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Reduced Invasive and Metastatic Potentials of *KAI1*-transfected Melanoma Cells

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***KAI1* is a metastasis suppressor gene for human prostate cancer. To reveal the effect of KAI1 on the *in vivo* metastasis of tumors other than prostatic cancer, we transfected a human KAI1 cDNA into highly metastatic B16-BL6 murine melanoma cells and established stable transfectant clones with different expression levels of KAI1 message. The following results were obtained with the use of those transfectants. (1) Cell aggregation assay revealed a significantly enhanced Ca^{2+}-independent aggregation of B16-BL6 cells by KAI1 cDNA transfection compared with mock transfectants ($P<0.01$). (2) The *in vivo* phagokinetic activity and invasive ability of KAI1 transfectants were clearly decreased as compared with those of mock transfectants ($P<0.01$). There was no significant effect of KAI1 expression on the *in vitro* or *in vivo* proliferation of B16-BL6 cells. (3) Lung colony formation of intravenously injected KAI1 transfectants in nude mice was significantly reduced as compared with mock transfectants or parental B16-BL6 cells ($P<0.01$). These data suggest that KAI1 expression gives rise to the suppression of invasive and metastatic potentials of B16-BL6 cells.**

Key words: *KAI1* gene — Invasion — Metastasis — Cell adhesion

KAI1 has recently been cloned as a metastatic suppressor gene for human prostate cancer, and has been mapped to human chromosome 11p11.2.[1)] The gene transfer of *KAI1* into rat AT6.1 prostate cancer cells resulted in a significant suppression of the pulmonary metastases with no effect on the growth rate of the primary tumor. The expression level of KAI1 mRNA in human cell lines derived from metastatic prostate cancers was undetectable or much smaller in amount as compared with that in normal prostate tissue. An immunohistochemical study showed that the expression of KAI1 protein was negatively related to the advanced clinical stage of prostate cancer.[2)] *KAI1* encodes a protein of 267 amino acids, which contains four hydrophobic, presumably membrane-spanning segments and a single major putative extracellular domain with three potential *N*-glycosylation sites. Thus, KAI1 belongs to a structurally distinct family of membrane glycoproteins named "transmembrane 4" (TM4) or "tetra spans transmembrane" family,[3–6)] of which most of the known members have been identified as leukocyte surface proteins.[4)] The *KAI1* gene was found to be identical to three cDNAs obtained from human leukocytes designated as R2,[7)] C33,[8, 9)] IA4[5)] and 4F9,[10)] which have been classified as CD82. Although several recent studies employing mAbs have suggested that some members of this family play putative roles in the regulation of cell aggregation and proliferation,[11–13)] the mechanism of metastasis suppression remains to be fully elucidated. KAI1 is expressed ubiquitously, not only in prostate tissue, but also in a wide variety of other tissues,[1)] suggesting that KAI1 may play a role in suppressing metastases of other tissue-derived cancers.

The aim of the current study was to address the issue of a suppressive effect of KAI1 on the invasive and metastatic properties of tumor cells other than prostatic cancer. We have introduced a KAI1 cDNA into highly metastatic murine melanoma cells, B16-BL6. The resultant phenotypic changes of the stable transfectants *in vitro* and *in vivo* were examined and a suppressive effect of KAI1 on the invasion and metastasis of these cells was demonstrated.

MATERIALS AND METHODS

Tumor cell lines and culture conditions B16-BL6, a murine melanoma cell line with high lung-metastasizing ability, was a generous gift from Dr. I. J. Fidler (Houston, TX) and was maintained in Iscove's modified Dulbecco's medium (Gibco-BRL, Grand Island, NY) containing 10% FBS. Cell cultures were maintained and incubated in 5% CO_2-95% air at 37°C.

RT-PCR and Southern blot analysis of KAI1 mRNA expression of stable clones According to the nucleotide sequence of the R2 cDNA identified by Gaugitsch *et al.*,[7)] specific PCR primers were designed to recognize the full coding region of KAI1 cDNA: forward "*Hin*dIII-MGSACIK" primer, 5′-CC-AAGCTT-ATG-GGC-TCA-GCC-TGT-ATC-AAA-G-3′, primer 1; reverse "*Hin*dIII-

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The abbreviations used are: RT-PCR, reverse transcriptase-polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; mAb, monoclonal antibody.

