2, S5) were identified that reduced both mRNA (data not shown) and protein levels (Fig. 1*A*). This suppressant effect of KITENIN siRNA was greater in 293 cells than KM1214 cells. In addition, effective silencing of KITENIN expression was observed when S2 and S5 KITENIN siRNAs (mouse pSUPER-KITENIN S2, S5) designed to the murine KITENIN were stably transfected into CT-26 cells (Fig. 1*B*). KITENIN siRNA S2–transfected KM1214 cells showed significantly decreased *in vitro* motility and invasive potential by fibronectin compared with parent cells (data not shown).

To show the ability of pSUPER-KITENIN (siRNA) to suppress KITENIN expression *in vivo*, siRNAs were given to BALB/c mice, a syngeneic host of CT-26 cells, through tail vein with amounts of 100 μg in 200 μL volume. Biodistribution of siRNAs was assessed by observing the green fluorescent protein in tissue samples from liver, lung, and spleen obtained at 72 hours after injection. A substantial amount of fluorescence was observed in these tissues, and marked fluorescent expression was also found around blood vessels, indicating that a high amount was transfected into endothelial cells (Fig. 1*C*). *In vivo* KITENIN expression was examined by RT-PCR and Western blot analyses in the liver, lung, spleen, and kidney at 3 days, 1, 2, 3, and 4 weeks after i.v. siRNA injection. Expression of KITENIN decreased 3 days after injection. Potent inhibition of KITENIN mRNA expression was observed at 1 week and peaked at 2 weeks, especially in lung and liver. KITENIN mRNA expression recovered to control level after 3 weeks (Fig. 1*D*). However, the suppression of KITENIN proteins were observed from 3 days after injection and persisted until 3 weeks (Fig. 1*E*). These results indicate that the silencing effect of single siRNA injection on the KITENIN expression in various tissues persists for 2 weeks and suggests that the effect of KITENIN siRNA could be maintained if given i.v. at weekly intervals.

Targeted suppression of KITENIN expression via intravenous KITENIN short interfering RNA injection renders mice resistant to tumor challenge. To examine whether i.v. administration of KITENIN siRNA affects progression of experimental colon cancer, 50 μg of both KITENIN siRNA S2 and S5 mixed with FuGENE 6 were given i.v. into the tail vein of syngeneic mice at 1 week before, simultaneously, and 1 week after the injection of CT-26 cells. KITENIN siRNA was given weekly for 1 month. After a single injection, tumor necrosis was observed beginning 2 days, but new tumor mass had grown around the necrotic tumor tissues after 4 days. However, the tumor size was substantially regressed in the KITENIN siRNA injection group compared with the control vector group, regardless of injection timing (Fig. 2*A*). Interestingly, normal tumor growth was observed during 2 weeks after injection of CT-26 cells in mice in which i.v. siRNA delivery was started 1 week before the tumor challenge. However, substantial tumor regression was observed after the fourth injection, which was given at 2 weeks after the tumor challenge. Remarkably, complete regression of established tumor after four deliveries of KITENIN siRNA was observed in one mouse, starting at 1 week after the injection of CT-26 cells.

Next, to mimic the clinical situation, we observed whether i.v. siRNA injection affects tumor growth and metastasis of experimental colon cancer after the tumor is already formed. KITENIN siRNA was given weekly five times starting at 1 or 2 weeks after the injection of CT-26 cells. The administration of KITENIN siRNA substantially delayed the growth of established tumor in all mice in comparison with control vector–injected group, especially after 1 month of tumor cell injection (Fig. 2*B*). The results were similar between both injection groups, despite the difference in how soon the injections started after tumor formation. After 1 month, regression of the established tumor was observed in all mice, and 10 of 13 mice survived at 56 days in the KITENIN siRNA group. In the vector group, the tumor grew quickly after 1 month, and no regression of tumors was observed. Only 5 of 20 mice survived at 56 days in the vector group (Fig. 2*C*). Thus, KITENIN siRNA delivery significantly prolonged the survival of mice more than the vector alone (77 ± 12.2 versus 25 ± 9.9 days, $P < 0.01$). Internal organs were examined by H&E staining for metastases of adenocarcinoma cells in the mice receiving KITENIN siRNA or vector ($n = 13$ versus $n = 20$). After 6 weeks, liver and lung metastases were detected in all control mice (Fig. 2*D, top columns*), whereas liver or lung was still intact in the mice ($n = 12$) with KITENIN siRNA (Fig. 2*D, bottom columns*). In one mouse that received KITENIN siRNA, some liver metastasis was detected, but metastatic focus was very small compared with those of vector group. Thus, the metastasis to liver or lung was inhibited in the KITENIN siRNA-delivered mice compared with vector group. These results suggest that *in vivo* inhibition of KITENIN expression renders mice resistant to tumor challenge.

