[CANCER RESEARCH 59, 5255–5263, October 15, 1999]

A Chimeric Cell Adhesion Molecule Mediates Homing of Lymphocytes to Vascularized Tumors[1]

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ABSTRACT

To facilitate tumor colonization by adoptively transferred cells of the immune system, we created a chimeric cell adhesion molecule that mediates tumor-specific homing by binding to the integrin $\alpha v\beta 3$ on angiogenic endothelial cells. A high-affinity cell adhesion molecule for integrin $\alpha v\beta 3$ was generated by fusing the disintegrin kistrin to the transmembrane adhesion molecule CD31/PECAM-1. This chimeric cell adhesion molecule, termed KISS31, mediates adhesion of lymphoid cells to soluble recombinant integrin $\alpha v\beta 3$ and to endotheliomal monolayers *in vitro*. KISS31-expressing lymphoid cells accumulate in angiogenic tumors in two *in vivo* models, in B16/129 melanoma xenografts on the chick chorioallantois and in s.c. growing Lewis lung carcinoma in mice.

Our data indicate that expression of KISS31 on lymphoid cells confers tumor-specific homing. This is, to our knowledge, the first example of an experimental mechanism that targets living cells to tumors by redirecting their homing pattern.

INTRODUCTION

Endothelial CAMs[3] can be constitutively expressed in a tissue-specific manner or regulated by inflammatory cytokines and thereby serve as addressins to recruit leukocyte subsets to specific sites and tissues in the body (1–3). Endothelial CAMs are also involved in lymphocyte recirculation through blood and tissue as part of continued immune surveillance for neoplastic cells. However, it has been shown that certain melanomas and carcinomas repress the expression of CAMs on adjacent blood vessels, thereby hampering the immigration of leukocytes from the bloodstream into the tumor. Moreover, angiogenic ECs of tumor vasculature may be unresponsive to inflammatory stimuli and thus fail to up-regulate CAMs that promote the transmigration of leukocytes, including potential effector cells (4). Therefore, the angiogenic tumor vasculature is often deficient in CAMs, which may help the tumor escape immune surveillance (5–7).

Angiogenesis, the sprouting of new blood vessels from preexisting vasculature, is tightly controlled during development, organ growth and remodeling, wound healing, and the menstrual cycle. Once a tissue or organ is established, the blood vessels become quiescent, with the ECs having a very slow turnover. In contrast to this steady state, there are pathological situations, such as diabetic retinopathy or tumor progression, where angiogenesis is induced and new blood vessels are formed. Angiogenesis in an adult organism outside the reproductive tract can therefore be a strong indication of tumor growth and progression. Indeed, the degree of vascularization is an important prognostic factor for cancer therapy because it determines the metastatic potential of the tumor (8–11).

ECs modulate the surface expression of numerous receptors and adhesion molecules when they switch from the quiescent to the angiogenic phenotype to respond to the different stimuli that regulate their proliferation and migration (12). Integrin $\alpha v\beta 3$ is an important cell adhesion molecule that is up-regulated on ECs during angiogenesis (13, 14), and angiogenic ECs depend on survival signals delivered by engaged integrin $\alpha v\beta 3$ (15–18). Moreover, the level of $\alpha v\beta 3$ expression on angiogenic ECs has been shown to be a prognostic indicator in breast cancer (19).

To create a novel ligand-receptor system that would enable leukocytes to adhere to angiogenic ECs and to immigrate to the tumor, we investigated the possibility of using $\alpha v\beta 3$ as a tumor-specific addressin. Because leukocytes do not express high affinity ligands for integrin $\alpha v\beta 3$, we generated a novel chimeric CAM, termed KISS31, that consists of the leukocyte CAM CD31/PECAM-1 with the disintegrin kistrin fused at the NH_2 terminus.

Disintegrins are a family of snake venom peptides of 60–70 amino acids in length and characteristically contain an RGD-motif that binds with high affinity to integrins (20–22). The disintegrin kistrin is unique in the fact that it is only bound by integrins $\alpha v\beta 3$ and $\alpha IIb\beta 3$, unlike other members of the disintegrin family, which are more promiscuous in their binding of integrins (21, 23–25).

Here, we tested KISS31-expressing lymphoid cell lines for their ability to adhere to soluble recombinant integrin $\alpha v\beta 3$ in solid phase adhesion assays and to $\alpha v\beta 3$-expressing endothelioma monolayers *in vitro*. In addition, KISS31-transfected cells were investigated for their ability to home to tumors in two *in vivo* models, B16 melanoma xenografts on the chorioallantois in the chick and a carcinoma mouse model. Our results demonstrate that we have created a novel homing-addressin pair of CAMs that promotes the immigration of leukocytes into vascularized tumors.

MATERIALS AND METHODS

Soluble Recombinant Mouse Integrin $\alpha v\beta 3$

The cDNA encoding the ectodomain for mouse integrin αv was amplified by PCR from mouse placenta cDNA using Pfu polymerase (Invitrogen, San Diego, CA) and the following oligonucleotides: 5′primer, 5′-ATTATGGATCCACCATGGCTGCTCCCGGGCGCCTGCT; and 3′primer, 5′-ATATTAGGGCCCCTGAATGCCCCAGGTGATGTTAG. The PCR product was digested with *Bam*HI and *Apa*I (primer internal sites, underlined), cloned into the corresponding site of pcDNA3/FLAG in-frame with the flag-tag cDNA, and sequenced. The expression vector pcDNA3/FLAG was is a modified pcDNA3 (Invitrogen) and was generated as follows. Two oligonucleotides encoding the flag sense (5′-CGACTACAAGGACGACGATGACAAGTAAGGCC) and antisense (5′-TTACTTGTCATCGTCGTCCTTGTAGTCGGGCC) cDNA were annealed and cloned into the *Apa*I site of pcDNA3, thereby reconstituting only the *Apa*I site 5′ of the inserted flag cDNA. cDNAs to be expressed as flag-tagged soluble molecules were then cloned into the multiple cloning site of pcDNA3/FLAG in-frame with the flag-tag cDNA.

Full-length cDNA for mouse integrin $\beta 3$ was cloned into the *Bam*HI/*Xho*I site of pcDNA3, the cDNA encoding the transmembrane and cytoplasmic region excised with *Eco*RV/*Xho*I, and the open *Xho*I site blunted with mung bean nuclease. Religation of the vector resulted in a cDNA encoding the ectodomain of the $\beta 3$ cDNA chain, followed by three more amino acids (Ala-Cys-Ile) and a stop codon. Sr$\alpha v\beta 3$ was produced by transient transfection of 293T cells using the calcium phosphate precipitation method (26). Flag-

Received 4/30/99; accepted 8/18/99.

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[1] Supported by Grant 31-49241.96 from the Swiss National Science Foundation, Grant KFA 412-1-1997 from the Schweizerische Krebsliga, and a grant from the Fondation Gabriella Giorgi-Cavaglieri.