TCA-YKPVKS" primer, 5′-CC-AAGCTT-TCA-GTA-CTT-GGG-GAC-CTT-GCT-G-3′, primer 2. One microgram of total RNA was reverse-transcribed with random nanomers and avian myeloblastosis virus RT using an RT-PCR kit (Takara, Otsu) following the conditions recommended by the manufacturer. The template cDNAs were amplified with Taq polymerase in the presence of primers 1 and 2 by using a standard PCR protocol.[14] The thermocycling parameters used in the PCR were as follows: denaturation, 30 s at 94°C; anealing, 30 s at 60°C; extension, 1.5 min at 72°C. These reactions were repeated for 30 cycles. The PCR products were electrophoresed through a 2% agarose gel and blotted onto nylon membranes (Hybond-N, Amersham, Arlington Heights, IL) in alkaline solution (0.25 *M* NaOH, 0.4 *M* NaCl) by capillary transfer. The full-length KAI1 cDNA probe was labeled with [α-^{32}P]dCTP using a random primer labeling kit (Takara). The filters were hybridized with ^{32}P-labeled KAI1 cDNA probe as previously described,[15] and exposed to Kodak XAR films at −70°C with an intensifying screen. Similarly, β-actin was amplified and hybridized with ^{32}P-labeled β-actin cDNA probe to provide an internal control.

Constructs The eukaryotic expression vector (pRc/CMV) was obtained from Invitrogen (San Diego, CA). The vector contains the CMV promoter, which drives high-level expression of mammalian genes, and the neomycin-resistance gene as a selectable marker. In order to clone the full-length cDNA encoding human KAI1 into the expression vector pRc/CMV, the entire coding region of KAI1 cDNA was amplified from total RNA derived from normal colonic mucosa by RT-PCR using primers 1 and 2 as described above. The resulting 804 bp PCR product was digested with *Hin*dIII, and subcloned into the *Hin*dIII site of pRc/CMV vector. Confirmation of the constructs was obtained by dideoxy sequencing.

Transfection procedures and selection of stable clones Sense cDNA of KAI1 was introduced into B16-BL6 cells. Transfections were initiated following the Lipofection (Gibco-BRL) technique as described previously.[15] Following transfection, surviving clones in the selective media containing 700 μg/ml Geneticin (Gibco-BRL) were characterized by RT-PCR analysis for their expressions of KAI1 mRNA, and clones with different levels of KAI1 expression were used for the functional experiments described. Similarly, a clone transfected with the vector (pRc/CMV) alone was used as the control.

Cell aggregation assay Cell aggregation experiments were carried out following the procedure of Rojas *et al*.[16] Adherent tumor cells were made into single cell suspensions by a 5-min incubation at 37°C with 5 m*M* EDTA in PBS lacking Ca^{2+} and Mg^{2+}, and by 2 passes through a 27-gauge needle. Twenty-four-well plates were used for the assay of cell aggregation. The cells were washed three times with Puck's saline (5 m*M* KCl, 140 m*M* NaCl, 8 m*M* $NaHCO_3$, pH 7.4), resuspended at a concentration of 1×10^5 cells/ml in 1 ml of Puck's saline plus 0.8% FBS and 50 U/ml deoxyribonuclease I (Sigma Co., St. Louis, MO), the addition of which prevents non-specific aggregation mediated by DNAs derived from dead cells, and then incubated in an atmosphere of 5% CO_2-95% air at 37°C with agitation at 70–80 rpm using a gyratory shaker. Samples were taken at 1 h and 2 h after incubation, and both the total cell number (A) and the number of cells remaining as single cells (B) were counted under a microscope on 3 predetermined fields. The results are shown as the percentage of cells that formed aggregates; (A−B)/A×100 (%). To determine the Ca^{2+} dependence of cell aggregation, cells were resuspended at 1×10^5 cells/ml of Puck's saline plus 0.8% FBS and 50 U/ml DNase I with either 5 m*M* Ca^{2+} or 0 m*M* Ca^{2+}. Aggregation assays were performed in the same manner as described above.