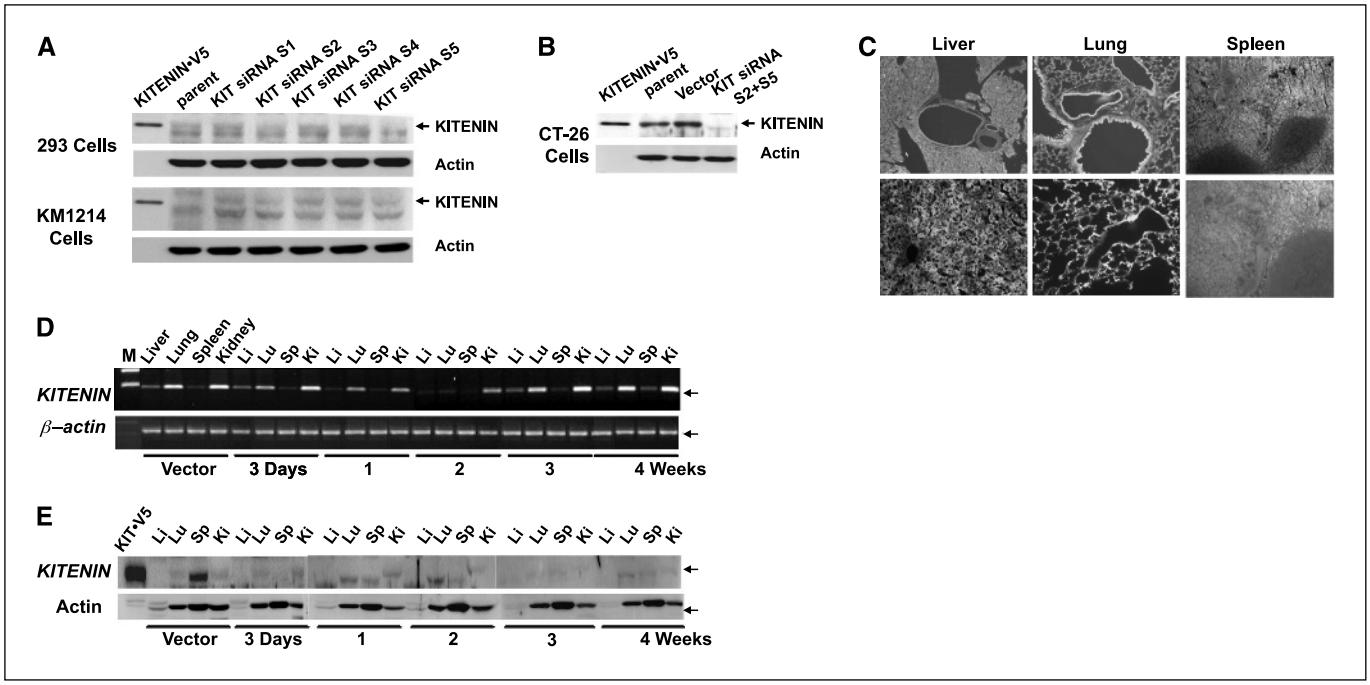

Figure 1. A pSUPER vector–based suppression of KITENIN expression in cultured cells and *in vivo* distribution and inhibition of KITENIN. *A,* silencing of KITENIN expression in 293 (*top*) or KM1214 (*bottom*) cells using pSUPER vectors producing siRNA to KITENIN (pSUPER-KITENIN). The indicated pSUPER-KITENIN (S1-S5) was transiently transfected into 293 or KM1214 cells for 48 hours. KITENIN expression (*arrow*) was markedly decreased in the S2 (KIT siRNA S2)– or S5 (KIT siRNA S5)–transfected cells. *In vitro* translated KITENIN (KITENIN·V5, ~65 kDa) was used as a positive control. Actin was used as an evidence for the protein loading control. *B,* efficient silencing of KITENIN in the CT-26 cells using pSUPER-KITENIN. KITENIN (*arrow*) was nearly absent in the CT-26 cells stably cotransfected with KIT siRNA S2 and S5. *C,* tissue distribution of green fluorescent protein at 3 days after i.v. injection of KIT siRNA S2 or S5. Representative images ($n = 4$; *top*, low-power field; *bottom*, high-power field) in mouse liver, lung, and spleen, which showed substantial amount of the fluorescence and a marked uptake was localized around the blood vessels. *D* and *E, in vivo* inhibition of KITENIN expression by i.v. pSUPER-KITENIN injection. Mice were i.v. injected with both KIT siRNA S2 and S5, and total RNAs (*D*) or proteins (*E*) were isolated from liver, lung, spleen, and kidney following 3 days, 1, 2, 3, and 4 weeks after injection. The decreased KITENIN mRNAs peaked at 2 weeks (*D*), whereas decreased proteins persisted until 3 weeks (*E*).

Intravenous injection of KITENIN short interfering RNA into syngeneic mice allows the generation of a tumor-specific immune response *in vivo*. The necrosis of established tumors observed beginning 2 days after a single i.v. KITENIN siRNA injection prompted us to investigate whether resistance to tumor challenge induced by selective KITENIN blockade could be associated with an enhancement of immune response *in vivo*. For analyzing cytokine production and T-cell accumulation, we obtained serum and tumor tissue daily for 6 days from mice given single injection of KITENIN siRNA or control vector given at 10 days after CT-26 cell challenge. At first, we examined the duration of tumor regression effect after single injection. The part of tumor tissue was black colored at 1 day after i.v. KITENIN siRNA (Fig. 3*A, top first column*). At 2 days after injection, tumor size was decreased, and crust formation was observed (Fig. 3*A, top second column*). These effects seemed to persist until 3 to 4 days after single injection, then tumor growth started again after 5 days (Fig. 3*A, top fifth column*). However, injection of vector alone did not affect the progression of the established tumor (Fig. 3*A, bottom columns*).

We measured tumor volume and then amounts of various immune cytokines by ELISA in the serum collected from each mouse. IL-2 increased significantly in the systemic circulation from 2 days after i.v. KITENIN siRNA injection, and this effect was observed until 4 days (Fig. 3*B*). In addition, IFN-γ increased significantly from days 2 to 5 after injection (Fig. 3*C*). In contrast, no significant differences were found in IL-1, IL-4, IL-5, and IL-6 levels between the experimental groups (data not shown). Accumulation of immune T cells was observed in the KITENIN siRNA injected regressed tumor tissues. $CD4^+$ helper T cells (Fig. 4*E*) and $CD8^+$ cytotoxic T cells (Fig. 4*F*) were increased in the regressed tumor tissues from 2 days and peaked at 4 days (Fig. 4*I* and *M, J* and *N*) after single injection. In addition, apoptotic cells containing segmented nuclei were found around the $CD8^+$ T cells (Fig. 4*N*). These results indicate that the effects of a single i.v. KITENIN siRNA injection on the immune response and tumor growth persisted for about 4 days. In addition, KITENIN siRNA was effective at promoting tumor cell destruction accompanied by extensive necrosis and apoptosis, whereas the integrity of the tumors was completely preserved following treatment with control vector. Thus, *in vivo* silencing of KITENIN is effective in stimulating T1-type antitumor response *in vivo*, as shown by increases in serum immune cytokines, such as IL-2 and IFN-γ levels. It also increases cytotoxic T-cell infiltration in the tumor tissue and thereby delays tumor growth. These results indicate a positive functional correlation between suppressed KITENIN levels in tumor tissues and antitumor T-cell responses *in vivo*.

Semiweekly injections of KITENIN short interfering RNA enhance its antitumor effect. Either 50 μg of both KITENIN siRNA S2 and S5, or 100 μg of pSUPER vector mixed with FuGENE 6, was given i.v. for 4 weeks in BALB/c mice with two injections per week started at 1 week after the injection of CT-26 cells. We compared the tumor volumes of semiweekly injection group with those of vector alone or weekly injection group. Compared with the control group, *in vivo* tumor progression was markedly delayed after the 4th injection of KITENIN siRNA, which was corresponding 2 weeks from the first injection (Fig. 5*A*). Notably, almost complete

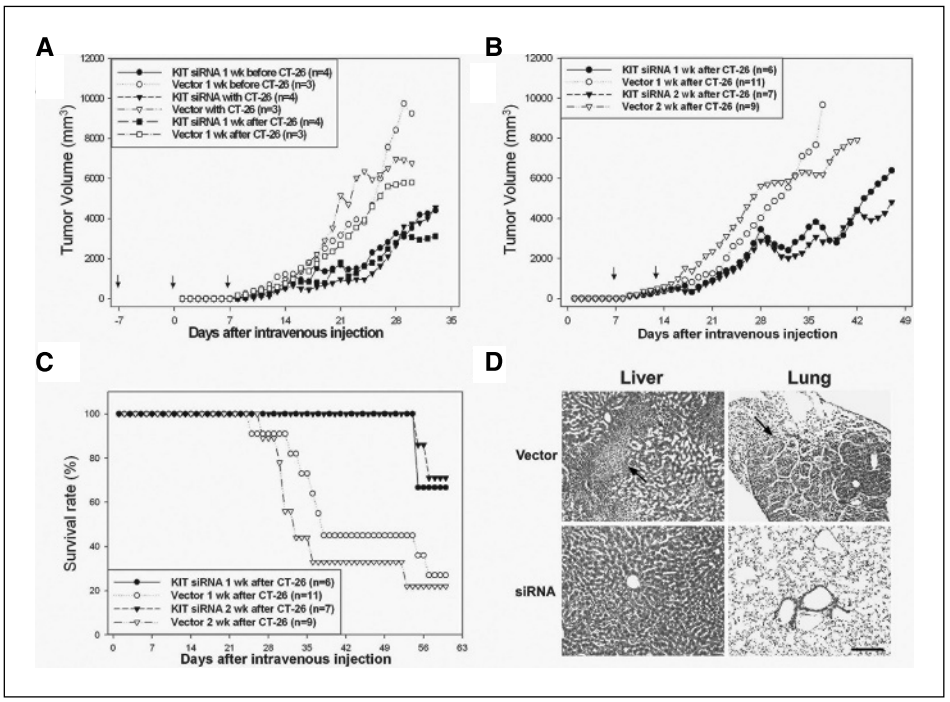

Figure 2. Effect of *in vivo* KITENIN gene silencing on progression of established tumor, survival, and distant metastases in experimental colon cancer of mice. *A,* effect of i.v. pSUPER-KITENIN injection on *in vivo* progression of experimental tumor. A mixture of pSUPER-KITENIN (S2 and S5) with FuGENE 6 was given into the tail vein of mice weekly for 1 month at 1 week before, simultaneously, and 1 week after the injection of CT-26 cells (*arrows*). *Points,* mean tumor volumes. *B,* effect of i.v. pSUPER-KITENIN injection done after tumor formation on progression of experimental tumor. pSUPER-KITENIN was given weekly five times starting at 1 and 2 weeks after the injection of CT-26 cells (*arrows*) in which the tumor was just and already formed, respectively. *C,* survival curves of tumor-bearing mice given pSUPER-KITENIN or vector alone. *D,* pSUPER-KITENIN delivery suppresses distant metastases in the mouse colon cancer model. After 6 weeks, liver and lung metastases were detected in all mice in control group (n = 20, *top, arrows*), whereas liver or lung was still intact in the mice (n = 12 of 13, *bottom*) with pSUPER-KITENIN. *Bar,* 50 μm.

