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# Suppression of Colorectal Cancer Liver Metastasis and Extension of Survival by Expression of Apolipoprotein(a) Kringles

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## ABSTRACT

The formation of hepatic metastases in colorectal cancer is the main cause of patient death. Current therapies directed at hepatic metastasis of colorectal cancer have had minimal impact on outcome. Therefore, alternative treatment strategies for liver metastasis require development. The present study was performed to evaluate the application of cDNA of LK68 encoding apolipoprotein(a) kringles IV-9, IV-10, and V as possible candidates for gene therapy treatment of this life-threatening disease. The murine colorectal cancer cell line CT26 was transduced *ex vivo* with LK68 cDNA via retroviral gene transfer, and an experimental model of hepatic metastasis was established by injecting LK68-expressing and control cells into the spleens of BALB/c mice. Expression of LK68 did not affect the growth characteristics and viability of transduced CT26 cells *in vitro*. LK68 produced from CT26 cells substantially inhibited the migration of endothelial cells *in vitro*. *In vivo*, substantial suppression of liver metastasis and prolonged survival were observed in mice bearing LK68-expressing CT26 cells, compared with controls. LK68-expressing liver metastases were restricted to smaller sizes and displayed decreased microvessel density and increased tumor cell apoptosis. Our data collectively indicate that LK68 suppresses angiogenesis-dependent progression of prevascular micrometastases to macroscopic tumors and their growth, which are clinically accessible and biologically relevant therapeutic targets. We propose that antiangiogenic gene therapy with LK68 is a promising strategy for the treatment of colorectal cancer liver metastasis.

## INTRODUCTION

Colorectal carcinoma is the third leading cause of cancer-related deaths worldwide. Most colorectal cancer patients develop metastasis to the liver during the course of the disease. Once liver metastases have developed, the natural course of the disease is associated with poor prognosis, resulting in the death of ~80% of patients (1). Surgery is the primary treatment option for isolated metastases, but only 20% to 25% of patients displaying hepatic metastases are suitable for resection (2), and postoperation recurrence is frequent. Therefore, the development of a new treatment modality for liver metastasis of colorectal cancer is urgently required.

Tumor growth essentially requires the formation of new blood vessels (a process known as angiogenesis) to supply tumors with nutrients and oxygen and allow the removal of waste products (3, 4). Moreover, metastatic spread of solid tumors depends on vascularization of the primary mass. Therefore, blockage of tumor angiogenesis may potentially suppress both tumor growth and metastasis. In this regard, the angiogenic process is a promising target to develop novel therapeutic modalities for the treatment of cancer. Numerous endogenous antiangiogenic inhibitors, such as angiostatin and endostatin,

have been identified that display significant efficacy against a variety of tumors in preclinical settings (5, 6).

Lipoprotein(a) is a lipoprotein particle associated with the development of atherosclerosis and coronary heart disease (7). Lipoprotein(a) is structurally distinct from low-density lipoprotein due to the presence of apolipoprotein(a) [apo(a)], which is covalently linked to apoB-100 protein of low-density lipoprotein by a disulfide bond (8). Apo(a) is a glycoprotein composed of multiple kringle domains and a protease-like domain homologous to plasminogen (9). Ten types of kringles homologous to plasminogen kringle 4, designated KIV-1 to KIV-10, and one kringle (KV) homologous to plasminogen kringle 5 are observed in apo(a). Each KIV domain is present as a single copy, with the exception of KIV-2, which varies in number from 3 to 42 between apo(a) alleles (10).

The kringle domain consists of approximately 80 amino acids with conserved rigid triple disulfide bonds and appears to be an independent folding unit. Kringle domains are present in many proteins with a surprisingly diverse array of functions, including growth factors, proteases, and coagulation factors (hepatocyte growth factor, plasminogen, prothrombin, urokinase, and so forth). These domains are thought to play an important role in specific protein-protein interactions that provide specificity to and facilitate the regulation of their parent proteins. Moreover, many kringle domains have been identified as inhibitors of angiogenesis (11). Similarly, although the physiologic role(s) of the apo(a) kringle domains has yet to be elucidated, current evidence suggests that these domains inhibit angiogenesis *in vitro* (12) and suppress tumor growth *in vivo* (13). Recently, we demonstrated that cryptic apo(a) kringle domain LK68, which contains KIV-9, KIV-10, and KV, inhibits the proliferation and migration of endothelial cells *in vitro* (14), in part by interfering with the activation of extracellular signal-regulated kinase 1 and 2 in endothelial cells via a protein tyrosine phosphatase-dependent pathway (15). Systemic administration of recombinant LK68 proteins suppresses angiogenesis-dependent growth of human colon (HCT-15) and lung (A549) tumors *in vivo*.

For antiangiogenic cancer therapy, chronic administration of antiangiogenic proteins is required (16) because micrometastases may remain dormant but viable for a long period of time. In addition, the relatively short half-life *in vivo* and high therapeutic doses of recombinant proteins pose manufacturing and economic constraints on their widespread clinical use (17, 18). The peak/trough kinetics resulting from bolus administration of recombinant proteins may be insufficient to maintain prolonged therapeutic levels in the tumor mass. Antiangiogenic gene therapy constitutes an attractive solution to overcome these problems (19–21) because this approach ensures sustained high levels of therapeutic molecules *in situ* simply via a few injections of the necessary antiangiogenic genes.

The present study was performed to analyze the therapeutic potential of gene therapy with LK68 in the treatment of life-threatening hepatic metastasis of colorectal cancer. Our data show that LK68 expression in colorectal cancer cells significantly suppresses hepatic metastasis by inhibiting tumor angiogenesis, consequently improving overall survival rates.