Phagokinetic track motility assay Cell motility was determined on the basis of phagokinetic tracks on a gold particle-coated plate. Briefly, uniform carpets of gold particles were prepared on cover slips coated with bovine serum albumin as described by Albrecht-Buehler.[17] The cover slips were rinsed extensively to remove nonadhering gold particles before cell plating. Colloidal-gold-coated coverslips were placed in 35-mm tissue culture dishes containing 2 ml of culture medium, and then 5×10^3 freshly trypsinized cells were added to each plate and left in an incubator for 24 h. Photographs were taken, and the areas from which gold particles had been cleared by a single cultured cell were traced on semitransparent paper of uniform thickness. The tracings (as well as paper squares representing known areas) were then cut out and weighed. The particle-free areas of at least 40 individual cells were measured, and the average value was calculated.

Cell invasion assay This assay was performed based on the methods described by Albini *et al.*,[18] Repesh,[19] and Parish *et al*.[20] We used 6-well Biocoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA) that consist of Cell Culture Inserts (Falcon, Oxnard, CA) containing an 8 mm pore size polycarbonate membrane that has been coated with a solubilized tissue basement membrane "Matrigel" (Collaborative Research, Bedford, MA) at 100 μg/cm^2. For quantification, cells that had migrated to the lower surface were counted under a microscope in 5 predetermined fields at a magnification of ×200.

MTT assay *In vitro* cell growth was measured using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method.[21, 22] The ratio of absorbance at 590 nm to absorbance at 630 nm (reference wavelength) was measured by a multiwell scanning spectrophotometer (Titertek Multiskan, Flow Laboratories).

***In vivo* growth and experimental metastasis assay** To investigate the effect of KAI1 on *in vivo* growth rate,

1×10^6 cells in 0.1 ml of HBSS (Gibco BRL) were injected subcutaneously into the flank region of nude mice (n=5 per transfectant). Tumor sizes (as maximum diameter) were monitored 10 and 20 days after inoculation of tumor cells.

To produce experimental pulmonary metastasis, unanesthetized BALB/c nude mice were injected with 1×10^4 viable tumor cells suspended in 0.1 ml of HBSS into the lateral tail vein. After three weeks, the mice were autopsied, and the lungs were removed, washed, and fixed in neutralized 10% formaldehyde/PBS. The number of tumor colonies in each lung was determined under a dissecting microscope.

Statistical evaluation Differences between control and experimental groups were evaluated by means of Student's *t* test, for which the Statworks program was used. The criteria of statistical significance were set at the 95% (P<0.05) and 99% (P<0.01) levels.

RESULTS

Transfection of KAI1 cDNA and selection of stable clones To clarify whether expression of *KAI1* gene plays a suppressive role in the *in vivo* metastatic process of tumor cells, we transfected a KAI1 cDNA into B16-BL6 cells, a highly metastatic melanoma cell line which offers a representative experimental metastatic model. Fig. 1 showed the expression of KAI1 mRNA in clones randomly selected on the basis of neomycin resistance. Of those KAI1 transfectants, clone no. 2 (Fig. 1, lane 2) with

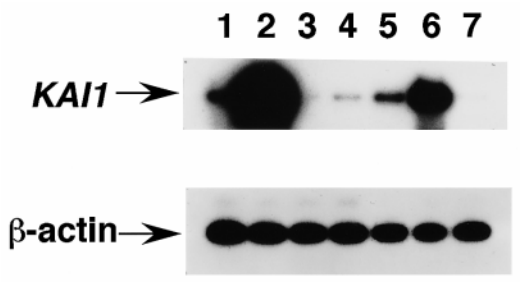

Fig. 1. Detection of KAI1 mRNA in KAI1 and mock transfectants by RT-PCR. Each amplified product was electrophoresed on a 2% agarose gel. After transfer to nylon membranes, the filter was hybridized with a ^{32}P-labeled full-length human KAI1 cDNA as a probe, and exposed to an X-ray film. The result showed the 804 bp band predicted for primer pair 1 and 2, as described in "Materials and Methods." The upper panel shows the results for clones of B16-BL6 cells transfected with KAI1 cDNA (lanes 1 to 6) and with vector (pRc/CMV) alone (lane 7). Expression of β-actin mRNA is shown in the lower panel to control for equal RNA amounts.