regression of established tumor with removal of crust and new epithelial tissue in the tumor area was observed in two of six mice. Whereas, the entire necrotic tumor mass disappeared from the back skin in two of six mice, and a part of the necrotic tumor mass sloughed from the back in the two remaining mice after the fourth injection of KITENIN siRNA (Fig. 5*B*). Moreover, after 2 weeks, progression of tumor was more suppressed than that of the group receiving four weekly KITENIN siRNA injections (Fig. 5*A*). However, the tumor grew very slowly again in all mice, despite further KITENIN siRNA injections. After the eighth injection of KITENIN siRNA, all mice had regrowing tumor mass, but it is still smaller than that of control group (Fig. 5*C*). This result suggests that the proliferation of tumor cells overwhelms the ability of the host immune response to suppress the tumor growth, or effects of KITENIN siRNA were down-regulated after repeated injections.

90K protein interacts with KITENIN and suppressed KITENIN in the tumor cells augments 90K release. To examine how KITENIN siRNA causes tumor regression and inhibition of metastasis, we searched for proteins capable of association with KITENIN by a yeast two-hybrid screen using the full-length of KITENIN as bait. By screening a mouse testis cDNA library, we isolated and sequenced several positive clones that revealed specific interaction with KITENIN. Two cDNA clones were identified as 90K and PKCI proteins.

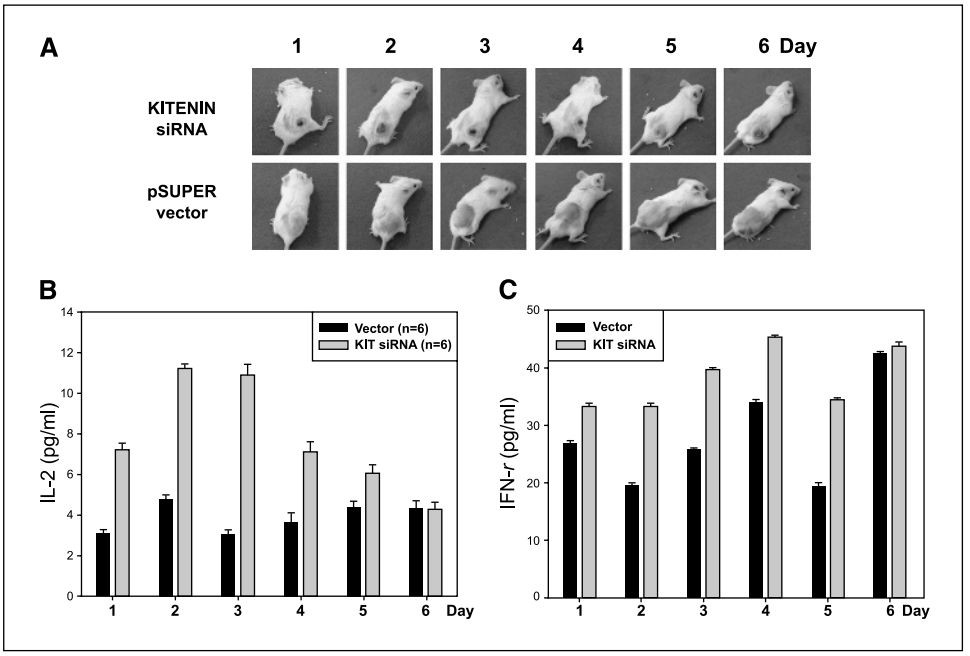

Figure 3. Effects of a single i.v. pSUPER-KITENIN injection on growth of established tumor, and serum cytokine levels of syngeneic mice. *A,* duration of regression effect on the established tumor by a single pSUPER-KITENIN injection. At 2 days after injection, tumor size was decreased and crust formation was observed, but tumor growth started again after 5 days. One of three representative experiments. *B* and *C, in vivo* silencing of KITENIN increases serum immune cytokines. From days 1 to 6 after injection, blood was pooled from two mice after measuring tumor volume, and amounts of various cytokines were measured. *B,* serum IL-2 was higher from 2 days after pSUPER-KITENIN than that of control group and this effect was observed until 5 days. *C,* IFN-γ increased from days 2 to 5 after pSUPER-KITENIN more than that of control group. *Columns,* means (n = 6) for each ELISA; *bars,* ±SE.

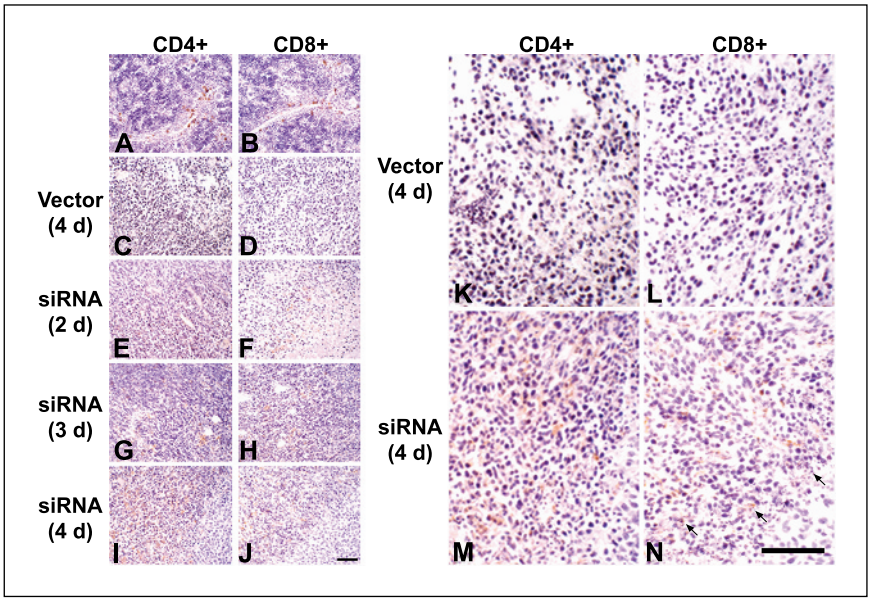

Figure 4. Sequential T-cell infiltration in the established tumor tissue of syngeneic mice following a single i.v. pSUPER-KITENIN injection. Representative images (*A-J*, low-power field; *K-N*, high-power field) of $CD4^+$ (*A, C, E, G, I, K*, and *M*) and $CD8^+$ (*B, D, F, H, J, L*, and *N*) T cells ($n = 3$). A mouse spleen specimen was included as a positive control for $CD4^+$ (*A*) and $CD8^+$ (*B*) T cells. *C-J,* accumulation of $CD4^+$ and $CD8^+$ T cells in the regressed tumor tissues started from 2 days (*E* and *F*), peaked at 4 days (*I* and *J*), and was maintained until 7 days after pSUPER-KITENIN injection compared with those at 4 days after vector injection (*C* and *D*). In high-power field (*K-N*), $CD4^+$ helper (*M*), and $CD8^+$ cytotoxic (*N*) T cells were increased in the pSUPER-KITENIN delivered regressed tumor tissues at 4 days compared with those of vector injection (*K* and *L*). In addition, apoptotic cells containing segmented nuclei (*arrows*) were found around the $CD8^+$ T cells (*N*). *Bar,* 50 μm.