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**Note:** H-K. Yu and J-S. Kim contributed equally to this work.

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## MATERIALS AND METHODS

**Construction of Vectors and Expression of LK68 in CT26 Cells.** LK68 cDNA was amplified by polymerase chain reaction using pET11a/LK68 (14) as the template with the following oligonucleotide primers: 5'-GCGGC-CCAGCCGGCCAAAAGCCCTGTGGTCCAGGATTGC-3' (LK68-*Sfi*I) and 5'-CCGCTCGAGTTAAGAGGATGCACAGAGAGGGATATC-3' (LK68-*Xho*I). The amplified cDNA fragment spanning nucleotides 12,052 to 12,975 (9) was cloned into the corresponding sites of an expression vector, pSecTag (Invitrogen, Carlsbad, CA). The DNA fragment containing LK68 cDNA and the immunoglobulin  $\kappa$  chain secretion signal was amplified by polymerase chain reaction with primers IgkEcoRI (5'-CCGAATTCCTAGCCACCATTGGAGACAGACACTCTG-3') and LK68-*Xho*I, digested with *Eco*RI and *Xho*I, and cloned into a predesigned retroviral expression vector, pLXSN (Clontech, Palo Alto, CA). The resultant plasmid, pLXSN-LK68, was used for subsequent transient transfection into a packaging cell line (PT67) to generate recombinant retroviruses containing the LK68 gene, using the DOSPER liposomal reagent (Roche Diagnostics GmbH, Mannheim, Germany). Empty pLXSN was additionally used as a control. CT26 cells were exposed to retroviral vector-containing culture supernatant, and colonies of stable cells resistant to 1 mg/mL G418 (Calbiochem, La Jolla, CA) were selected and further characterized. CT26 cells transduced with recombinant viruses containing pLXSN or pLXSN-LK68 were designated CT-Vec and CT-LK68, respectively.

**Cell Proliferation Assay.** Parental (CT-Mock), CT-Vec, or CT-LK68 cells ( $1 \times 10^5$  cells) were seeded onto 6-well culture plates in triplicate and incubated with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were harvested by treatment with 0.5% trypsin/5.3 mmol/L EDTA-4Na (Life Technologies, Inc., Rockville, MD) and resuspended in PBS, and viable cells were counted every 24 hours using the trypan blue dye exclusion method for 5 days.

**Flow Cytometry.** CT-Mock, CT-Vec, or CT-LK68 cells were grown in DMEM supplemented with 10% FBS until the cells reached 80% confluence. The apoptosis assay was performed using the ApoAlert annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (Clontech). Briefly, cells were harvested, and  $5 \times 10^5$  cells were added to a 12-mm test tube. After washing twice with annexin buffer, cells were resuspended in 5  $\mu$ L of annexin V/FITC solution, incubated at 4°C for 15 minutes, and washed with annexin buffer. Propidium iodide solution (10  $\mu$ L of 10  $\mu$ g/mL) and 190  $\mu$ L of annexin buffer were added, followed by analysis with FACScalibur apparatus (Becton Dickinson Immunocytometry Systems, San Jose, CA). The results were processed under CellQuest software, analyzed by dual parameter analysis for propidium iodide staining (FL2) versus annexin V/FITC staining (FL1), and presented as dot plots. Cells grown in the presence of 10  $\mu$ g/mL Taxol (Sigma, St. Louis, MO) were used as an experimental control.

**Western Immunoblotting.** Confluent cultures of CT-Vec or CT-LK68 cells were incubated in PFM protein-free medium (HyClone, Logan, UT) for 72 hours on 6-well culture plates (Nalge Nunc International, Rochester, NY). Conditioned media were harvested from each clone and concentrated using trichloroacetic acid solution (Sigma). Concentrated proteins were separated on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Bio-Rad, Richmond, CA). After soaking for 1 hour in blocking buffer (5% nonfat dry milk in PBS), the membrane was incubated for 1 hour with polyclonal mouse antibodies against recombinant LK68, followed by horseradish peroxidase-conjugated goat antimouse IgG (KPL, Gaithersburg, MD). Immunoreactive bands were detected using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Freiburg, Germany), according to the manufacturer's instructions. Recombinant LK68 proteins produced from Chinese hamster ovary cells were used as a control.

**Animals.** Male BALB/c mice, 6–8 weeks of age, were purchased from Charles River Japan, Inc. (Yokohama, Japan). Animals were maintained under specific pathogen-free conditions. All mice were fed a commercial diet, given water *ad libitum*, and subjected to a 12-hour light/12-hour dark cycle.

**Animal Model for Experimental Liver Metastasis.** Two groups of mice were anesthetized intraperitoneally with 2.5% Avertin (Sigma). The spleen was exteriorized through a left lateral flank incision. CT-Vec or CT-LK68 cells ( $5 \times 10^4$ ) in 100  $\mu$ L of PBS were injected into spleen parenchyma using a 27-gauge needle. The peritoneum and skin were closed in two layers with metal clips. Mice that did not display visible "paling" of the spleen and severe

bleeding were used in subsequent studies. Mice were used in survival experiments ( $n = 10$  mice per group) or sacrificed at 6, 12, and 14 days ( $n = 5$  mice per group at each time point) after tumor cell injection. Liver samples were collected and subjected to histologic and immunohistochemical examination. All surgical procedures and care administered to the animals were in accordance with institutional guidelines.