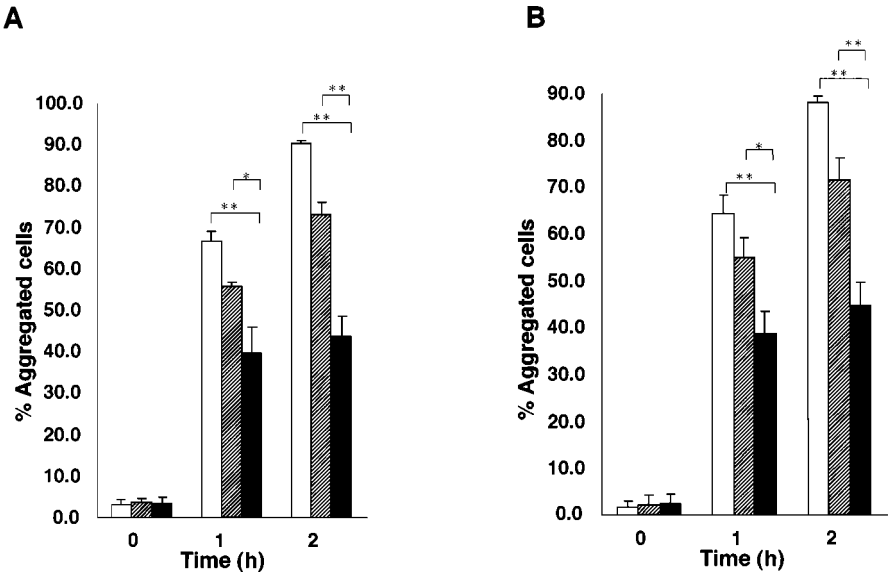

Fig. 2. Effect of *KAI1* gene expression on cell aggregability of B16-BL6 melanoma cells. KAI1 or mock transfectants (1×10^5 cells) were incubated in Puck's saline in the presence (A) or absence (B) of 5 mM Ca^{2+}. The degree of aggregate formation was examined under a microscope, and the result was expressed as a percent of total cell numbers forming aggregates at determined time points, as indicated. The percent values represent the mean±SD of triplicate determinations. Application of Student's *t* test revealed significant differences between KAI1 transfectants and control cells (*P<0.05, **P<0.01). Open column, BL6.KAI-H; oblique-lined column, BL6.KAI1-L; closed column, BL6.CMV-CON.