To confirm the interaction of KITENIN with 90K in mammalian cells, we did an immunoprecipitation assay using CT-26 cell lines. Cell lysate and *in vitro* translated 90K (90K·V5, 90K-V5 fusion protein) were reacted with anti-V5 (Fig. 6*A*) or anti-KITENIN antibody (Fig. 6*B*) and blotted with anti-KITENIN or anti-V5 antibody. KITENIN protein (Fig. 6*A*) and 90K protein was observed (Fig. 6*B*). We also confirmed that 90K protein interacts with the extracellular region of KITENIN with pull-down assay (Fig. 6*C*). To analyze the role of the interaction between KITENIN and 90K on a functional *in vivo* antitumor response, stably 90K-expressing CT-26 cell lines were established. CT-26 cells were stably transfected with pcDNA-90K cDNA (CT-26/90K), and also CT-26/90K cells were stably transfected with pREP4-antisense-KITENIN cDNA (CT-26/90K/antisense KITENIN). 90K expression in selected clones was then analyzed by RT-PCR analysis, because 90K protein is secreted into extracellular space with glycosylated form and antibody against human 90K did not detect the mouse protein (data not shown). To examine whether there are associations between 90K levels and KITENIN expression, we measured the quantity of 90K secreted in supernatants of CT-26/90K and CT-26/90K/antisense KITENIN cells. Supernatants were obtained by centrifugation of culture medium collected from CT-26/90K or CT-26/90K/antisense KITENIN cells 2 days after passage. The 90K band ($\sim$90 kDa) was detected in the culture medium of CT-26/90K cells but not from cell extracts (Fig. 6*D*) indicating that newly synthesized 90K·V5 was secreted into the extracellular space. The secreted 90K was markedly increased in the CT-26/90K/antisense KITENIN cells compared with CT-26/90K cells, indicating that 90K secretion is increased by antisense KITENIN. In addition, endogenous 90K secretion was increased significantly in the culture medium of 24 and 48 hours from stably pSUPER-KITENIN S5 transfected KM1214 cells compared with KM1214/vector cells (Fig. 6*E*). These results indicate that reduced KITENIN permits 90K secretion from tumor cells.

Coculture with 90K secreting tumor cells increases interleukin-1 and interleukin-6 secretions in Raw 264.7 cells. To investigate whether 90K protein in the culture medium is also derived from immune T cells as well as nonimmune tumor cells, RT-PCR analysis of 90K mRNA was done in the stably antisense KITENIN–transfected Jurkat cells. *KITENIN* expression decreased as a result of stable antisense KITENIN construct transfection; however, *90K* expression also decreased at the same time (Fig. 7*A*). In addition, 90K was measured in the conditioned medium from stably antisense KITENIN–transfected Jurkat cells. Consistent with

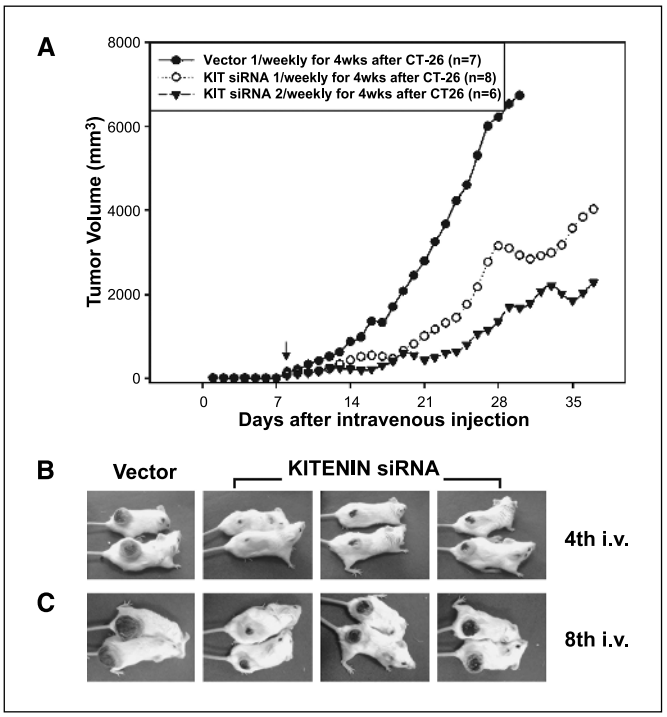

Figure 5. Antitumor effects of pSUPER-KITENIN were enhanced by increasing injection frequency. pSUPER-KITENIN (S2 and S5) or control vector was given twice weekly at 1 week after the injection of CT-26 cells (*an arrow*). *Points,* mean tumor volumes. *A, in vivo* progression of established tumor was markedly suppressed after the fourth injection of pSUPER-KITENIN compared with that of the vector group. *B,* after the fourth injection of pSUPER-KITENIN, the nearly complete regression of established tumor with removed crust in the tumor area was observed in two of six mice. *C,* after the eighth injection of pSUPER-KITENIN, all mice had regrowing tumor mass, which is still smaller compared with that of vector group.

result of the RT-PCR analysis, secretion of endogenous 90K decreased in the culture medium (Fig. 7*B*). These results indicate that 90K protein in the culture medium is derived from nonimmune tumor cells.

To examine whether secreted 90K could augment the immune response, Raw 264.7 cells were double cultured with CT-26/90K or CT-26/90K/antisense KITENIN cells using a Transwell apparatus. After 48 hours, supernatants were harvested and assayed for the accessory cell–derived cytokines, IL-1, IL-2, IL-4, IL-5, IL-6, and IFN-γ. Double culture of accessory cells with CT-26/90K/antisense KITENIN cells revealed that the production of IL-1 (Fig. 7*C*) and IL-6 (Fig. 7*D*) was increased by 2- and 4-fold compared with that of double culture with CT-26/p cells. In contrast, no significant differences were found in IL-2, IL-4, IL-5, and IFN-γ levels between double cultured cells (data not shown). To examine whether the purified 90K protein will also show the similar effects on Raw 264.7 cells, concentration of IL-1 and IL-6 were measured in the cultured medium harvested at 24 hours after adding graded amounts of purified 90K. The amounts of IL-1 (Fig. 7*E*) and IL-6 (Fig. 7*F*) in medium were increased by dose-dependent manner by 90K protein. These results confirmed that suppression of KITENIN induced 90K secretion from CT-26 tumor cells, and secreted 90K protein stimulated accessory cells to increase the secretion of IL-1 and IL-6. In addition, this result of immune cytokine secretions was parallel with that of 90K secretion from genetically engineered CT-26 cells (Fig. 6*B*). However, IL-1 (Fig. 7*C*) and IL-6 (Fig. 7*D*) were increased more in the double culture with CT-26/p cells than those of Raw 264.7 cells only, suggesting that the ability to stimulate the cytokine secretion could not be attributed solely to the 90K protein itself. Nonetheless, we conclude that the culture of accessory cells with 90K protein from tumor cells is sufficient to stimulate the secretion of IL-1 and IL-6.