**Histology and Immunohistochemistry.** Tumor-bearing livers were dissected, fixed with 10% neutral buffered formalin overnight, embedded in paraffin, and sectioned into 4- $\mu$ m slices. Sections were stained with hematoxylin and eosin (H&E) and subjected to immunohistochemical analysis. All tissue histology and necrosis areas were analyzed by H&E staining. Sections subjected to immunohistochemistry were cleared with xylene and rehydrated with ethanol. Slides used to detect the von Willebrand factor (vWF) and proliferating cell nuclear antigen (PCNA) were boiled in 0.01 mol/L citrate buffer (pH 6.0) for 10 minutes. Endogenous peroxidase was blocked with peroxidase blocking solution (DAKO, Carpinteria, CA) for 30 minutes. Sections were incubated overnight at room temperature with rabbit polyclonal anti-vWF antibody (DAKO) and monoclonal anti-PCNA antibody (DAKO). Next, samples were incubated with biotinylated goat antirabbit antibody for 30 minutes. After incubation with labeled streptavidin-biotin kit peroxidase (DAKO), stains were visualized with chromogen Nova Red (Vector Laboratories, Burlingame, CA).

**Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Staining.** Liver sections were processed in parallel for terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) using the ApopTag Red *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY), according to the manufacturer's instructions. Sections were incubated with 40  $\mu$ g/mL proteinase K for 10 minutes at 37°C to digest proteins. Intergen proprietary equilibration buffer was applied to sections at room temperature for 5 minutes, followed by incubation with DNA strand break labeling solution containing terminal deoxynucleotidyl transferase at 37°C for 1 h. Sections were washed in stop/wash buffer at room temperature for 10 minutes, followed by three 1-minute washes with PBS. Next, sections were incubated with anti-digoxigenin-alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, IN) at 37°C for 30 minutes. Tissues were counterstained with nuclear fast red (DAKO).

**Statistical Analysis.** Data are presented as means  $\pm$  SE. Statistical significance was calculated by Student's *t* test, with the exception of *in vivo* survival experiments, in which we used log-rank analysis of a Kaplan-Meier survival curve.  $P < 0.05$  was statistically significant.

## RESULTS

**Generation of Recombinant Colorectal Cancer Cells That Express LK68.** A cDNA sequence encoding human apo(a) kringle, LK68, was genetically fused to an immunoglobulin  $\kappa$  leader sequence to facilitate secretion of expressed protein and subsequently cloned into pLXSN retroviral vector to produce pLXSN-LK68, which induces transgene expression under control of the Moloney murine leukemia virus 5'-long terminal repeat promoter (Fig. 1A). LK68 expression was analyzed by Western blotting of culture supernatants prepared from transduced cells with anti-LK68 polyclonal antibody (Fig. 1B). LK68-immunoreactive proteins with molecular masses of 50,000 to 52,000 daltons were present in the CT-LK68 supernatant, whereas no signal was detected in supernatant fractions from CT-Vec or parental cells (CT-Mock). The molecular mass of LK68 produced in CT26 cells was identical to that of LK68 from Chinese hamster ovary cells but substantially higher than that of *Escherichia coli*-derived LK68 (~37,000 daltons), probably due to the presence of N- and O-linked glycosides (data not shown). The G418-resistant stable CT-LK68 clones showed variable level of LK68 expression. The concentration of LK68 in 72-hour conditioned medium (CM) secreted by  $1 \times 10^6$  cells of CT-LK68 (clone 7), showing the highest level of LK68 expression, was 80 ng/mL. Unless otherwise stated, CT-LK68 represents CT-LK68 clone 7 in the following experiments.

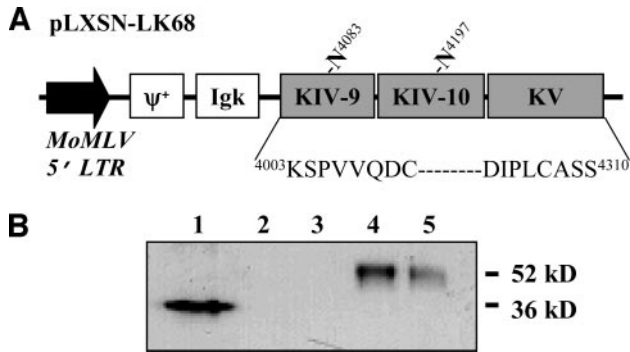


Fig. 1. Construction of a LK68 vector and expression of the protein in CT26 cells. *A*, schematic representation of the retroviral expression vector pLXSN-LK68. A truncated apo(a) kringle domain containing KIV-9, KIV-10, and KV (designated LK68) is depicted in gray boxes. LK68 constitutes the amino acid residues of apo(a) from Lys<sup>4003</sup> to Ser<sup>4310</sup>. Two predicted N-glycosylation sites (Asn<sup>4083</sup> and Asn<sup>4197</sup>) are depicted. Black arrow, the 5'-long terminal repeat promoter derived from the Molony murine leukemia virus.  $\Psi$ , the retroviral packaging signal. *Igk*, immunoglobulin  $\kappa$  leader sequence. *B*, Western blot displaying LK68 expression. Lane 1, LK68 produced from *E. coli*; Lane 2, CM from CT-Mock; Lane 3, CM from CT-Vec; Lane 4, CM from CT-LK68; Lane 5, LK68 produced from Chinese hamster ovary cells. Molecular mass markers are shown on the right.