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[3] The abbreviations used are: CAM, cell adhesion molecule; EC, endothelial cell; ICAM, intercellular adhesion molecule; VCAM, vascular endothelial cell molecule; PECAM, platelet/endothelial CAM; FACS, fluorescence-activated cell sorting.

tagged molecules were purified from the supernatant using a M2 anti-flag affinity column (Eastman Kodak Co., New Haven, CT) and then competitively eluted with flag peptide, according to the manufacturer's instruction. Eluted sr$\alpha v\beta 3$ was separated from flag peptides by fast protein liquid chromatography.[4]

KISS31

For the generation of KISS31, we used a full-length cDNA clone of mouse CD31/PECAM-1 obtained by PCR of mouse placenta cDNA using the 5′primer 5′-ATTAAAGCTTCCACCATGCTCCTGGCTCTGGGACTCA and the 3′primer 5′-TATTAGGGCCCTTAAGTTCCATTAAGGGAGCCTT. The PCR product (a 2.2-kb fragment) was cloned into pcDNA3 (*Hin*dIII/*Apa*I), and the sequence was confirmed. The cDNA for kistrin was created by annealing and ligation of a set of 12 overlapping sense and antisense oligonucleotides, designed according to the published protein sequence. The 5′ end of the kistrin cDNA additionally included the sequence encoding the CD31 signal peptide, and the 3′ end contained a *Pin*AI restriction site, followed by a stretch of 20 nucleotides homologous to the mature CD31 protein. This kistrin cDNA was used as a mega forward primer for the CD31 cDNA to create a fusion product containing the kistrin cDNA inserted between signal peptide and mature CD31 protein. To avoid steric problems, the cDNA for the murine CD8α hinge region was amplified by PCR (5′oligo, 5′-TATAATACCGGTGAACTCTACTACTACCAAGCCAGTG; 3′oligo, 5′-ATATTAACCGGTCCGAAGTCCAATCCGGTCCCCTTCAC) and inserted into the *Pin*AI site 3′ of the kistrin cDNA. For all PCR reactions, Pfu polymerase (Stratagene AG, Basel, Switzerland) was used.

Soluble KISS31 (SKI-1)

The ectodomain of KISS31 was expressed as flag-tagged soluble molecule by cloning the cDNA for the ectodomain of KISS31 (*Hin*dIII/*Nhe*I fragment) into the *Hin*dIII/*Xba*I site of pcDNA3/FLAG. The sites for *Nhe*I and *Xba*I are compatible and keep the flag-cDNA in-frame with the inserted cDNA. SKI-1 was produced by transient transfection of 293T cells using the calcium-phosphate precipitation method (27). Soluble flag-tagged protein was purified from supernatant with an anti-flag M2 affinity gel, eluted with 0.1 M glycine (pH 3.2; Eastman Kodak Company, New Haven, CT), and then dialyzed against PBS, according to the manufacturer's instructions.

Soluble murine cell adhesion molecules CD31, ICAM-1, and VCAM-1 have been described previously (28).

Transfection of Lymphoid Cells

The mouse myeloma cell line J558L, the B-cell line B300-19, and the thymoma EL4 were transfected with cDNA for KISS31 or CD31 by electroporation. Briefly, 10^7 cells were electroporated with 20 μg of *Pvu*I-linearized plasmid at 280 V, 960 μF and then subcloned in 96-well plates. G418-resistant clones were analyzed for surface expression of KISS31 or CD31 by FACS analysis using the anti-CD31 antibody GC51.

Antibodies and Reagents

Cyclic RGD peptide (H-2574) was purchased from Bachem AG (Bubendorf, Switzerland). Biotinylated hamster-anti-mouse integrin $\beta 3$ chain and biotinylated hamster-anti-mouse integrin αv chain are from PharMingen (Hamburg, Germany); FITC-conjugated anti-rat IgG and FITC-conjugated streptavidin are from Jackson ImmunoResearch Laboratories, Inc. Mouse anti-flag (M2) was from Eastman Kodak, FITC-conjugated goat-anti-mouse IgG was from Becton Dickinson, and rabbit-anti-mouse thrombocyte antibody CV5H.7 was from Inter-Cell technologies (Hopewell, NJ).

FACS Analysis

Staining for integrin αv and $\beta 3$ was carried out as follows. Briefly, cells were incubated in PBS/0.5% BSA on ice with biotinylated primary antibodies, followed by a FITC-conjugated streptavidin second reagent. For analysis of SKI-1 binding, purified SKI-1 was used at a concentration of 1 μg/ml. Bound SKI-1 was revealed with the M2 mouse antibody (which binds to the flag-tag), followed by incubation with a FITC-conjugated anti-mouse reagent or with the anti-CD31 antibody, GC51, followed by incubation with a FITC-conjugated anti-rat reagent. Fluorescence intensity was analyzed with a FACScalibur (Becton Dickinson).

In Vivo Homing in Chicken Eggs

Implantation of B16 Melanoma Cells. The chorioallantoic membranes of 10-day-old fertilized white Leghorn chicken eggs, incubated at 37°C and 60% relative humidity, were used as recipients. For implantation of B16 melanoma cells, a hole was made in the shell above the air sac with an 18-gauge needle on day 10. The position of the embryo was determined by candling, and a small triangle (1 cm^2) of the egg shell above the embryo was cut out by using a dental drill. By carefully lifting up a section of the underlying egg membrane, the chorioallantoic membrane dropped down, thereby creating a false air sac. Five μl of DMEM containing 5×10^5 B16/129 melanoma cells (cultured in DMEM with 10% FCS) was placed onto the chorioallantois, and the opening in the egg shell was sealed with adhesive tape. The eggs were incubated for 5 days.

Injection of Chromium-labeled Cells. For ^{51}Cr labeling, up to 10^7 EL4 cells were incubated with 400 μCi of ^{51}Cr (Amersham, Buckinghamshire, United Kingdom) in 500 μl of FCS for 30 min at 37°C. Free ^{51}Cr was then removed by four washes in DMEM containing 10% FCS. The labeling efficiency was between 3 and 5 cpm/cell. For injection of the labeled cells, a second opening was cut into the shell directly above a large vein, and 5×10^5 ^{51}Cr-labeled EL4/KISS or EL4/CD31 cells were injected i.v. in 200 μl of PBS using a 25-gauge needle. Two h later, the eggs were opened, the tumor and organs (liver spleen, heart, and gut) of the embryo were collected and weighed, and their radioactivity was counted in a gamma counter.

In Vivo Homing in Mice

Cell Labeling. B300/KISS31 and B300/CD31 were labeled with PKH67green- and PKH26 red-fluorescent linker kit (Sigma-Aldrich Chemie, Steinheim, Germany), respectively, according to the manufacturer's protocol. After labeling, cells were cultured for 24 h in DMEM/10% FCS to allow recovery from the labeling procedure (which may affect the adhesion of the cells to specific substrates immediately after labeling[5]). The cells were then washed twice in PBS and resuspended at 5×10^7 per ml each. The two cell suspensions were subsequently mixed, and 200 μl (*i.e.*, containing 5×10^6 cells of each cell type) were injected into mice as follows.