Combination of 90K expression and KITENIN suppression reduces tumor growth and metastasis *in vivo*. To investigate whether overexpression of 90K affects colon tumor progression, we established cell lines overexpressing 90K with or without expressing antisense KITENIN cDNAs. RT-PCR analysis showed that *90K*

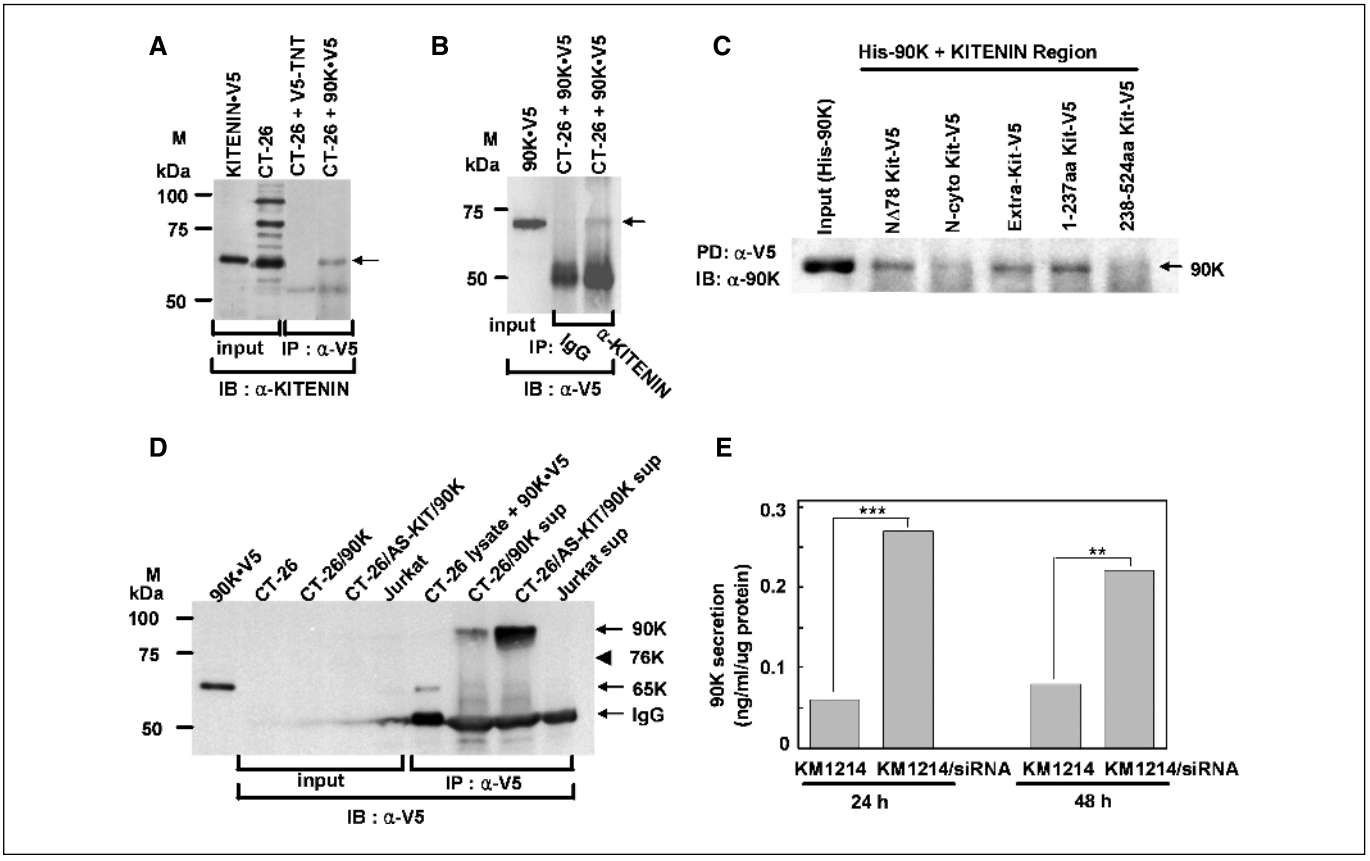

Figure 6. Direct interaction of 90K with KITENIN and the effect of antisense KITENIN on 90K release. *A* and *B,* immunoprecipitation (*IP*) analysis of the 90K in the CT-26 cells. Cell lysate mixed with *in vitro* translated 90K·V5 was incubated with protein A/G agarose beads and anti-V5 (*A*) or anti-KITENIN antibody (*B*). The immunoprecipitates were resolved by SDS-PAGE and blotted with anti-KITENIN or anti-V5 antibody. *Arrow,* ~65-kDa KITENIN (*A*) or *in vitro* translated ~65-kDa 90K·V5 protein (*B*). *C,* identification of KITENIN region required for interaction with 90K. Indicated region of V5-tagged KITENIN protein was purified using *in vitro* translation system and mixed with purified His-90K protein. These mixtures were incubated with protein A/G agarose beads and anti-V5 antibody or control mouse IgG. The immunoprecipitates were blotted with anti-90K antibody. The presence of interactions between 90K and roughly whole region of KITENIN devoid of partial NH_2-terminal region (NΔ78 Kit-V5), and extracellular region of KITENIN (Extra-Kit-V5), and roughly whole region of KITENIN devoid of COOH-terminal region (1-237 aa Kit-V5) indicate that extracellular portion of KITENIN interacts with 90K. N-cyto Kit-V5 is full NH_2-terminal cytoplasmic region; 238-524 aa Kit-V5 is full COOH-terminal cytoplasmic portion. *D,* immunoblot analysis of cell lysate and conditioned medium harvested from stably 90K-transfected CT-26 cells. Each cell lysate was immunoblotted with anti-V5 antibody. In addition, culture medium (*sup*) from each cell was immunoprecipitated with anti-V5 antibody and immunoblotted with anti-V5 antibody. Jurkat cells were included as a negative control for immunoprecipitation. Note that the molecular size of *in vitro* translated 90K (*arrow*) and endogenous precursor (*arrowhead*, in CT-26/AS-KITENIN/90K cells) of the glycosylated 90K protein are ~65 and ~76 kDa, respectively. This 76-kDa protein is not detected in the culture medium. *E,* increased endogenous 90K secretion in the culture medium from stably pSUPER-KITENIN S5–transfected KM1214 cells. **, $P < 0.01$ and ***, $P < 0.001$ (significant difference in 90K secretion between groups).

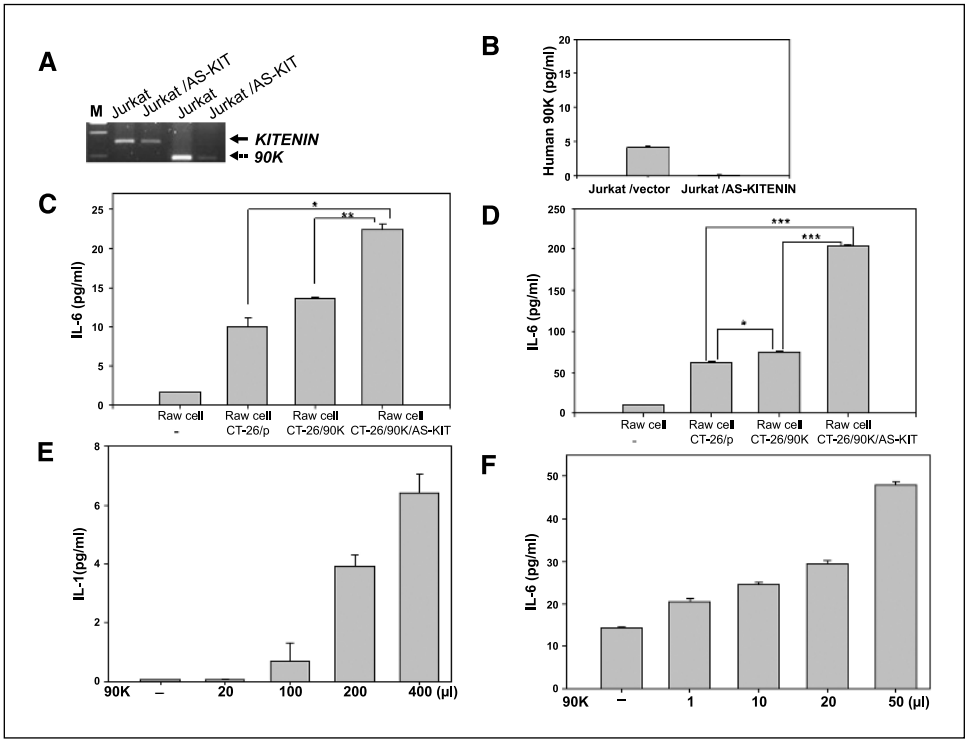

Figure 7. The 90K protein in the culture medium is derived from nonimmune tumor cells and stimulates the secretion of cytokines from accessory cells. *A,* RT-PCR analyses of KITENIN and 90K mRNAs in stably antisense KITENIN–transfected Jurkat cells. *B,* measurement of 90K protein in the conditioned medium from stably antisense KITENIN–transfected Jurkat cells. *C* and *D,* measurement of cytokines in the double-cultured accessory cells/tumor cells. The amounts of IL-1 (*C*) and IL-6 (*D*) in double-cultured medium of Raw 264.7 cells and CT-26/AS-KITENIN/90K cells were increased significantly more than those of Raw 264.7 cells and CT-26/p cells or Raw 264.7 cells and CT-26/90K cells. *Columns,* means (n = 3) for each ELISA; *bars,* ±SE. *, $P < 0.05$ (significant difference in IL-1 or IL-6 secretion between groups). *E* and *F,* measurement of IL-1 or IL-6 in the accessory cells by treatment of purified 90K protein from supernatant of CT-26/90K/AS-KITENIN cells. The amounts of IL-1 (*E*) and IL-6 (*F*) in the cultured medium of Raw 264.7 cells were increased after adding graded amounts (μL) of 90K. *Columns,* means (n = 3) for each ELISA; *bars,* ±SE.