**Characterization of *In vitro* Growth Kinetics and Apoptosis of Genetically Modified CT26 Cells.** To determine whether the expression of LK68 in CT26 cells affects cell growth, we examined the *in vitro* growth kinetics of control and LK68-expressing CT26 cells by viable cell counting. There were no significant differences in cell growth among CT-Mock, CT-Vec, and CT-LK68 cells (Fig. 2A). In addition, we analyzed tumor cell viability and/or apoptosis by flow cytometry after staining CT26 cells with annexin V and propidium iodide. Cell viability was similar between CT-LK68 and control cells, such as CT-Mock and CT-Vec (Fig. 2B). In all cases,  $\geq 97\%$  of cells retained viability. CT-Mock cells treated with cytotoxic agents such as Taxol (10  $\mu\text{g}/\text{mL}$ ) were used to define the dead cell fraction. Moreover, there was no significant clonal variation because three different stable clones of CT-Vec or CT-LK68 revealed similar growth kinetics and cell viability (data not shown). The results indicate that LK68 gene delivery and expression by a retroviral vector do not intrinsically affect the growth characteristics and viability of transduced cells.

**Inhibition of Endothelial Cell Migration by LK68 Derived from CT-LK68 Cells.** To analyze the antiangiogenic activity of LK68 produced by CT-LK68 cells, we performed an *in vitro* wound migration assay according to a previous report (14). Human umbilical vein endothelial cells (HUVECs) migrated readily to the denuded area in response to filtered CM prepared from CT-Vec cells (Fig. 3A). However, on treatment of HUVECs with CM from CT-LK68, an approximate 50% reduction in cell migration was observed, compared with controls ( $P < 0.05$ ; Fig. 3B). To determine whether the decreased migration of HUVECs is mediated by LK68 present in CM, the migration assay was performed in the presence of rabbit anti-LK68 antisera or preimmune rabbit sera. HUVEC migration recovered to control levels after pretreatment with anti-LK68 antibodies ( $P < 0.05$ ). In contrast, control antibodies had no influence on migration patterns, clearly signifying that LK68 is responsible for the observed antimigratory effects (Fig. 3B).

**Suppression of Liver Metastasis after Intrasplenic Injection.** To determine the effect of LK68 expression in colorectal cancer cells on hepatic metastasis, CT-Vec and CT-LK68 cells were injected into the spleens of syngeneic BALB/c mice. Livers from both groups of animals ( $n = 5$  mice per group at each time point) were collected at postoperation day (POD) 6, 12, and 14. Macroscopically, no significant differences were observed until POD 12 in terms of shape, size, liver weight, and number of metastasized surface nodules. At POD 14,

control animals displayed enlarged livers covered with numerous tumor nodules throughout and with a whitish and irregular surface caused by extensive tumor growth, whereas livers from mice injected with CT-LK68 cells were smaller in size, displaying a macroscopically normal appearance with a few surface nodules (Fig. 4A). The suppressive effect on liver metastasis was somewhat proportional to the expression levels of LK68 proteins in transduced clones (data not shown). The number of tumor nodules on the surface of livers from mice that received injection with CT-Vec and CT-LK68 showing the highest expression of LK68 were  $125 \pm 14$  and  $46 \pm 3$ , respectively ( $P < 0.001$ ; Fig. 4B). A significant decrease in liver weight was additionally observed ( $P < 0.001$ ). Specifically, the mean liver weights were  $1.78 \pm 0.22$  and  $1.37 \pm 0.05$  g for CT-Vec- and CT-LK68-injected mice, respectively (Fig. 4C). Moreover, similar inhibition of liver metastasis was also observed in mice that received intrasplenic injection with LK68-expressing LS174T human colorectal cancer cells (data not shown). These results may indicate that suppression of colorectal cancer liver metastasis by LK68 is not specific to the CT26 cell line, but rather representative of colorectal cancer in general.

To further characterize the antimetastatic effects of LK68, histologic examination of H&E-stained liver tissues was performed. The extent of liver metastasis was assessed in terms of number and size of metastases. The formation of neovessels within hepatic metastases was reported to be required for tumors to grow beyond a critical size

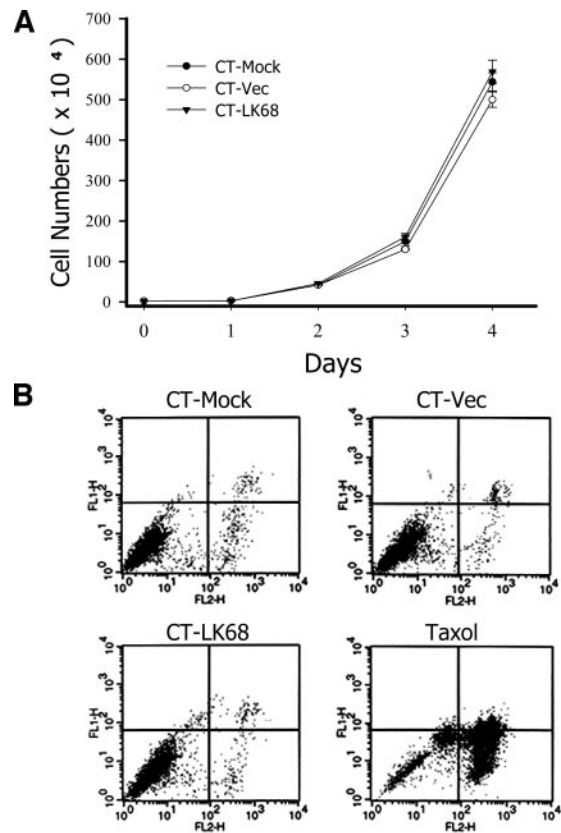


Fig. 2. Effects of LK68 expression on the growth and viability of CT26 cells *in vitro*. *A*, CT-Mock, CT-Vec, or CT-LK68 cells were incubated in DMEM supplemented with 10% FBS, and the numbers of viable cells over 5 days were counted using the trypan blue dye exclusion method. Results are representative of three separate experiments (mean  $\pm$  SE). *B*, CT-Mock, CT-Vec, or CT-LK68 cells were grown in DMEM supplemented with 10% FBS and stained with annexin V/FITC and propidium iodide. Apoptosis was analyzed by flow cytometry, as described in Materials and Methods. FL1 on the Y axis and FL2 on the X axis represent annexin V/FITC staining and propidium iodide staining, respectively.