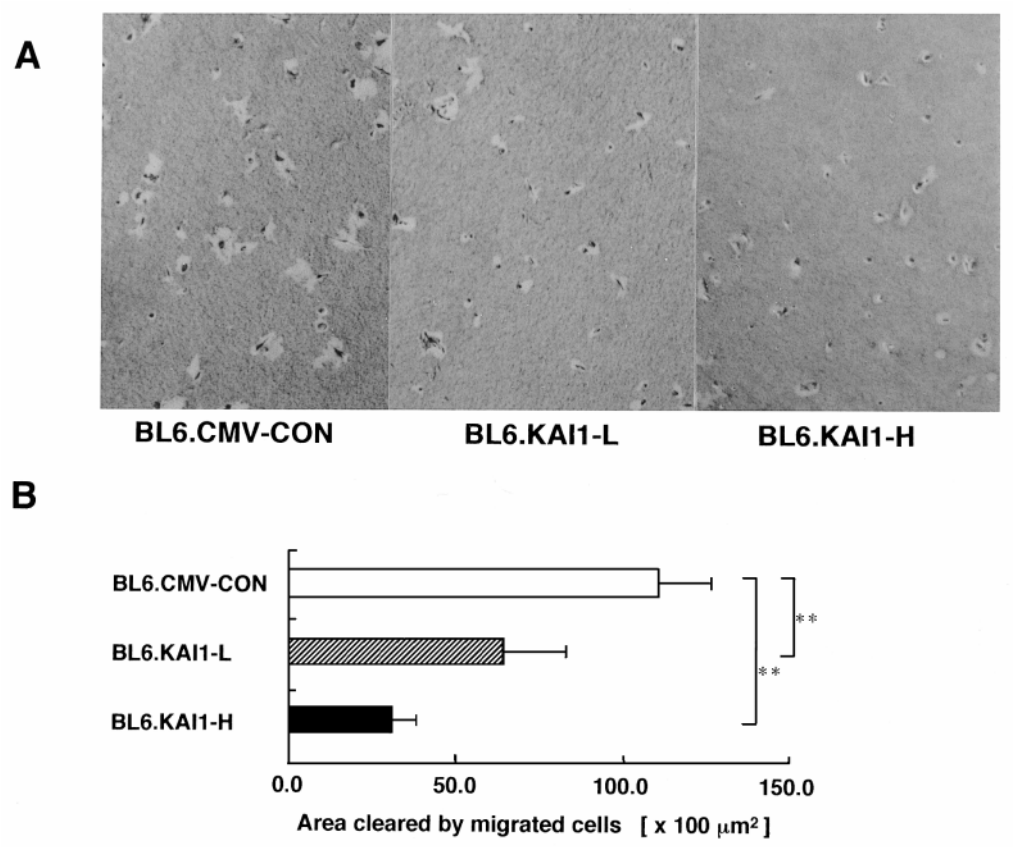

Fig. 3. Effect of *KAI1* gene expression on *in vitro* motility of B16-BL6 melanoma cells as determined by phagokinesis in a colloidal gold-clearing assay. Transfectants were seeded onto colloidal gold-coated coverslips in 35-mm culture dishes and incubated for 24 h. Then, the tracks produced by individual cultured cells were photographed, and the particle-free areas of 40 cells were measured. (A) Representative photomicrographs (original magnification ×40) for each clone (BL6.CMV-CON, BL6.KAI1-L and BL6.KAI1-H) are displayed. (B) The results of the phagokinetic track assay (means±SD, n=40) were plotted. The difference is statistically significant (**P<0.01).

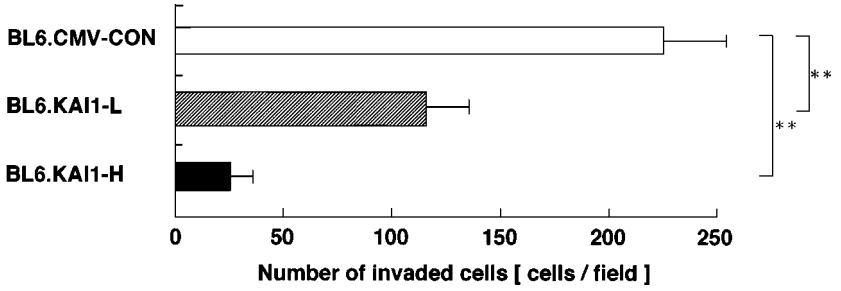

Fig. 4. Effect of *KAI1* gene expression on *in vitro* invasiveness of B16-BL6 melanoma cells. The cells (1.5×10^4) were incubated using Transwell chambers equipped with reconstituted basement membrane-coated filters. After 24 h, the number of cells which had migrated through the membrane and stuck to the lower surface of the filter was counted under a microscope in 5 pre-determined fields at a magnification of ×200. The bars represent the standard deviation of the mean of three independent experiments. Asterisks indicate a significant decrease (**P<0.01) in the invasive ability of KAI1 transfectants as compared with control cells.