Injection of Tumor-bearing Mice. Anesthesized C57/BL6 mice were injected s.c. with 5×10^5 LLC-1 cells (Lewis lung carcinoma, obtained from the European Collection of Cell Cultures, Salisbury, United Kingdom). Two to 3 weeks later, mice bearing tumors of ~0.5–1 cm in diameter received injections of a mixture of 10^7 labeled KISS31/CD31 cells into their tail vein, and then were killed 4 h later with CO_2. Animal experiments were carried out in accordance with the Swiss veterinary office. For platelet depletion, mice received an intramuscular injection of an anti-thrombocyte antibody 24 h before injection of the cells. Tumors and control organs were embedded in OCT Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, the Netherlands) and frozen on dry ice. Cryosections of 7 μm were prepared and air dried, and fluorescent cells were counted using a Zeiss microscope equipped with a double filter for both dyes (Zeiss, Oberkochem, Germany).

Adhesion Assays

Purified soluble $\alpha v\beta 3$ was coated onto microtiter plates (Nunc Maxisorb; Nunc, Polylabo, Switzerland) at the indicated concentrations in 50 μl of PBS at 4°C for a minimum of 12 h. The wells were then blocked with 0.5% BSA for 1 h at room temperature, and unbound proteins were washed off. Fifty μl of adhesion buffer (DMEM, 1% BSA, and 20 mM HEPES) were added to the each well with or without specific blocking agents.

Cells to be used in the adhesion assay were labeled with calcein AM (Molecular Probes, Eugene, OR). Briefly, the cells were washed twice in serum-free DMEM, resuspended at a concentration of up to 10^7 cells/ml in OptimemI (Life Technologies, Inc.) containing 10 μM calcein AM, and then incubated for 30 min at 37°C. After two washes in adhesion buffer, the cell

[4] For analysis of sr$\alpha v\beta 3$, see G. Wiedle, F. P. Ross, and B. A. Imhof. Superactivation of integrin $\alpha v\beta 3$ by low antagonist concentrations, submitted for publication.

[5] Unpublished data.

concentration was adjusted to 2×10^6 cells/ml, and 100 μl of this cell suspension were added per well. After a 15-min incubation on ice, the plates were transferred to 37°C for 30 min. Unbound cells were removed by three to four washes with prewarmed adhesion buffer, and the number of bound cells was quantified using a CytofluorII fluorescence reader (Stehlin, Basel, Switzerland).

For adhesion assays on tend.1 monolayers, 2×10^4 tend.1 cells/well were plated onto 96-well tissue culture plates (Nunc) 48 h before the experiment. Before the addition of labeled cells, the medium was removed, and 2×10^5 labeled J558/KISS31 or J558/CD31 in 200 μl of DMEM (with or without cyclic RGD) were then added to the wells. The plate was returned to the incubator for 30 min. Washing and quantitation of bound cells was carried out as described above.

RESULTS

Generation of KISS31

To create a CAM with high affinity for integrin $\alpha v\beta 3$ that would mediate cell binding to angiogenic, $\alpha v\beta 3$-expressing ECs, we looked for a naturally existing ligand that could be converted to a membrane anchored surface receptor. Because an active, high-affinity conformation may not be achieved by simply expressing complex RGD-containing molecules on a cell surface, we created a novel chimeric cell adhesion molecule that contains a disintegrin moiety as a ligand for integrin $\alpha v\beta 3$.

To convert the disintegrin kistrin to a membrane-bound adhesion molecule, the cDNA was ligated to the cDNA of the transmembrane cell adhesion molecule CD31/PECAM-1. Expression of this construct results in a chimeric CD31 with an NH_2-terminal kistrin moiety, the chimeric cell adhesion molecule termed KISS31 (Fig. 1).

The KISS31 cDNA was transfected into various lymphoma cell lines, including EL4, J558L, and B300-19, and surface expression of KISS31 was determined by FACS analysis using the monoclonal anti-CD31 antibody GC51. For all experiments, cells stably expressing KISS31 were compared with control cells that express PECAM-1/CD31 either from a transgene (J558; Fig. 1*C*), endogenously (B300-19) or both (EL4), not shown, to control for the expression of the CD31 moiety. The mean fluorescence intensity of the EL4 lines stained with GC51 was 23.8 (wild-type), 92.7 (CD31-transfected), 96.2 (KISS31-transfected), and 5.3 (isotype-matched control staining of wild-type cells). B300-19 staining with GC51 gave mean fluorescence intensity values of 36.2 (wild-type), 93.8 (KISS31-transfected), and 4.5 (isotype-matched control staining of wild-type cells).

KISS31 Mediates Binding to Integrin $\alpha v\beta 3$ *in Vitro*

To confirm that the kistrin moiety in KISS31 retains its affinity for integrin $\alpha v\beta 3$, we tested the binding of KISS31-expressing cells to recombinant soluble mouse $\alpha v\beta 3$ (rs$\alpha v\beta 3$) in a solid phase adhesion assay. Adhesion of KISS31-expressing cells, as shown for J558/KISS31, increases with the amount of coated rs$\alpha v\beta 3$ in a dose-dependent manner, whereas control cells transfected with wild-type CD31 (J558/CD31) do not adhere (Fig. 2*A*). Adhesion of J558/KISS31 to rs$\alpha v\beta 3$ is integrin specific, because cell binding can be blocked by the addition of EDTA or high concentrations of the competitive antagonist cyclic RGD. Moreover, expression of KISS31 does not confer cell adhesion to soluble recombinant ICAM-1 and VCAM-1, two major adhesion molecules that are expressed on the vascular endothelium (Fig. 2*B*). Because J558 cells are devoid of endogenous integrins, including the receptors for ICAM-1 and VCAM-1, $\alpha L\beta 2$ and $\alpha 4\beta 1/\alpha 4\beta 7$, respectively, wild-type cells have no tendency to adhere to these CAMS either. Moreover, the lack of binding of J558/KISS31 to recombinant soluble CD31 indicated that the CD31 moiety in KISS31 has lost its capacity to interact homotypically with CD31.

To verify KISS31 cell binding to wild-type $\alpha v\beta 3$ in a more physiological context, we performed adhesion assays on monolayers of tend.1, an endothelioma cell line that expresses endogenous $\alpha v\beta 3$. J558/KISS31 cells bound tightly to the apical surface of tend.1 monolayers, and this adhesion could again be blocked by the $\alpha v\beta 3$ antagonist cyclic RGD (Fig. 3). In contrast, J558/CD31 control cells do not bind to tend.1 monolayers under these conditions, indicating that J558/KISS31 binding is mediated by the kistrin moiety and not by a CD31-CD31 homotypic interaction between CD31 expressed by tend.1 and the CD31 backbone of KISS31.