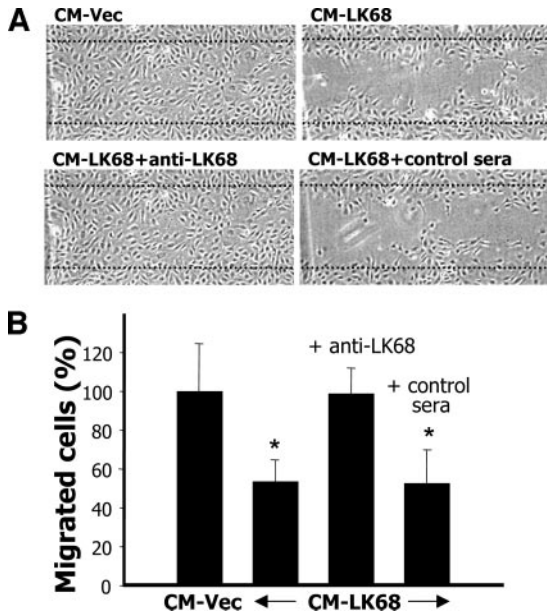


Fig. 3. Inhibition of HUVEC migration by LK68. Confluent HUVECs were scraped and incubated with CM from CT-LK68 in the presence of either rabbit anti-LK68 antisera or preimmune control sera, and migration of HUVECs into the denuded area was monitored over 8 hours. CM from CT-Vec was used as a control. *A*, representative photomicrographs of migrated cells. The dotted lines indicate the area occupied by the initial wound. *B*, Cells that migrated to the denuded area were counted and expressed as percentage of migrated cells treated with CM from CT-Vec. Results are representative of three separate experiments (means  $\pm$  SE). \*,  $P < 0.05$ .

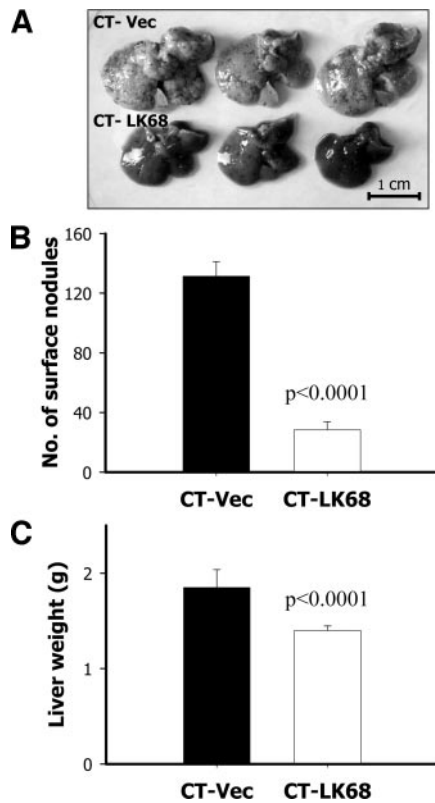


Fig. 4. Macroscopic evaluation of the effects of LK68 expression in CT26 cells on liver metastasis. CT-Vec and CT-LK68 cells were injected into the spleens of BALB/c mice. Fourteen days later, mice were sacrificed, and liver metastases were examined. *A*, representative photograph showing livers obtained from mice that received injection with CT-Vec and CT-LK68, as indicated. Scale bar, 1 cm. *B* and *C*, quantitative analysis of the number of surface tumor nodules and weights of tumor-bearing livers from mice that received injection with CT-Vec and CT-LK68 cells. These experiments were repeated three times with similar results. Data are expressed as means  $\pm$  SE.

(200  $\mu$ m in diameter; ref. 22). On the other hand, it was reported that tumors below  $\sim$ 1 mm in diameter are supplied sufficient nutrients by diffusion (23). In view of this finding, we graded metastases sizes into three levels, specifically, small (<200  $\mu$ m in diameter), medium (200  $\mu$ m to 1 mm in diameter), and large (>1 mm in diameter), representing prevascular metastases, metastases in progression to a vascularized state, and macroscopic vascularized metastases, respectively. Relatively few metastases were observed in both groups of animals at POD 6 and POD 12. However, a significant increase in total metastasis number was detected in livers from control animals at POD 14, compared with livers bearing CT-LK68 cells ( $P < 0.001$ ; Fig. 5A). The sizes of liver metastases were substantially enlarged in a time-dependent manner in control organs. Livers containing CT-LK68 cells were mainly occupied by small-sized metastases at all time points (Fig. 5). The results collectively indicate that expression of LK68 affects the hepatic metastasis of colorectal cancer cells, primarily at the level of angiogenesis-dependent growth of micrometastases.