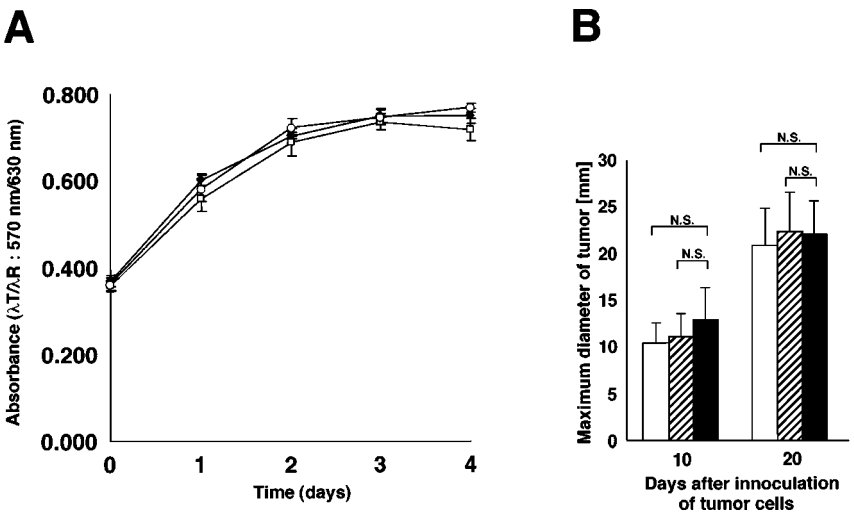

Fig. 5. Effect of *KAI1* gene expression on *in vitro* (A) and *in vivo* (B) growth rates of B16-BL6 melanoma cells. (A) Viable tumor cells (1×10^4 per well) were seeded in 96-well tissue culture plates and incubated at 37°C for a four-day period, and the number of viable cells was quantified by MTT assay. Closed diamonds stand for BL6.KAI1-H; open squares, BL6.KAI1-L; open circles, BL6.CMV-CON. Data represent the mean±SD of quadruplicate cultures. (B) Cells (1×10^6) were injected subcutaneously into nude mice (n=5 per each transfectant). Maximum diameters (mm) of tumors were measured on the days indicated. Each value represents the mean±SD (n=5). NS stands for no significance. Open columns, BL6.KAI-H; oblique-lined columns, BL6.KAI1-L; closed columns, BL6.CMV-CON.

high KAI1 expression (designated as BL6.KAI1-H) and clone no. 6 (Fig. 1, lane 6) with low KAI1 expression (designated as BL6.KAI1-L) were chosen and subjected to further detailed analyses, while clone no.7 (Fig. 1, lane 7) which was transfected with a control vector pRc/CMV alone was selected as the control, designated as BL6.CMV-CON. No cellular morphological change accompanied the gene transfer of *KAI1*.

Effect of KAI1 on B16-BL6 cell aggregation The involvement of KAI1 in intercellular adhesiveness of B16-BL6 cells was assessed using KAI1 transfectants. Single cell suspensions were prepared and allowed to aggregate in saline with either 0 or 5 m*M* Ca^{2+} at 37°C as described in "Materials and Methods." As shown in Fig. 2A, the percentage of aggregated cells in BL6.KAI1-H and BL6.KAI1-L clones was significantly increased at 1 h and 2 h after incubation as compared with that in control BL6.CMV-CON cells. Fig. 2B shows the results of aggregation assay in the absence of Ca^{2+}; the percentages of cell aggregates formed by BL6.KAI1-H and BL6.KAI1-L clones were also significantly higher than that by control BL6.CMV-CON cells. These results showed that cell aggregation of B16-BL6 cells was enhanced by KAI1 expression in a Ca^{2+}-independent manner.