Cross-linking of surface CD31 can generate intracellular signals that lead to increased cell adhesion via activation of integrins (see "Discussion"). We therefore tested whether the observed binding of KISS31 can be mediated solely by the kistrin moiety or whether it depends on activation of other CAMs upon KISS31 engagement. Thus, binding of KISS31 to $\alpha v\beta 3$ expressed on the cell surface was further assayed by FACS analysis using a soluble, transmembrane truncated form of KISS31, SKI-1 (Fig. 1*A*). SKI-1 was incubated with tend.1 cells or with KLN205 carcinoma cells, and bound SKI-1 was detected with the anti-flag tag antibody M2 (for tend.1) or with the anti-CD31 antibody GC51 (for KLN205, which does not express CD31). Binding of SKI-1 to tend.1 could also be revealed with GC51 and was seen as an increase in surface CD31 expression (endogenously expressed CD31 plus integrin bound SKI-1, not shown). The results in Fig. 4 show that SKI-1 binds to both cell lines, and that SKI-1 (like KISS31) binding to cells does not depend on CD31-CD31 homotypic interactions or alternative adhesion mechanisms activated by KISS31 cross-linking. In contrast, using the same technique, there was no binding of SKI-1 to cells that do not express integrin $\alpha v\beta 3$, such as J558L or Lewis lung carcinoma (LLC-1, not shown).

Thus, we demonstrate that KISS31 can be expressed on lymphoid cells and act as a high-affinity adhesion molecule for integrin $\alpha v\beta 3$. To further investigate its potential in creating tumor homing specificity by immobilizing circulating lymphoid cells in angiogenic blood vessels, we tested the homing behavior of KISS31-transfected cells in two different tumor models.

KISS31 Transfected Cells Preferentially Home to Angiogenic Tumors *in Vivo*

***In Vivo* Homing in Chicken Eggs.** In the first *in vivo* model, we xenografted mouse B16/129 melanoma cells on the chorioallantois of day 10 chicken embryos. Five days after grafting, 5×10^5 ^{51}Cr-labeled EL4/KISS31 or EL4/CD31 cells were injected into the embryonic blood circulation. Two h later, the tumors and several control organs of the embryos were taken and weighed, and their specific radioactivity was counted. For each individual organ, the radioactivity recovered per mg of weight was calculated as a percentage of the total radioactivity recovered from all organs. This calculation was performed for each injected egg. A comparison was then made of the values obtained for eggs injected with EL4/KISS 31 and EL4/CD31 cells. Results are expressed as a ratio of KISS31 recovered activity *versus* CD31 recovered activity from each organ. For spleen, liver, gut, and heart, the amount of radioactivity (as a percentage of total) counted per mg of organ was equivalent for EL4/KISS31 and EL4/CD31 injected eggs, *i.e.*, a ratio of 1 was recorded, indicating an equal trapping of both cell types in these tissues (Fig. 5*A*). However, in the B16 melanoma grafts, this ratio increased to 2.8 for KISS31 cells compared with CD31 control cells, demonstrating a specific accumulation of KISS31-expressing cells in the tumor. Because the tumor vasculature in this assay is of chicken origin, the retention of KISS31 cells in the tumor is evidently dependent on the interaction of the kistrin moiety with chicken integrins.

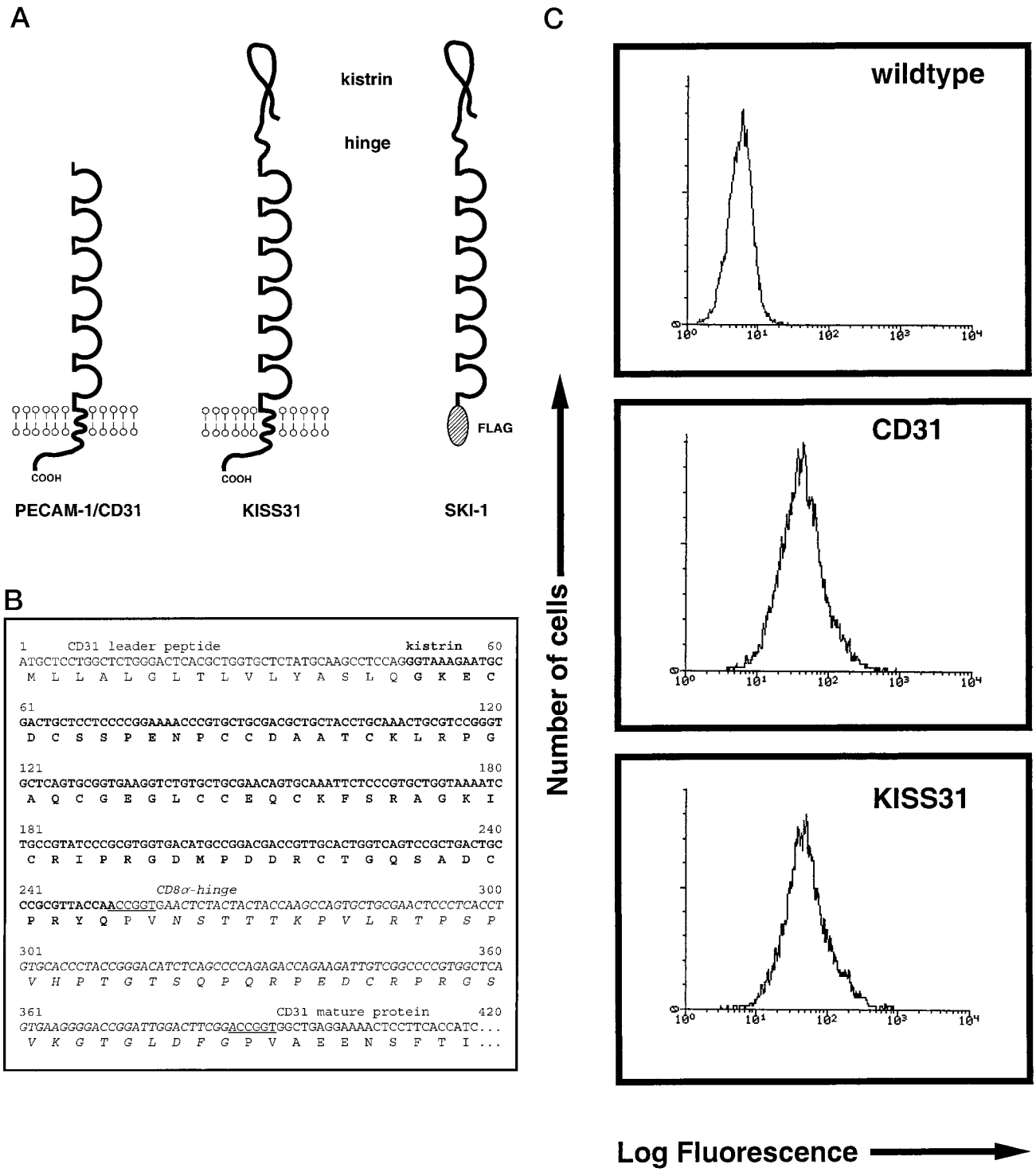

Fig. 1. Generation and expression of KISS31 and its soluble form, SKI-1. *A*, schematic representation of CD31/PECAM-1 (*left*) and the chimeric molecules KISS31 (*middle*) and SKI-1 (*right*). SKI-1 is identical to the extracellular domain of KISS31, but the transmembrane and cytoplasmic domains have been replaced by the flag-tag. *B*, cDNA sequence and protein translation of the NH_2-terminal chimeric portion of KISS31. The sequence consists of the CD31 leader peptide, the kistrin moiety (position 49–252, *boldface*), the CD8α hinge region (position 259–387, *italic*), and continues with the CD31 mature protein. Introduced *Age*I sites are *underlined*. *C*, FACS analysis of wild-type J558 cells (*upper panel*), CD31 (*middle panel*), and KISS31 (*lower panel*) transfected J558 cells. Surface expression of transfected molecules was detected using the anti-CD31 antibody GC51. Wild-type J558 does not express endogenous CD31.