**Suppression of Tumor Angiogenesis and Induction of Tumor Apoptosis in Liver Metastases Generated by LK68-Expressing Colorectal Tumor Cells.** To investigate the mechanism of LK68-mediated inhibition of liver metastasis *in vivo*, liver samples isolated at POD 14 were examined histologically and immunohistochemically for tumor cell proliferation, apoptosis, and angiogenesis. Histologic examination of H&E-stained liver tissues revealed that very large areas of the liver contained metastases in controls bearing CT-Vec cells, compared with those containing CT-LK68 cells (Fig. 6A and B). Tumor cell proliferation and apoptosis were examined by immunostaining tissue sections using antibodies against PCNA (Fig. 6C and D) and *in situ* labeling of fragmented DNA in apoptotic cells using TUNEL technique (Fig. 6E and F), respectively. The proliferative index and apoptotic index were determined by counting the number of positive cells in liver metastases, which were expressed as a percentage of total cells. Proliferative indices were similar in liver metastases generated by CT-Vec ( $64.35 \pm 1.96\%$ ) and CT-LK68 ( $64.52 \pm 3.27\%$ ; Fig. 7A). Interestingly, apoptotic indices were significantly increased in liver metastases generated by CT-LK68 ( $5.37 \pm 3.33\%$ ), compared with CT-Vec ( $0.48 \pm 0.47\%$ ;  $P < 0.001$ ;

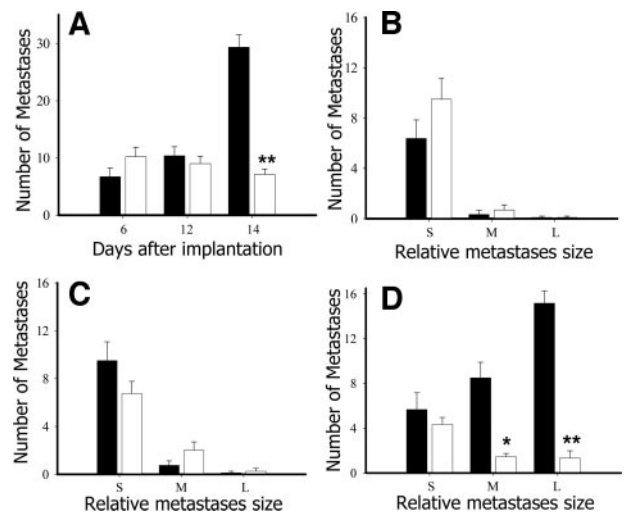


Fig. 5. Histologic examination of the effect of LK68 expression on the number and size of liver metastases. Mice received intrasplenic injection with CT-Vec (■) or CT-LK68 (□) cells. Livers were isolated at 6, 12, and 14 days after tumor cell administration, and histologic examination of H&E-stained tissue sections was performed. *A*, quantitative analysis of the total number of metastases in tumor-bearing livers. *B-D*, distribution of the sizes of metastases in livers at 6 (*B*), 12 (*C*), and 14 days (*D*) after tumor cell administration. Metastatic foci were subdivided into three groups according to diameter: small (S), <200  $\mu$ m; medium (M), 200  $\mu$ m to 1 mm; large (L), >1 mm. \*,  $P < 0.0005$ . \*\*,  $P < 0.0001$ .

Fig. 7B). There were no significant effects on proliferation and apoptosis in the normal regions of liver tissues from both groups of mice, indicating that intratumoral expression of LK68 is not toxic to normal liver tissues. We immunostained tumor-bearing liver tissues with an endothelial-specific antibody raised against vWF to determine the effects of LK68 expression on tumor angiogenesis (Fig. 6G and H). A significant reduction in the number of vWF-positive blood vessels was observed in LK68-expressing liver metastases, compared with control tumors (Fig. 7C;  $P < 0.001$ ). The results indicate that LK68 expression in colorectal cancer cells reduces angiogenesis and induces apoptotic death of tumor cells that metastasized into the liver with no toxicity to normal liver tissues.

**Extension of Survival by LK68 Gene Delivery.** To analyze whether the suppression of liver metastasis by LK68 expression prolongs overall survival, mice ( $n = 10$  mice per group) received intrasplenic injection with CT-Vec or CT-LK68 cells, and the fraction of surviving animals was monitored over time. As depicted in Fig. 8, host survival was significantly improved in animals bearing CT-LK68 cells, compared with control animals (log-rank test,  $P < 0.001$ ). The median survival was 24.5 and 35 days for groups of animals injected