Effect of KAI1 on B16-BL6 cell migration Phagokinetic motility of the KAI1 transfectants was determined by a gold-particle coating method as described in "Materials and Methods." BL6.KAI1-H, BL6.KAI1-L, and BL6.CMV-CON cells were plated on gold particle-coated substrates, and the average areas of the tracks cleared by these transfectants were measured. A graphical representa-

Table I. Suppressive Effect of KAI1 on Pulmonary Colony Formation of B16-BL6 Melanoma Cells[a)]

	No. of metastatic nodules of a pair of lungs	Mean±SD	
BL6.KAI1-H	8, 6, 7, 10, 7, 6	(7.3±1.5)	
BL6.KAI1-L	25, 19, 21, 33, 27, 22	(24.5±5.0)	*b)*
BL6.CMV-CON	30, 25, 29, 18, 21, 28	(25.2±4.8)	*c)*
B16-BL6 parental cells	27, 23, 25, 35, 27	(27.4±4.6)	

a) Aproximately 1×10^5 viable B16/BL6 cells transfected with KAI1 cDNA (BL6.KAI1-H) were injected intravenously into the tail veins of nude mice. Mice were autopsied at the 21st day after injection and the number of metastatic foci on the lung surface was determined using a dissecting microscope. Similar results were obtained from three separate experiments.
b, *c*) A significant reduction (P<0.01) was observed in the number of pulmonary metastatic foci by BL6.KAI1-H cells, compared with control BL6.CMV-CON cells and parental B16-BL6 cells by Student's *t* test.

tion of these differences is shown in Fig. 3A. The average areas of the particle-cleared zones for BL6.KAI1-H and BL6.KAI1-L cells (calculated from 40 randomly selected cells) after 24 h incubation (Fig. 3B) were significantly smaller (32.0±6.3 mm^2 or 64.7±19.9 mm^2, respectively) than that of BL6.CMV-CON cells (110.5±18.9 mm^2). This assay demonstrated that the expression of *KAI1* gene suppressed the random motility of B16-BL6 cells in an expression level-dependent manner.

Effect of KAI1 on B16-BL6 cell invasion Fig. 4 shows the results of a comparative analysis of the invasive ability of KAI1 transfectants, BL6.KAI1-H and BL6.KAI1-L, and control BL6.CMV-CON cells through reconstituted basement membrane. In this assay, the cells in the upper chamber must adhere to and penetrate the Matrigel-coated filters in order to enter the lower chamber. The mean numbers±SD of three separate experiments of these transfectants attached to the lower surface of the filters were as follows: BL6.KAI1-H, 25.6±15.8 cells per field; BL6.KAI1-L, 117.8 ±25.1; BL6.CMV-CON, 225.8±33.7. The invasiveness in KAI1 transfectants, BL6.KAI1-H and BL6.KAI1-L, was significantly suppressed depending on the expression level of KAI1 mRNA as compared with control BL6.CMV-CON cells. These results indicate that the enhanced expression of *KAI1* gene in B16-BL6 cells reduced their invasive capability.

Effect of KAI1 expression on B16-BL6 cell proliferation We examined by MTT assay whether the *KAI1* gene expression affects the cell proliferation of B16-BL6 cells before *in vivo* experiments. As shown in Fig. 5A, all of BL6.KAI1-H, BL6.KAI1-L and BL6.CMV-CON revealed a similar pattern of growth curves over the period tested, indicating no significant effect of KAI1 expression. To examine the effect of KAI1 on *in vivo* growth, these three transfectants were then subcutaneously injected into nude mice, and the maximum tumor diameter was monitored. The *in vivo* assay also showed no significant difference of growth rate among those transfectants (Fig. 5B).

Suppressive effect of KAI1 on the pulmonary colony formation of melanoma cells Preliminary *in vivo* experiments were performed to see the effect of KAI1 on the metastatic potential of B16-BL6 cells using experimental metastasis assays, in which pulmonary colonies are formed by tumor cells injected intravenously into the tail vein.[23] We monitored lung colony formation subsequent to intravenous injection of KAI1 transfectants into nude mice, which were used to eliminate the possible effects of altered sensitivities of the transfectant clones to host T cell-dependent immune responses. As shown in Table I, BL6.KAI1-H cells produced significantly fewer pulmonary colonizations (represented by the mean number) than did control BL6.KAI1-CON cells or parental B16-BL6 cells, whereas no significant difference was observed between BL6.KAI1-L cells and control cells. The diameters of pulmonary metastatic foci formed by BL6.KAI1-H cells were not different from those of BL6.CMV-CON cells.