***In Vivo* Homing in Mice.** In the second *in vivo* model, mice with s.c. growing Lewis lung carcinoma of about 0.5 cm in diameter received i.v. injections of a mixture of B300/CD31 and B300/KISS31 that had been labeled with a red (PKH26) or green (PKH67) fluorescent dye, respectively. Four h later, the mice were killed, and the tumors along with several control organs were taken to prepare cryosections. Using a fluorescent microscope equipped with a double filter for both dyes, we determined the number of green B300/KISS31 and red B300/CD31 control cells by counting 30–40 sections of each tissue. This method allowed us to calculate the relative numbers of KISS31 cells *versus* coinjected control cells in different tissues of the same animal. Taking the heart as a baseline, the ratio in the kidney

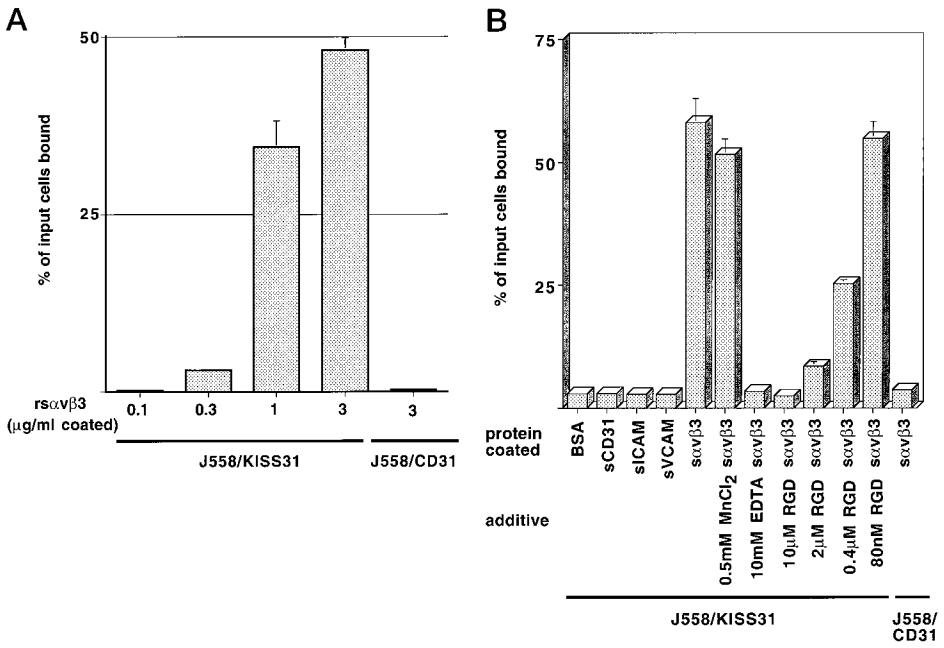

Fig. 2. Solid phase adhesion assay with J558/KISS31 and J558/CD31 cells on soluble recombinant adhesion molecules. *A*, binding to increasing amounts of coated soluble recombinant $\alpha v\beta 3$. Cell binding did not increase at concentrations above 3 μg/ml of coated integrin, indicating saturation. *Bars*, SE. *B*, blocking of cell adhesion to soluble $\alpha v\beta 3$ with EDTA and cyclic RGD antagonists and binding to control soluble recombinant adhesion molecules CD31, ICAM-1, and VCAM-1. *Bars*, SE.

was only slightly increased, whereas in the tumors, it was increased by >3-fold (Fig. 5*B*). Thus, expression of KISS31 mediates a 3-fold higher retention of the injected cells in the tumor as compared with other organs.

Because it was possible that the injected KISS31 cells might bind to integrins $\alpha v\beta 3$ and $\alpha IIb\beta 3$ on circulating platelets, we tested whether a lower platelet count at the time of injection might further increase the tumor homing potency of KISS31. We therefore repeated the experiment with tumor-bearing mice that had received an intramuscular injection of an anti-thrombocyte antibody 24 h prior to injection of the KISS31/CD31 cell mix. The anti-thrombocyte antibody induces a pronounced decrease in circulating platelets of up to 90% at the injected dose (29–31). Indeed, in these animals, the tumor specificity of B300/KISS31 could be further increased to a ratio of more than 5:1 compared with B300/CD31 control cells, indicating that KISS31 may be partially quenched by its ligands $\alpha v\beta 3$ and $\alpha IIb\beta 3$ on platelets (Fig. 5*B*). By lowering the amount of these competing ligands, homing of KISS31 cells was further increased, resulting in a 5-fold higher tumor homing rate (Fig. 5*B*).

Taken together, these data demonstrate the generation of a novel chimeric cell adhesion molecule, KISS31, that can serve as a high-affinity cell surface ligand for $\alpha v\beta 3$ integrin. KISS31-expressing cells bind to both $\alpha v\beta 3$ in a soluble recombinant form and $\alpha v\beta 3$ expressed on the cell surface. Finally, KISS31 cells home specifically to vascularized tumors in two different *in vivo* models, B16 melanoma growing on the chorioallantoic membrane in the chicken egg and Lewis lung carcinoma (LLC-1) growing s.c. in mice.

DISCUSSION

We have generated a novel chimeric CAM, termed KISS31, by fusing the soluble disintegrin kistrin to the transmembrane molecule PECAM-1/CD31. KISS31 can be expressed on lymphoid cell lines, and KISS31-expressing cells bind to soluble recombinant $\alpha v\beta 3$ and to $\alpha v\beta 3$-expressing endothelioma cells with high specificity and affinity. Moreover, a KISS31-expressing lymphoid cell line was assayed *in vivo* for its ability to home to solid tumors *in vivo* via binding to $\alpha v\beta 3$ integrin on the angiogenic tumor vasculature. Our results indicate that KISS31 expression on lymphoid cells confers tumor-specific homing.

Extravasation of leukocytes from blood into tissue is mediated and controlled by a multistep process (1–3). Because each step in this process depends on a preceding step, lack or insufficient expression of only a single adhesion molecule on the ECs can be sufficient to interrupt this cascade and impede transmigration. For example, tumor-induced down-regulation of endothelial ICAM-1 and VCAM-1 prevents the tight adhesion of circulating cells (5–7), and this can render the endothelial lining an impermeable barrier for patrolling cells of the immune system.