increased in the CT-26/90K and CT-26/antisense KITENIN/90K cells (Fig. 8*A*). To examine the effects of 90K expression with or without suppressed KITENIN on *in vivo* tumor growth, CT-26/p, CT-26/90K, CT-26/antisense KITENIN, and CT-26/90K/antisense KITENIN cells were injected s.c. in syngeneic mice at a density of 1×10^6 cells. All of the mice inoculated with above CT-26 cells formed tumors; the mice inoculated with CT-26/90K/antisense KITENIN cells showed the most delayed tumor growth among them (Fig. 8*B*). However, tumor sizes obtained from CT-26/90K cells were similar to those from CT-26/p cells, indicating that released 90K from CT-26/90K cells was not sufficient to suppress tumor growth. In addition, the mice inoculated with CT-26/90K cells (Fig. 8*C, middle columns*) did exhibit lung and liver metastases at 4 and 6 weeks after injection, respectively, just like the mice with CT-26/p cells (Fig. 8*C, top columns*), indicating that released 90K alone does not inhibit metastasis. However, there were no metastatic foci in the lung and liver of the mice with CT-26/antisense KITENIN/90K cells (Fig. 8*C, bottom columns*). Therefore, we confirmed that the association between 90K protein and KITENIN is responsible for immune augmentation and thereby for the regression of established tumor observed but not for the metastasis.

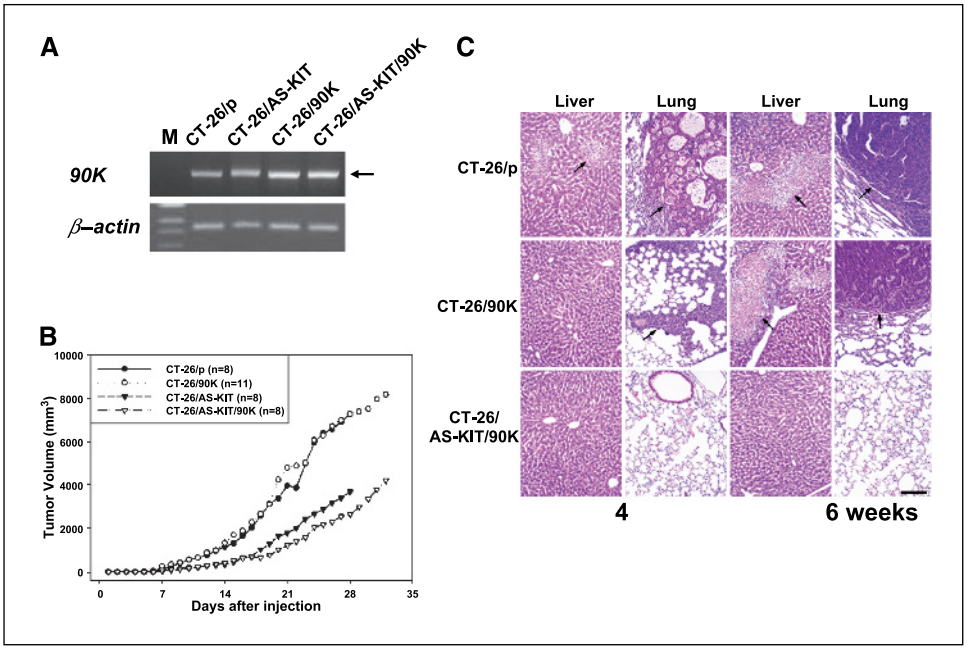

Figure 8. Effect of 90K expression in addition to suppressed KITENIN on *in vivo* tumor progression and metastases. *A,* establishment of cells expressing 90K with or without AS-KITENIN cDNAs. RT-PCR analysis showed that *90K* expression (*arrow*) was higher in the CT-26/90K and CT-26/AS-KITENIN/90K cells than corresponding cells. *B,* effect of 90K expression with or without reduced KITENIN on *in vivo* tumor progression. Tumor growth was induced by s.c. injection of CT-26/p, CT-26/90K, CT-26/AS-KITENIN, or CT-26/AS-KITENIN/90K cells in BALB/c mice (n = 8 or 11 per group). *Points,* mean tumor volumes. *C,* expression of 90K alone did not influence the distant metastases in mouse tumor model. Four weeks after cell injection (n = 12 per group), lung metastasis was detected in mice injected with CT-26/parent cells (*top left, arrows*) and with CT-26/90K cells (*middle left*) but not with CT-26/AS-KITENIN/90K cells (*bottom left*). Six weeks after injection, liver and lung metastases were still not detected in mice injected with CT-26/AS-KITENIN/90K cells (*bottom right*) but with CT-26/90K cells (*middle right*). *Bar,* 50 μm.

KITENIN interacts with protein kinase C inhibitor, and protein kinase C inhibitor increases invasiveness of CT-26 cells *in vitro*. For immunoprecipitation to confirm the interaction of KITENIN with PKCI, CT-26 cell lysate or CT-26 cell lysate mixed with *in vitro* translated PKCI was reacted with anti-KITENIN (Fig. 9*A*) or anti-V5 antibody (Fig. 9*B*) and blotted with anti-PKCI or anti-KITENIN antibody. PKCI protein or KITENIN protein appeared as a ~13-kDa (Fig. 9*A*) or a ~65-kDa band (Fig. 9*B*), indicating an association of PKCI with KITENIN in mammalian cells. In subsequent pull-down assay, we confirmed that the COOH-terminal region of KITENIN interacts with PKCI protein (Fig. 9*C*).

The forced expression of KITENIN in the CT-26/p cells resulted in the increased expression of PKCI, whereas the expression of PKCI protein was reduced in the CT-26/antisense KITENIN cells compared with CT-26/vector cells (Fig. 9*D*). This result indicates that KITENIN directly or indirectly causes the increased expression of PKCI. Transwell invasion assays were used to investigate whether the PKCI affects the *in vitro* invasive potential of CT-26 cells, as KITENIN does. Cell motility was measured using fibronectin as a chemotactic factor. We established CT-26 cell lines overexpressing PKCI or stably expressing antisense PKCI cDNAs. Western blot analysis showed that PKCI expression was increased in the CT-26/PKCI cells, whereas reduced in the CT-26/antisense PKCI cells compared with CT-26/vector cells (Fig. 9*E*). Compared with CT-26/vector cells, CT-26/PKCI cells showed significantly increased *in vitro* motility and invasive potential induced by fibronectin, whereas significantly decreased in CT-26/antisense PKCI cells (Fig. 9*F*). This result indicates that PKCI is involved in the invasiveness of CT-26 cells into extracellular matrix.