DISCUSSION

To investigate further the hypothesis that KAI1 is a suppressive regulator of the metastatic process, we produced KAI1 transfectants with different expression levels of KAI1 mRNA using B16-BL6 murine melanoma cells that offer a representative *in vivo* metastasis model, and we analyzed these KAI1 transfectants for changes in the characteristics of cell aggregation, motility, invasiveness and proliferation, all of which are essential steps for the establishment of metastasis.

In cell aggregation assay, KAI1 transfectants exhibited increased aggregability, depending on the degree of KAI1 mRNA expression, as compared with control cells. The C33 antigen, which is identical to KAI1, has been identified as the target antigen of a mAb inhibitory to HTLV-1-induced syncytium formation.[8] This antigen is reported not to be the HTLV-1 receptor itself, but to be related to cell adhesion during the process of HTLV-1-induced syncytium formation of human T cells. In addition to these observations, our results indicate that up-regulation of *KAI1* gene expression may be involved in enhanced cell-to-cell adhesion, suggesting that detachment of tumor cells from the primary lesion might be a step at which KAI1 would play a suppressive role during the metastatic cascade. Both phagokinetic track assay (Fig. 3) and *in vitro* invasion assay (Fig. 4) revealed that the transfection of KAI1 cDNA into B16-BL6 cells significantly suppressed their motility and invasive ability, and its suppressive action was related to the expression level of KAI1 mRNA. Furthermore, the results of *in vitro* and *in vivo* growth assays (Fig. 5) showed that expression of the *KAI1* gene had no significant influence on the growth rate of melanoma cells.

Based on those findings, we performed preliminary *in vivo* experiments using athymic nude mice. Experimental metastasis assay with BL6.KAI1-H cells indicated that KAI1 expression decreased their lung colony formation. Unexpectedlly, there was no significant difference of pulmonary colony formation between BL6.KAI1-L and control cells even though significant effects of KAI1 expression were observed in *in vitro* cell aggregation, cell motility and invasion assays. The expression level of KAI1 mRNA in BL6.KAI1-L was almost equivalent to the endogenous expression levels of colon cancer BM314 cells and gastric cancer MKN74 cells as determined by RT-PCR (data not shown). We have found that the *in vitro* cell motility and invasion of BM314 cells was significantly reduced by the antisense suppression of KAI1 (Takaoka *et al.*, to be published elsewhere). These find-

ings suggest that the threshold of KAI1 expression for suppressing experimental metastasis may be between the expression level of KAI1 in BL6.KAI1-H and that in BL6.KAI1-L, which is considered to be sufficiently high to influence the *in vitro* functions of B16-BL6 cells. However, further *in vivo* experiments are required to reveal if such high-level expression of KAI1 in B16-BL6 cells is indispensable for manifestation of *in vivo* effects. It should be noted that the size of metastatic nodules of BL6.KAI1-H was not different from that of the control cells despite the smaller number. Since the *in vivo* growth rate of BL6.KAI1-H cells was almost identical to that of BL6.KAI1-L or control cells (Fig. 5B), it is not plausible that the reduced number of pulmonary colonies of BL6.KAI1-H cells was simply due to higher cell aggregability or loss of the transfected *KAI1* gene during *in vivo* growth. The decreased motility and invasiveness of BL6.KAI1-H cells may be relevant to the reduced colony formation. These factors may reduce the probability that B16-BL6 cells can pass through the complicated processes leading to successful transplantation into the lung.

Several recent clinicopathological reports support the idea that KAI1's metastasis suppression could act on other kinds of tumors, as well as prostatic cancer. A study on expression analysis by RT-PCR[24] showed that the high-level expression of the KAI1 gene is associated with good prognosis of patients with non-small cell lung cancer, especially those with adenocarcinoma. Northern blot analyses by another group[25] demonstrated reduced expression of KAI1 mRNA in pancreatic cancer tissues with advanced tumor stages, and suggested that a decreased level of KAI1 mRNA expression may be involved in lymph node metastasis. These observations, including our data, suggest that KAI1's suppressive action in metastasis may not be limited to prostatic cancer, but may be effective in other tumor systems.

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