Because angiogenic ECs depend on survival signals delivered through $\alpha v\beta 3$, its expression is unequivocally linked to tumor progression and expansion (15–18). A tumor that induces $\alpha v\beta 3$ down-regulation would consequently prevent its vascularization and thereby hamper its own growth and progression. Interestingly, two recent reports showed that mice deficient in integrin αv or $\beta 3$ show normal vasculogenesis, angiogenesis, and remodeling of the capillary plexus (32, 33). Although these findings contradict earlier observations that integrin $\alpha v\beta 3$ plays a crucial role in cell migration and survival under normal conditions, the absence of αv or $\beta 3$ during development may lead to induction of a compensatory pathway of angiogenesis. In addition, it is not known whether tumor angiogenesis is dependent on the same mechanisms as angiogenesis that occur during development.

The question of accessibility of $\alpha v\beta 3$ on ECs was first addressed by Conforti *et al.* (34), who showed that HUVECs express $\alpha v\beta 3$ in an active form on their luminal surface. Even more important was the finding that integrin $\alpha v\beta 3$ on angiogenic ECs can serve as a ligand for circulating phages that display an RGD ligand, and that angiogenic tumor endothelium can be detected by i.v. injected anti-$\alpha v\beta 3$ antibodies (35, 36). Consequently, studies have been carried out to investigate whether $\alpha v\beta 3$ integrin can be used as a target for antiangiogenic antagonists to prevent vascularization of the tumor, thereby starving it. Alternatively, accumulation of cytotoxic drugs in the vicinity of the tumor by linkage of the cytotoxic agent to anti-$\alpha v\beta 3$ antibodies allowed a reduction in the effective drug dose in chemotherapy (37, 38). These experiments demonstrate that angiogenic ECs express $\alpha v\beta 3$ integrin and that it is accessible for circulating protein ligands.

To overcome the potential problems associated with leukocyte

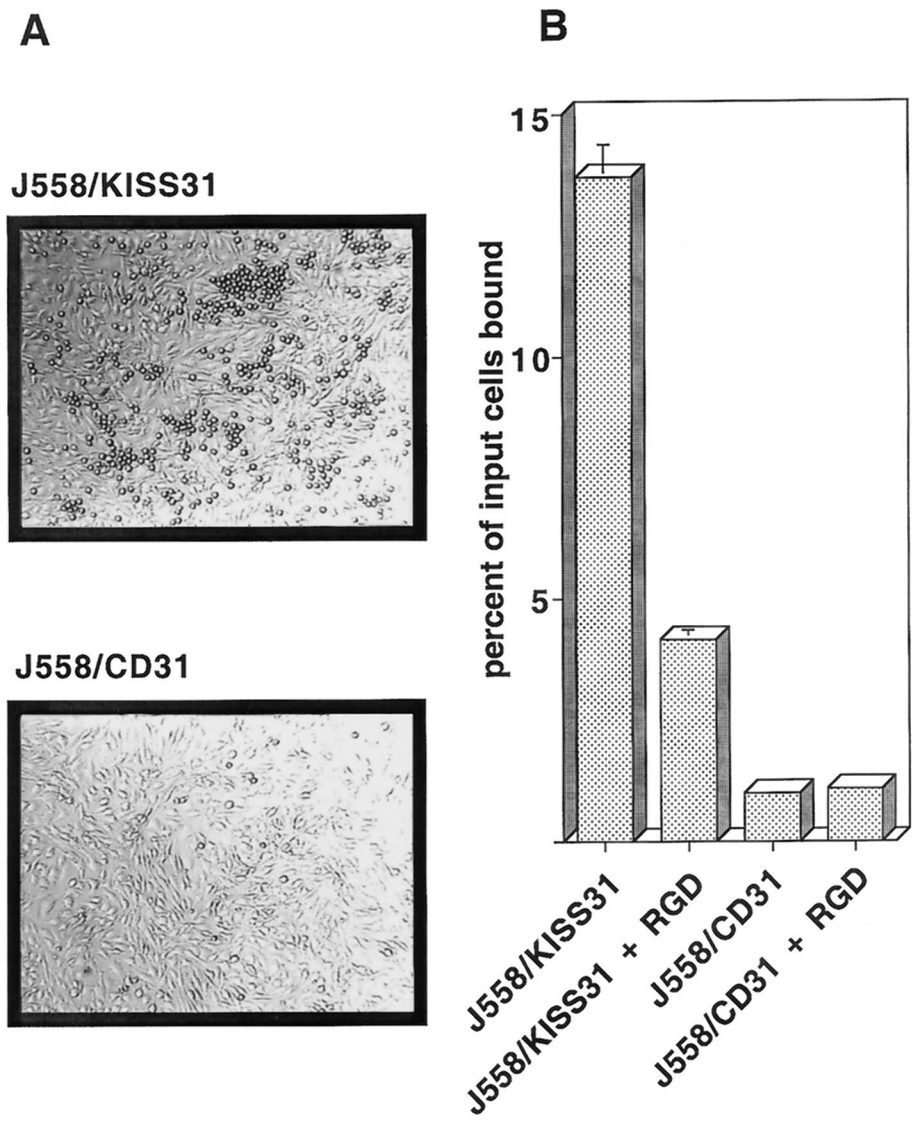

Fig. 3. Adhesion of J558/KISS31 and J558/CD31 cells to tend.1 endothelioma monolayers. *A*, phase contrast of a representative assay; ECs appear in the monolayer as spindle shaped, whereas the adherent J558 appear as circles. *B*, quantitation of bound cells and integrin-specific blocking of adhesion by cyclic RGD antagonists (2 μM). Representative assays are shown with quadruple samples per condition. *Bars*, SE.

migration to vascularized tumors, we created a novel CAM that allows adoptively transferred effector cells to adhere to and cross the tumor vasculature via binding to the $\alpha v\beta 3$ integrin, a process not involving the classical endothelial CAMs that may be unfavorably modulated by the growing tumor.

Physiological ligands of $\alpha v\beta 3$ integrin are components of the extracellular matrix, such as vitronectin, fibronectin, von Willebrand factor, osteopontin, thrombospondin, and fibrinogen (39). Their common $\alpha v\beta 3$-binding site consists of an Arg-Gly-Asp (RGD) motif that, depending on the protein context, can be exposed as a high-affinity ligand or may be cryptic and inaccessible (40). Therefore, the affinity of $\alpha v\beta 3$ for its ligands strongly depends on the conformation of this RGD motif. To obtain a cell surface receptor that binds integrin $\alpha v\beta 3$ with high affinity and which is able to mediate cell adhesion under conditions of flow, we used the soluble disintegrin kistrin as a binding moiety. Kistrin has a known conformation, with its RGD motif exposed on the apex of an elongated loop, making it a constitutively high-affinity ligand (24, 41). A further advantage of kistrin is its relative specificity for $\beta 3$ integrins, because it reportedly does not bind to the other RGD-binding integrins, $\alpha v\beta 5$ and $\alpha 5\beta 1$, and only weakly to $\alpha v\beta 1$ (25). On the other hand, consistent with its presence in snake venom, kistrin is not species specific but binds to human and porcine integrins (21, 25). This broad functionality is an important feature because it allows the application of kistrin-containing chimeric molecules to different experimental systems *in vivo*.