Ablation of KITENIN induces a distorted actin phenotype. Previously, we found that the transfection of antisense KITENIN cDNA into CT-26/p or CT-26/KAI1 cells was associated with development of a longer process than on CT-26/p or CT-26/KITENIN cells (11, 14). Thus, we speculated that the morphologic changes caused by reduced KITENIN might be caused by changes in the cytoskeleton. Therefore, we examined the cellular morphology of CT-26/antisense KITENIN cells by confocal microscopy. Western blot analysis showed that KITENIN expression was nearly absent in the CT-26/antisense KITENIN cells (Fig. 10*A*). In CT-26/vector cells, actin was arranged in a radial pattern from the nucleus to the periphery of the cytoplasm (Fig. 10*B*) and showed a continuous filamentous shape (Fig. 10*C*). However, in the antisense KITENIN–transfected CT-26 cells, the arrangement of filamentous

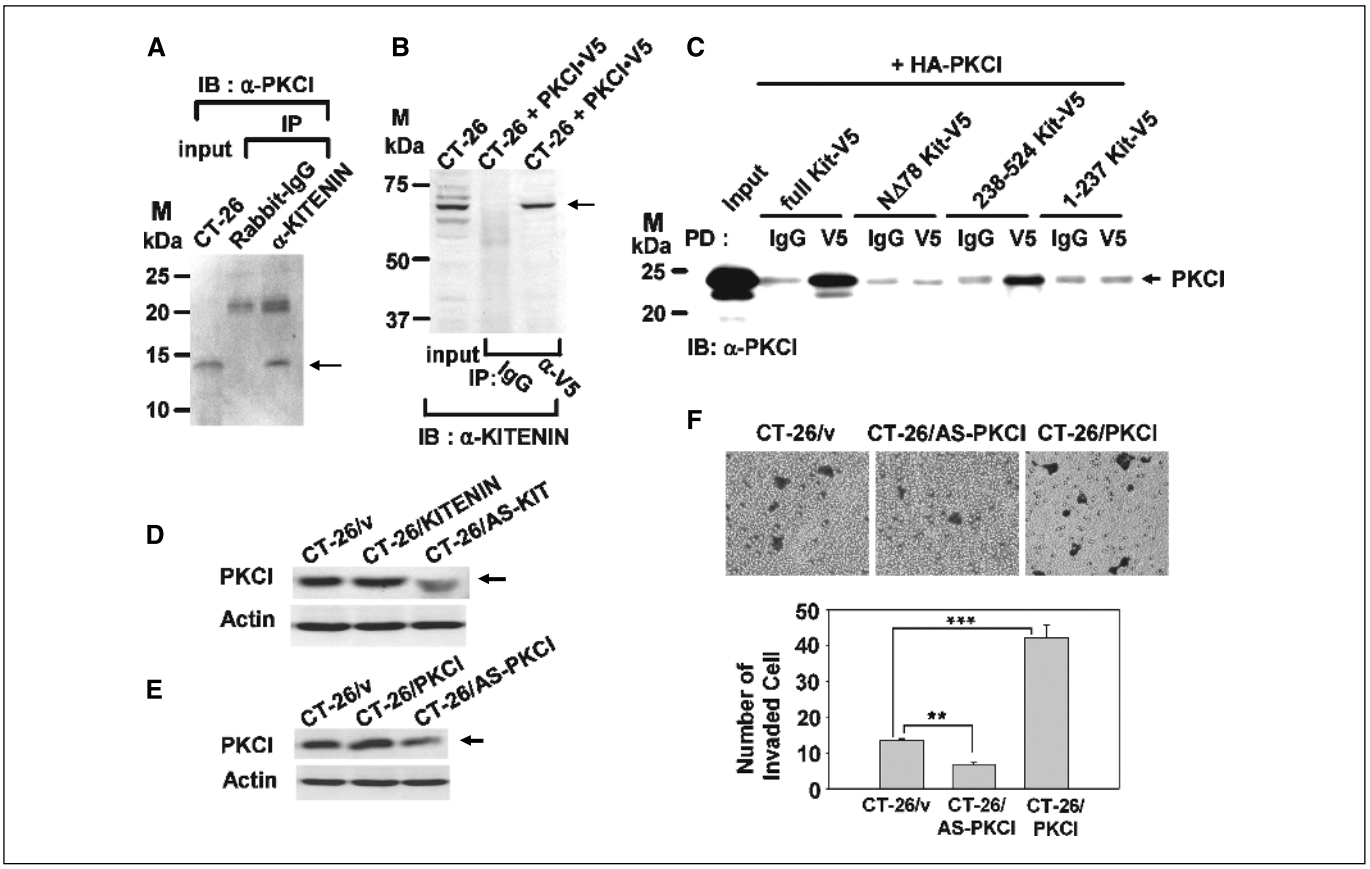

Figure 9. Direct interaction of PKCI with KITENIN and PKCI involvement in the invasiveness of tumor cells. *A* and *B,* immunoprecipitation (*IP*) analysis of PKCI that interacted with KITENIN. CT-26 cell lysate was incubated with protein A/G agarose beads and anti-KITENIN antibody (*A*) or with PKCI·V5 protein, protein A/G agarose beads, and anti-V5 antibody (*B*) and blotted with anti-PKCI or anti-KITENIN antibody. *Arrow,* ~15-kDa PKCI (*A*) or the ~65-kDa KITENIN (*B*). *C,* identification of KITENIN region required for interaction with PKCI. Indicated region of V5-tagged KITENIN protein was mixed with purified HA-PKCI protein. These mixtures were incubated with protein A/G agarose beads and anti-V5 antibody or control mouse IgG. The immunoprecipitates were blotted with anti-PKCI antibody. *Arrow,* ~23-kDa HA-PKCI protein. The presence of strong interaction between PKCI and 238-524 Kit-V5 indicates that PKCI interacts with COOH-terminal cytoplasmic region of KITENIN. *D,* relationship between expression levels of KITENIN and PKCI. The expression level of PKCI (*arrows*) was examined in KITENIN-expressing CT-26 cell lines. *E,* establishment of CT-26 cell lines expressing changed PKCI. *F,* effect of PKCI expression on *in vitro* invasiveness of colon cancer cells. Compared with CT-26/vector cells, CT-26/PKCI cells had greater invasive potential induced by fibronectin, whereas lesser in CT-26/AS-PKCI cells (*top*). *Columns,* mean of the mean number of migrated cells per field; *bars,* ±SE (*bottom*). *, significant difference in cell migration among the CT-26 cell groups.

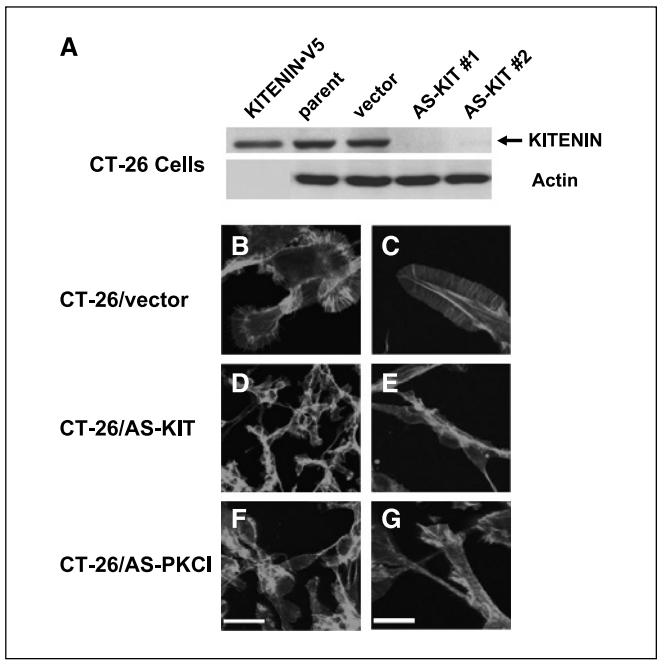

Figure 10. Effects of antisense KITENIN and antisense PKCI cDNA transfection on microfilament arrangement. *A,* establishment of cell lines expressing AS-KITENIN. KITENIN (*arrow*) was nearly absent in the CT-26/AS-KITENIN cells. *B-G,* actin in CT-26 cells was visualized. Control vector–transfected CT-26 cells (*B* and *C*), anti-KITENIN cDNA–transfected cells (*D* and *E*), and anti-PKCI cDNA–transfected cells (*F* and *G*). Note that the absence of KITENIN expression disturbs the arrangement of the actin (*D*) and causes distortion (*E*), and that decreased PKCI expression also disturbs the arrangement of the actin (*F*) and causes distortion (*G*), just as in the CT-26/AS-KITENIN cells. *Bars,* 30 μm (*B, D,* and *F*) and 75 μm (*C, E,* and *G*).

actin was disrupted and distorted (Fig. 10*D* and *E*). Thus, decreased KITENIN seems to be related to the distorted actin, which may partially explain the absence of distant metastasis observed in the mice injected with CT-26/antisense KITENIN cells.