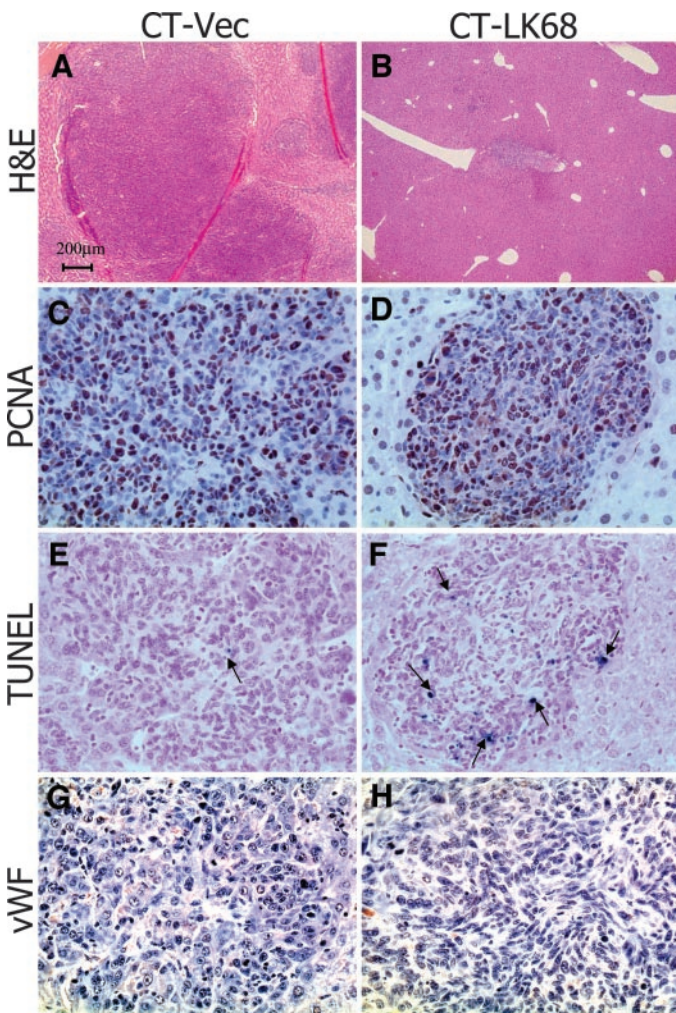


Fig. 6. Histologic and immunohistochemical examination of proliferation, apoptosis, and angiogenesis in tumors. Representative photomicrographs of tumor tissues from mice that received injection with CT-Vec (A, C, E, and G) or CT-LK68 cells (B, D, F, and H) are shown. A and B. Sections of tumor tissue were stained with H&E. Magnification,  $\times 40$ . Scale bar, 200  $\mu\text{m}$ . C and D. Tumor cell proliferation was determined by immunostaining of tumor tissues with anti-PCNA antibodies. Magnification,  $\times 400$ . E and F. Tumor cell apoptosis was examined by labeling fragmented DNA using TUNEL. Magnification,  $\times 400$ . Apoptotic cells are indicated by arrows. G and H. Immunostaining of tumor tissues using an antibody against vWF is depicted. Magnification,  $\times 200$ .

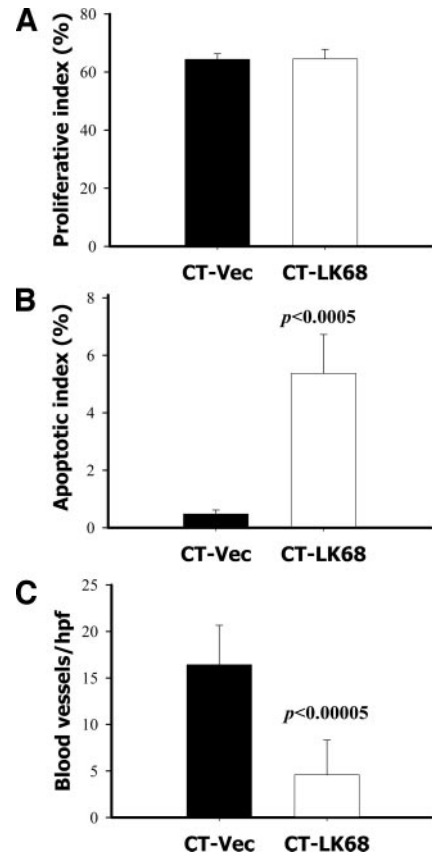


Fig. 7. Quantitative analysis of proliferation, apoptosis, and angiogenesis. A. The cell proliferative index was measured by counting cells stained with anti-PCNA antibody and expressed as a percentage relative to the total number of cells. B. The apoptotic index was measured by labeling fragmented DNA in apoptotic cells using TUNEL, counting cells, and expressing values as percentages relative to the total number of cells. A and B. Approximately 2,000 cells were counted at a magnification of  $\times 400$ . The values in each column represent means  $\pm$  SE. C. Tumor tissues were immunostained with anti-vWF antibodies, and blood vessels in at least 10 random high-power fields were counted. Each column represents means  $\pm$  SE.

with CT-Vec and CT-LK68 cells, respectively. Our data collectively indicate that inhibition of hepatic metastasis of colorectal cancer cells by LK68 gene delivery leads to substantial extension of survival.

## DISCUSSION

Antiangiogenesis therapy is a promising treatment for cancer, based on the finding that most tumors and their metastasis are angiogenesis dependent. Gene therapy can potentially expand the horizons of tumor angiogenesis therapy due to the production of high concentrations of therapeutic agents within a local area for a sustained period. Antiangiogenic gene therapy with angiostatin (24) or endostatin (25) induces significant anti-metastasis effects, supporting its potential use for the treatment of colorectal cancer metastatic to liver. Moreover, the idea that blocking angiogenesis is an effective strategy to treat human metastatic colorectal cancer can be further validated by the recent approval of bevacizumab (Avastin), a humanized monoclonal antibody against vascular endothelial growth factor, by the United States Food and Drug Administration as a first-line therapy for metastatic colorectal cancer. In the present study, we show that expression of LK68 in colorectal cancer cells substantially suppresses hepatic metastasis and has statistically significant survival advantages in animals, indicating that LK68 cDNA is a promising candidate as a gene therapy agent to treat hepatic metastasis of colorectal cancer.

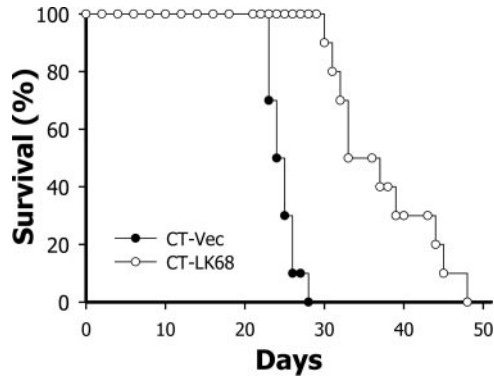


Fig. 8. Kaplan-Meier survival curve of mice bearing liver metastases. Two groups of mice ( $n = 10$  mice per group) received intrasplenic injection with CT-Vec or CT-LK68 cells, and the fraction of surviving mice was monitored over time. Differences in survival were statistically significant, as determined by log-rank analysis ( $P = 0.001$ ).