CD31/PECAM-1 was chosen as the backbone for KISS31 because its extracellular region consists of six immunoglobulin-homology domains that extend the fused kistrin moiety away from the cell surface and thereby serves as a spacer. Moreover, CD31 is a leukocyte signaling molecule that can activate physiological pathways involved in transendothelial migration, such as actin polymerization and activation of $\beta 1$ and $\beta 2$ integrins (42–48). Endogenous CD31 is a cell adhesion molecule that interacts with at least three CAMs: with itself in a homotypic manner; with integrin $\alpha v\beta 3$; and with CD38 (28, 49, 50). Because CD38 is only expressed on leukocytes, it does not interfere with the interaction of KISS31 cells with ECs. Interestingly, we did not observe any significant binding of KISS31-transfected cells to soluble recombinant CD31 or of CD31-transfected cells to soluble recombinant $\alpha v\beta 3$ (Fig. 2*B*). We therefore assume that the addition of the kistrin moiety abolishes homotypic interactions of the CD31 moiety in KISS31 with wild-type CD31, presumably attributable to steric hindrance. Furthermore, the interaction of CD31 with integrin $\alpha v\beta 3$ seems to be strongly determined by conformational constraints in the CD31 folding. Although two groups independently

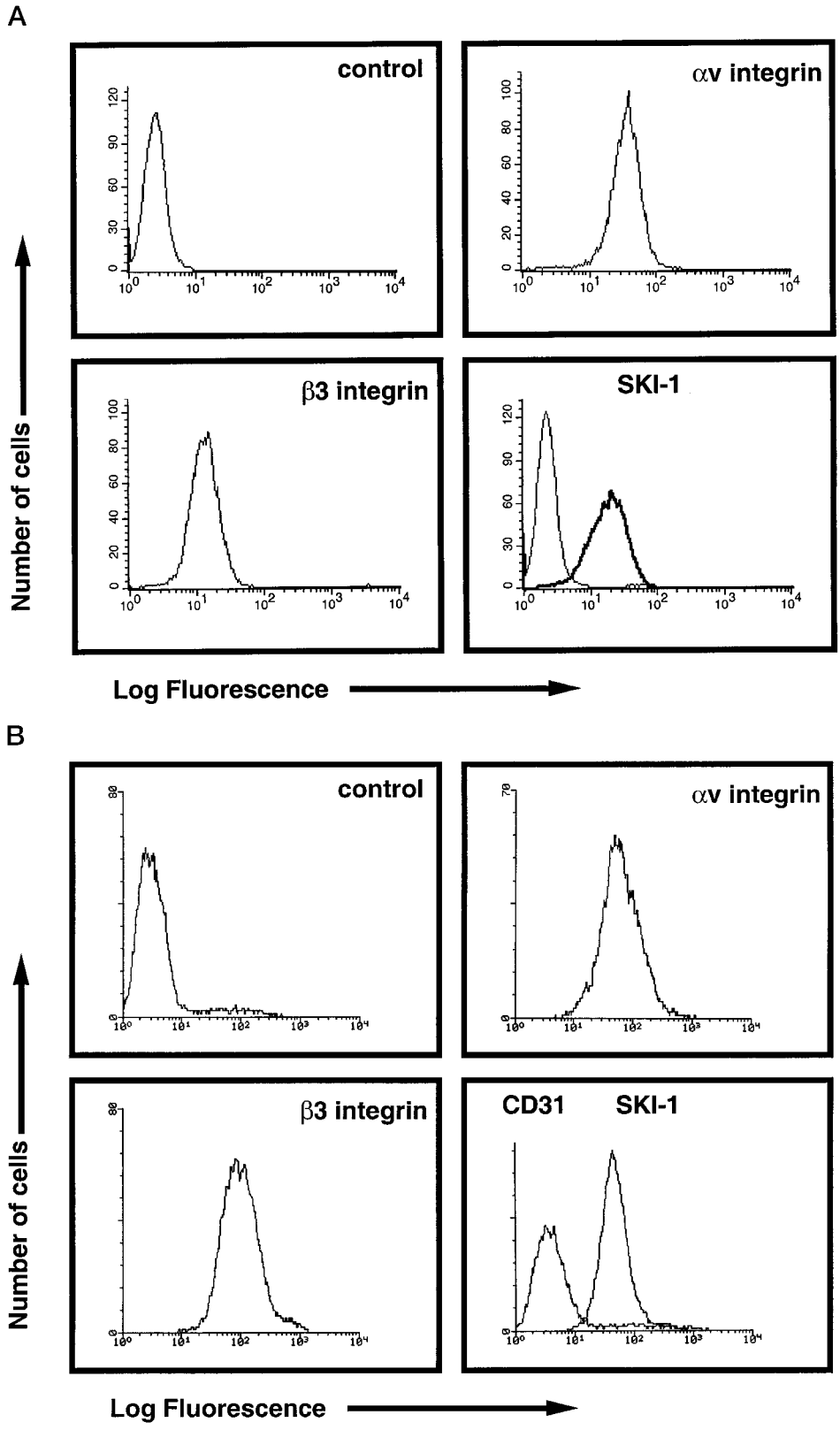

Fig. 4. FACScan analysis of SKI-1 binding to tend.1 ECs (*A*) and KLN205 carcinoma cells (*B*) and expression of integrin αv and β3 chains. Bound SKI-1 was revealed with the anti-flag antibody M2 on tend.1 (*A*) or with the anti-CD31 antibody GC51 on KLN205 (*B*). For the staining with SKI-1, the *thin histogram* depicts cells incubated with M2 and FITC-conjugated secondary reagent alone, whereas the *thick histogram* shows the staining after prior incubation of the cells with SKI-1.

found that cellular αvβ3 binds to certain forms of soluble recombinant CD31 (28, 51), we[6] and others (26) have not been able to show a direct interaction of cell surface CD31 with integrin αvβ3 in purified, membrane-bound, or recombinant soluble form. However, our unpublished data indicate that αvβ3 does interact with CD31 but acts in a *cis* fashion on the same cell rather than as a ligand on an opposing cell.[7]

To test the ability of KISS31 to redirect adoptively transferred lymphocytes to sites of tumor growth, we used two different *in vivo*

[6] G. Wiedle, unpublished observations.

[7] Wong *et al.*, manuscript in preparation.