Decreased PKCI also disrupts actin fiber arrangement. Because decreased KITENIN was related to the expression of distorted actin, we also examined the cellular morphology of CT-26/antisense PKCI cells (Fig. 9*E*) by confocal microscopy. Compared with CT-26/vector cells, the actin fiber arrangement was markedly disrupted and distorted after antisense PKCI transfection (Fig. 10*F* and *G*), just like in CT-26/antisense KITENIN cells.

Discussion

KITENIN is expressed by many different tumors, and its expression correlates with the acquisition of metastatic phenotypes (11). In this study, we observed that weekly or semiweekly injection of pSUPER-KITENIN for 1 month markedly inhibited the growth of established tumors and suppressed the distant metastases to liver and lung. Secretion of 90K, a KITENIN-interacting protein, was higher in culture medium from the antisense KITENIN cDNA–transfected CT-26 and KITENIN siRNA–transfected KM1214 colon cancer cells but not from the immune Jurkat/antisense KITENIN cells. However, normal tumor growth was observed during 2 weeks after injection of CT-26 cells in mice in which i.v. KITENIN siRNA delivery was started at 1 week before the tumor challenge, whereas tumor size had regressed after the fourth injection given at 2 weeks after the tumor challenge. These results suggested that 90K was released from established growing tumor tissue by KITENIN siRNA and might be associated with antitumor action of *in vivo* KITENIN ablation.

The 90K tumor-derived glycoprotein has been shown to have positive effects on the generation of cytotoxic effector cells from human PBMC, such as natural killer cells and lymphokine-activated killer cells. 90K is a soluble costimulatory molecule that acts at the level of the accessory cell, indirectly supporting T-cell activation through expression of adhesion molecules as well as on cytokine production (9). It binds to β1-integrin, collagen, and fibronectin as well as galectin-1, galectin-3, and galectin-7 (7, 16–18). Based on these reports, it seems that the released 90K protein from extracellular portion of KITENIN following KITENIN siRNA injection could be part of the host defense mechanisms facilitating the elimination of established growing tumors. To determine whether local production of 90K from CT-26 tumor cells causes immune reactions, we have examined the effects of 90K on cytokine production in Raw 264.7 cells, a mouse macrophage cell line. Coculture of Raw 264.7 cells with CT-26/antisense KITENIN/90K cells or treatment of Raw 264.7 cells with purified 90K protein revealed increased secretion of IL-1 and IL-6, which was also observed in a previous report (9). IL-1 supports the antigen-driven activation of T cells (19) and the production of lymphokines such as IL-9 (20). IL-6 exhibits a number of effects on T-cell activation, including stimulation of thymocyte proliferation, enhancement of cytotoxic T-cell differentiation in mixed lymphocyte reactions, and T-cell activation in conjunction with TCR cross-linking or mitogen stimulation (21). Increased secretion of proinflammatory cytokines such as IL-1 and IL-6 by 90K after KITENIN siRNA could result in a greater degree of infiltrating activated T-helper and effector cells. Indeed, we observed increased secretion of IL-2 and IFN-γ in the peripheral blood, and infiltration of $CD8^+$ as well as $CD4^+$ T cells in the regressed tumor tissues from mice injected with pSUPER-KITENIN.

In contrast, our results suggest that, after injection of KITENIN siRNA into syngeneic mice, nonimmune tumor cells or normal cells that express KITENIN (7) may initiate to increase the T-cell effector functions by secretion of 90K through ablating KITENIN expression, and that this mechanism may contribute to the generation of a tumor-specific T1-type response and thereby may serve as effective inhibitors to the spread of tumors within the host. Recently, rat 90K was reported to increase MHC class I, and both 90K and MHC class I were coordinately increased by introducing double-stranded polynucleotides into FRTL-5 rat thyroid cells, which support a role of 90K in immune self defense mechanisms of nonimmune cells (22).

However, 90K also possess the negative influence on the prognosis of various cancers (10). Several studies have shown that high 90K levels are associated with a poor prognosis and the presence of metastasis in breast cancer (23) and non–small cell lung cancer (24). It seemed that the negative influence of 90K on prognosis was related to binding with galectins, and that malignant cell transformation and cancer progression are associated with increased expression of galectin-1, galectin-3, and 90K (25). To explain these contradictory observations, it should be examined whether 90K protein is also produced by host cells in response to tumor progression and this endogenous 90K acts as ligands to tumor cells by binding to extracellular portion of KITENIN rather than acts to accessory cells, because the different origin of the protein may in turn have a differential influence on the course of various cancers.

The final result of an effective antitumor response can be determined by a delicate interaction between activating and

inhibitory regulatory pathways and that removal of inhibitory signals may be a particularly useful adjunct to other anticancer therapies (26). Interestingly, inhibition of cell proliferation was observed after transient transfection of KITENIN into Raw or Jurkat cells, which express a very low KITENIN level (data not shown). Thus, our results suggest that KITENIN may act as one of the negative regulators of T-cell activation and survival and confer immune privilege to tumor cells, whereas blockade of KITENIN *in vivo* stimulates the generation of tumor-specific type 1 T cell–mediated immune responses in syngeneic mice and thereby suppresses tumor cell proliferation. In addition, the mice inoculated with CT-26/90K/antisense KITENIN cells showed more delayed tumor growth than the mice with CT-26/antisense KITENIN, confirming that 90K protein is really responsible for immune augmentation by KITENIN siRNA injection. Therefore, our data provided evidences of anti-KITENIN/90K-mediated immunostimulation as a novel mechanism of tumor regression and indicated that ablation of KITENIN in the growing tumor cells by systemic delivery of anti-KITENIN siRNA has strong therapeutic potential to shift the balance in favor of an efficient T cell–mediated antitumor immune response.

In this study, we showed that rearrangement of actin and increased *in vitro* invasiveness to fibronectin are associated with KITENIN and PKCI. Thus, PKCI seems to be involved in the metastasis enhancing property of KITENIN. The PKCI protein was originally identified by its ability to inhibit the *in vitro* kinase activity of bovine brain PKC (27). However, previous study suggests that *in vivo* the ubiquitously expressed PKCI protein, although localized mainly in the nucleus, does not function as an inhibitor of PKC but rather acts as an enzyme in an unidentified pathway (28) or a regulator of gene transcription (29–31). Recently, it was reported that $PKCI^{-/-}$ mouse embryo fibroblasts display spontaneous immortalization during serial passage (32). However, it is unknown in this study how the interaction of KITENIN, membrane protein, and PKCI, the major function of which may reside in the nucleus, is related to increased invasiveness of tumor cells. This study indicates that released 90K alone is not involved in the inhibition of the distant metastasis by KITENIN ablation. Thus, we suppose that other signal transduction pathways should exist to link KITENIN with cytoskeletal proteins as well as balancing the KAI1's action by KITENIN, and that KITENIN activates this signaling pathway involving PKCI. These interactions may result in the formation of multimeric protein complexes that influence downstream signaling effectors involved in cell motility and invasion.

Acknowledgments

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