Angiogenesis is the net result of a dynamic balance between proangiogenic and angiostatic factors in the extracellular microenvironment of a tumor (26). Expression of LK68 in tumors may either increase the local concentrations of angiogenesis inhibitors or decrease the expression of angiogenic factors, such as angiogenin, basic fibroblast growth factor, and vascular endothelial growth factor (14), and consequently shifts the balance between angiogenesis and antiangiogenesis in favor of the latter, leading to suppression of tumor angiogenesis. This theory was confirmed by histologic and immunohistochemical findings showing that in LK68-expressing colorectal cancer liver metastases, microvessel density was substantially decreased and metastases were restricted to sizes smaller than  $\sim 200 \mu\text{m}$  in diameter. Notably, angiogenesis is required for liver metastases to grow beyond this size (22). Therefore, LK68 appears to suppress hepatic metastasis of colorectal cancer by inhibiting the development of new vasculatures in liver metastases, thus limiting the progression of prevascular micrometastases to a vascularized state and growth of vascularized metastases. Similarly, systemic administration of the angiogenesis inhibitor endostatin has been reported to inhibit the generation of hepatic micrometastases and macrometastases growth of colorectal cancer cells (27).

Expression of LK68 in colorectal cancer cells did not affect the growth kinetics and viability of cancer cells *in vitro*. However, interestingly, tumor cell apoptosis was considerably increased in LK68-expressing liver metastases *in vivo*, whereas no significant effects on tumor cell proliferation were observed. Similar results were observed in metastatic liver cancer treated by either gene transfer using adeno-associated virus vector encoding angiostatin (24) or a small molecule inhibitor of vascular endothelial growth factor receptor, SU5416 (28). The mechanism of induction of tumor cell apoptosis is currently unclear, but it appears to be associated with the inhibition of tumor angiogenesis by LK68, which may limit the supply of oxygen and nutrients to tumor cells. When the loss of adequate vasculature occurs, tumor cells supported by these vessels subsequently undergo apoptosis. Several studies show that angiogenesis inhibitors induce tumor cell apoptosis by decreasing the levels of paracrine tumor cell survival factors derived from endothelial cells, such as platelet-derived growth factor, interleukin 6, and heparin-binding epithelial growth factor, among others (29). However, the issue of whether LK68 directly inhibits the production of these paracrine factors by endothelial cells remains to be elucidated.

Metastasis is a highly selective and nonrandom process that consists of a series of steps, including escape of cells from a primary tumor into the blood or lymphatic circulation (intravasation), survival

of cells in the circulation, arrest in a secondary organ, escape from circulation into the surrounding tissue (extravasation), initiation and maintenance of growth, and vascularization of the metastatic tumor (30, 31). Any of these phases of metastasis may be a therapeutic target because the failure of any one step disrupts the entire metastatic cascade.

By the time primary colorectal cancers are detected, subclinical or clinically relevant liver metastases have already occurred (32). Thus, later steps offer more promising targets for therapy because these may not have occurred at the time of colorectal cancer diagnosis. Recent studies demonstrate that cancer cell arrest and survival in the microcirculation and the extravasation process are performed with very high efficiency (33, 34), whereas the initiation of growth to form micrometastases and formation of macroscopic metastases are considerably less efficient. Targeting an inefficient biological process is easier than targeting an efficient one because fewer cells are inhibited. In view of these considerations, antiangiogenesis therapy using LK68, which primarily targets the angiogenesis-dependent growth of metastasis, is a clinically accessible and biologically relevant therapeutic strategy for liver metastasis.

Two general strategies of antiangiogenic gene therapy have been proposed, specifically, tumor-directed gene therapy and systemic gene therapy. Because antiangiogenesis therapy displays a low toxicity profile and is designed to treat both tumor metastasis and growth, almost all therapeutic approaches to date have been systemic in nature. However, in the treatment of colorectal cancer metastatic to liver, tumor-directed, regional administration of angiostatic genes is advantageous because this ensures maximal levels of transgenic proteins *in situ* and minimal systemic toxicity, compared with systemic gene delivery. Moreover, the unique anatomic features of the liver facilitate regional treatment approaches (35).

As antiangiogenic drugs are directed against components of the developing vasculature, they may lead to stable disease rather than complete remission and have little or no effect on bulky end-stage tumors. Moreover, discontinuation of antiangiogenic therapy may allow the tumor or its metastases to resume growth (36–38). Hence, the patients most suitable for long-term antiangiogenic treatment are possibly those with “early metastatic” or “minimal residual” disease, such as the growth of dormant metastases occurring after surgical removal of a primary tumor (5, 39) or following the reduction of the primary tumor by ionizing radiation (40). The experimental model used in this study is appropriate for this patient category. In this setting, therapeutic antiangiogenesis may prolong the state of tumor dormancy by suppressing micrometastases that remain despite successful treatment of primary tumors.

Although antiangiogenic therapy using LK68 induces dormancy of micrometastases, complete eradication of dormant tumor cells that exist in the form of either nonproliferating solitary cells or preangiogenic micrometastases was not accomplished in this study. These dormant cells pose a substantial future danger of metastasis recurrence. Accordingly, a combination of antiangiogenic therapy and conventional chemotherapy (41, 42) or radiotherapy (43) is an appropriate clinical approach. Such a combination with anti-growth therapies may improve suppressive effects because they directly target the growth of tumor cells, leading to remission of metastases and tumor cell eradication.

Our data clearly show that LK68 cDNA is a promising candidate for antiangiogenic gene therapy. In parallel with the development of gene delivery systems that present therapeutic genes specifically and efficiently to the tumor site, future application of antiangiogenic gene therapy with LK68 in clinical settings may provide an efficient way to treat colorectal cancer metastatic to the liver.

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