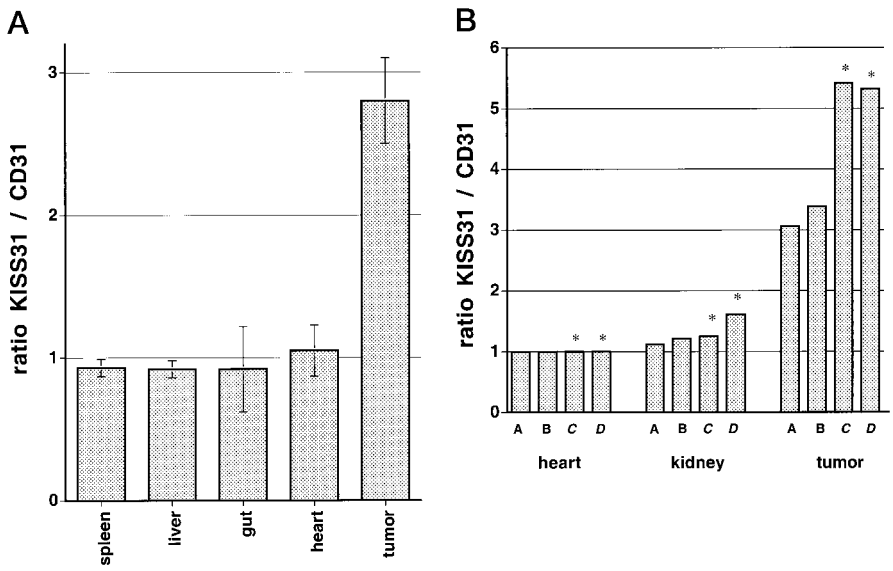

Fig. 5. Colonization of vascularized tumors by KISS31 cells. *A*, ratio of EL4/KISS31 *versus* EL4/CD31 in B16 melanoma xenografts growing on the chorioallantoic membrane and in control organs of the chicken embryo. Data are calculated from the mean values of three animals per group. Bars, SE. *B*, B300/KISS31 and B300/CD31 control cells were labeled with green or red fluorescent dyes, mixed in equal numbers, and injected into tumor-bearing mice. The ratio of B300/KISS31 *versus* B300/CD31 cells in three selected organs was determined by counting the fluorescent cells in at least 30 frozen sections per organ. Each *column* represents one mouse in a representative experiment including two untreated (*A* and *B*) and two platelet-depleted (*C* and *D*, *asterisks*) mice. The ratio KISS31/CD31 was normalized to obtain a value of 1 in the heart to facilitate comparisons among different organs and animals.

tumor models. The first model, a xenograft assay on the chorioallantoic membrane of the chicken embryo, is a model used frequently to study angiogenesis, and Brooks *et al.* (16) showed that tumor induced angiogenesis on the chorioallantois depends on integrin $\alpha v\beta 3$ expressed by the ECs of the chicken (52). Likewise, when we xenografted mouse B16/129 melanoma cells on the chorioallantois, they formed a solid tumor that became vascularized by the chicken host. KISS31-expressing EL4 lymphoma specifically accumulated in these tumors when injected i.v., but their distribution in other organs was identical to that of injected control cells expressing CD31. It is noteworthy that this accumulation is mediated by the interaction of KISS31 with integrins expressed by the vascular endothelium of the chicken. Together with the data from our solid phase adhesion assays of J558/KISS31 on soluble mouse $\alpha v\beta 3$, this confirms the earlier finding that kistrin binds $\alpha v\beta 3$ integrin of different species and adds mouse and chicken to this list. Our choice to use EL4 transfectants for these *in vivo* experiments, as compared with J558 transfectants for the *in vitro* experiments, is based on the CAM expression by these cells. Because J558L cells do not express endogenous integrins or CD31, they are an ideal tool for the *in vitro* characterization of KISS31, thereby avoiding contributions by other CAMs. For our *in vivo* homing studies, however, it was important to ensure a stable binding of KISS31 cells to the endothelium under shear stress. Therefore, we used cells that express $\beta 1$ integrins (EL4) or both $\beta 1$ and $\beta 2$ integrins (B300-19, see below) and are thus able to engage their integrins after initial immobilization by KISS31. *In vitro*, we obtained similar results with the different KISS31-transfected cell lines.

We also tested the homing specificity of B300/KISS31 cells in mice with a s.c. growing Lewis lung carcinoma. Again, KISS31 mediated specific homing of the injected cells to the tumors. Analysis of the cryosections of the tumor indicated that many of the labeled cells had clearly entered the tumor stroma, whereas a significant number of cells were still in contact with the vasculature. The possibility that accumulation of B300/KISS31 is based on a direct interaction with the tumor cells could be ruled out, because LLC-1 cells do not express the integrin subunits αv and $\beta 3$ and do not bind SKI-1 (not shown). In contrast to the chicken model, in mice we saw a generally higher number of KISS31 cells in all organs (spleen, heart, and kidney). This may be attributable to a low level expression of kistrin-binding integrins on all mouse ECs. In addition, it is possible that platelets in the circulation bind to the injected KISS31 cells, and that such "decorated" cells adhere to the endothelial lining by interaction of platelets with ECs via their respective CAMs. We therefore repeated the *in vivo* homing experiments in mice with a reduced number of platelets in the circulation by applying a platelet depletion protocol that results in a decrease (50–90% after 24 h of antibody injection) in circulating platelets. After such treatment, homing of B300/KISS31 to the tumors was further increased, resulting in a 5:1 ratio of KISS31 to CD31 cells. We believe that platelet $\beta 3$ integrins compete with $\alpha v\beta 3$ integrin on angiogenic ECs for KISS31, and that the integrin binding sites on KISS31 cells are partially occupied by circulating platelets. Transient reduction of platelet numbers in the circulation, therefore, leaves more KISS31 binding sites unoccupied and thus accessible to $\alpha v\beta 3$ integrin on the vascular endothelium. After platelet depletion, the low level of kistrin binding seen in the other organs does not increase, because the level of integrin expression by these quiescent ECs would probably be limiting in quiescent blood vessels. In contrast, the level of $\alpha v\beta 3$ integrin expression in the angiogenic tumor vasculature seems not to be a limiting factor for KISS31 cell binding, but rather the availability of kistrin sites on the injected cells. Therefore, a decrease in platelet numbers results in more KISS31 molecules available, and consequently, the adhesion of KISS31 cells to angiogenic ECs increases. Our data demonstrate that KISS31 expression is sufficient to induce homing of adoptively transferred cells to angiogenic tumors, and that the homing efficiency can be increased by short-term reduction of competing ligands on circulating platelets.

In conclusion, we demonstrate the generation of a novel chimeric cell adhesion molecule, KISS31, that mediates specific homing of adoptively transferred lymphoid cells to vascularized tumors. This new, modified migration pattern can be used to facilitate tumor colonization by cytolytic effector cells, thereby increasing the local concentration of cytolytic activity and launching an antitumor response. Furthermore, our data offer the possibility that chimeric CAMs with designed specificities can be used to target a tissue of choice with effector cells that would not migrate there under normal conditions.

ACKNOWLEDGMENTS

We thank Claude Magnin for expert technical assistance and Christian Vesin for help with the injections.

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