

Systemic Tumor Suppression by the Proapoptotic Gene *bik*¹

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Abstract

Metastatic breast cancer requires systemic treatment. We have developed a systemic gene therapy approach for breast cancer, consisting of a nonviral gene delivery system (SN) and a proapoptotic gene, *bik*. The transfection efficiency of SN carrying a reporter gene was 5–10 times higher than the common nonviral agents Fugene-6 and Lipofectamine in the presence of serum. The SN-*bik* gene complex induced significant apoptosis in four breast cancer cell lines *in vitro* as well as in orthotopic tumor tissues in nude mice. Systemically administrated SN-*bik* significantly inhibited the growth and metastasis of human breast cancer cells implanted in nude mice and prolonged the life span of the treated animals. This study demonstrates that SN-*bik* is a promising approach for further development as a potential therapeutic agent of cancer.

Introduction

The *bik* gene is a proapoptotic member in the Bcl-2 gene family. Loss of informative alleles on chromosome 22q where the *bik* gene is located may be related to the development of human breast and colorectal cancers (1, 2). The *bik* gene encodes a M_r 18,000 protein product, which contains a BH3 domain critical for its proapoptotic activities (3, 4). *bik* forms heterodimers with various antiapoptotic proteins, including Bcl-2 and Bcl-X_L (3, 4), the association of which hinders the function of the antiapoptotic proteins. *bik* triggers apoptosis through a *p53*-independent pathway (4), suggesting a broad potential of *bik* to target different types of cancer cells. However, a method for delivering the *bik* gene to human cells *in vivo* has not been reported.

Breast cancer is a metastatic disease. Drug delivery systems able to systemically target cancer cells are required for efficient treatment of breast cancer patients. Nonviral gene delivery systems have been attractive strategies for therapeutic application. Cationic lipids of high binding affinity with negatively charged DNA molecules are the most commonly used vehicle because of their minimal immunogenicity and low toxicity *in vivo*. However, the low DNA transduction efficiency and instability in the presence of serum has limited the application of lipid-based systemic delivery systems. In this report, we used a novel formulation of cationic lipid that resulted in enhanced biostability and tumor suppression function when used to deliver the proapoptotic *bik* gene to breast tumor cells *in vitro* and *in vivo*.

Materials and Methods

Cell Lines. Human breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-435, and MDA-MB-468 and the human non-small cell lung carcinoma cell line A549 were purchased from the American Type Culture Collection (Rockville, MD) and cultured according to the vendor's instructions.

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Expression Plasmids. *bik*, *luc*³-, *p53*-, and GFP-expressing plasmids were constructed by inserting the cDNAs of *bik*, *luc*, *p53*, and *GFP*, respectively, into the pcDNA3 vector containing a cytomegalovirus promoter.

Formulation. The gene delivery system, termed SN, was essentially a cationic liposome formulation composed of dipalmitoylethylphosphocholine, dioleoylphosphoethanolamine, dipalmitoylphospho-ethanoamine, and polyethyleneglycol. The DNA was entrapped in the liposome using the thin-lipid film hydration method and extrusion through a filter with 0.2- μ m-diameter pores (Gelman Sciences; Ann Arbor, MI) as described previously (5). The liposomal DNA particles were 60–170 nm in diameter.

Transfection. Cells were cultured for 24 h in six-well plates with 1 ml/well of DMEM/F12 medium with 10% FBS (Life Technologies, Inc., Gaithersburg, MD) until 60–70% confluence was reached. The liposomal DNA (SN-DNA or Lipofectamine-DNA complex) or nonliposomal DNA (Fugene-6-DNA complex) was directly added into the culture plates at a ratio of 2 μ g of DNA/10⁶ cells. Twenty-four h later, the transfection efficiency was determined by counting the GFP-positive cells under a fluorescence microscope and expressing the result as a percentage of total cells. Six random fields with >200 cells/field were counted for each sample. All experiments were repeated three times independently.

Colony Formation in Soft Agarose. The standard colony formation assay (6) was used to test whether transfection of *bik* in cationic liposomes inhibits colony formation of tumor cells in soft agarose. Briefly, cells of the human breast cancer lines MDA-MB-231 and MDA-MB-468 were transfected with SN-DNA. One day after the transfection, the cells (5×10^3 cells/well) were plated in six-well plates in culture medium containing 0.5% agarose overlying a 1% agarose bottom layer and cultured at 37°C with 5% CO₂. Five weeks later, the top layer of the culture was stained with *p*-iodonitrotetrazolium (1 mg/ml). Colonies >100 μ m in diameter were counted.

Apoptosis Assay. For *in vitro* studies, standard fluorescence-activated cell sorter analysis was used to determine the apoptosis of the cells. Briefly, the cells were transfected with SN-*bik* or other agents. Forty h after transfection, the apoptotic cells were assessed by flow cytometric detection of sub-G₁ DNA content after being stained with propidium iodide. Fields with >2000 cells in each were randomly selected, and the apoptotic *versus* nonapoptotic cells were counted. For *in vivo* studies, female nude mice (*nu/nu*, 7–8 weeks of age, 18–22 g; Harlan Sprague Dawley, Madison, WI) were inoculated with the human breast cancer cell line MDA-MB-231 in the MFPs at a dose of 2×10^6 cells/mouse (one tumor/mouse). Five weeks later, the tumor-bearing mice were randomly divided into two groups with three mice in each group. The mice in the treatment group received a single *i.v.* injection of SN-*bik*, 15 μ g of DNA/mouse. The mice in control groups received the same dose of SN-*luc*. One day after the injection, the tumors and other organs were resected. The tissues were fixed in 10% buffered formalin for 12 h and were then processed and embedded in paraffin. The slides were then deparaffinized, rehydrated, refixed in 4% formalin, and digested in 20 μ g/ml proteinase K solution for 15 min. The slides were washed in PBS and refixed in 4% formalin, washed again in PBS, and equilibrated in equilibration buffer. Biotinylated nucleotide mix and TdT enzyme were added and incubated for 1 h at 37°C; slides were washed in PBS, blocked in hydrogen peroxide, and incubated in streptavidin horseradish peroxidase. The slides were developed in 3,3'-diaminobenzidine and counterstained with Harris hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. The apoptosis index was defined by the percentage of brown cells among the total cells of each sample. Ten fields with >200 cells in each were randomly counted for each sample.

³ The abbreviations used are: *luc*, luciferase; GFP, green fluorescent protein; FBS, fetal bovine serum; MFP, mammary fat pad; RLU, relative luc unit; RES, reticuloendothelial system.

Ex vivo Tumor Inhibition. MDA-MB-231 and MDA-MB-468 cells were transfected by SN-*bik* or SN-*luc*. Twenty-four h after transfection, the cells were carefully trypsinized, harvested, and inoculated into the MFPs of nude mice (2×10^6 cells/tumor). The volume of the resulting tumor was measured weekly.

Tissue Distribution of the SN-delivered Reporter Gene. MDA-MB-231 cells were inoculated into MFPs of the nude mice. Five weeks later, the tumor-bearing mice received a single i.v. injection of SN-*luc* at a dose of 60 $\mu\text{g}/\text{mouse}$. One day after the injection, the mice were killed, and the tumors and other organs were resected and immediately frozen on dry ice. The tissues were homogenized after adding $1 \times$ lysis buffer (Promega Corp., Madison, WI) with a volume (μl) equivalent to five times the tissue weight (mg). The tissue suspension was centrifuged at $2500 \times g$ for 10 min after undergoing a freeze-thaw procedure. *luc* activity in the supernatant was determined with a luminometer (Promega). The *luc* activity in 100 mg of tissue was used to compare gene expression in different tissues. To compare the gene delivery capabilities, the commercial transfection kits Fugene-6 and Lipofectamine were used as controls. The carrier:DNA ratio was 2:1 for SN-*luc*, 5:1 for Fugene-6-*luc*, and 9:1 for Lipofectamine-*luc* complex.

Antitumor Activity Tests. To study tumor growth inhibition, female nude mice were inoculated with 2×10^6 of breast cancer cells/tumor into the MFPs. Two weeks later, when most tumors exceeded 4×4 mm, the tumor-bearing mice were randomly divided into three groups with 5 mice in each group. The mice in all treatment groups received i.v. injections of SN-*bik* twice a week for 3 weeks, at a dose of 15 μg of DNA/mouse. The mice in control groups were injected with the same dose of SN-*luc* or the same volume of PBS. The tumor volume was measured weekly. To assess animal survival and the increase in life span, the same tumor models and the same therapeutic treatments were used. The experiment was terminated on day 200 after tumor inoculation. To evaluate tumor metastasis, the nude mice inoculated with 2×10^6 MDA-MB-468 cells into their MFPs were randomly divided into three groups with 5 mice in each group. Two weeks after the inoculation, the mice were treated by SN-*bik*, SN-*luc*, or PBS as mentioned above. Autopsies were performed on sacrificed mice at week 10. The metastasis was identified in the peritoneal cavity, and the numbers of metastatic tumor nodules <1 mm in diameter were counted.

Statistical Analysis. All statistical tests used in this study are two-sided log-rank statistical tests.

Results

SN Efficiently Transfected Genes into Cancer Cells in Serum-supplemented Cultures. The formulated SN liposome was first tested for its gene delivery efficiency by transfection of a reporter gene, *GFP*, in cell culture. To mimic the systemic administration condition, transfection was performed in medium supplemented with serum. For comparison, SN transfection was done side by side with Fugene-6 and Lipofectamine, the most popular commercial transfection agents. Four different breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-468) were transfected in the presence of 10% FBS. The transfection efficiency of SN was 12–32-fold higher than that of Lipofectamine in all cell lines tested ($P < 0.001$; Fig. 1A). SN was 1.6–1.8-fold more efficient in MCF7, MDA-MB-231, and MDA-MB-468 cells ($P < 0.01$) and similar ($P > 0.05$) in MDA-MB-435 cells compared with Fugene-6. SN and the commercial transfection kits showed similar transfection efficiencies in serum-free cultures (data not shown).

SN Efficiently Delivered Gene to Orthotopic Breast Tumors in Mice via i.v. Injection. We further tested the gene delivery capability of SN by determining the tissue distribution after tail vein injection in nude mice. Nude mice with MDA-MB-231-derived tumors inoculated in the MFPs were given tail-vein injections of luciferase gene (60 μg) entrapped in SN (SN-*luc*). Twenty-four h after injection, mice were killed, protein extracts isolated from the tumors and different organs, and the relative luc activity was determined using a luminometer. For comparison, Fugene-6 and Lipofectamine were also included in the test. The results showed that overall SN was more efficient in sys-

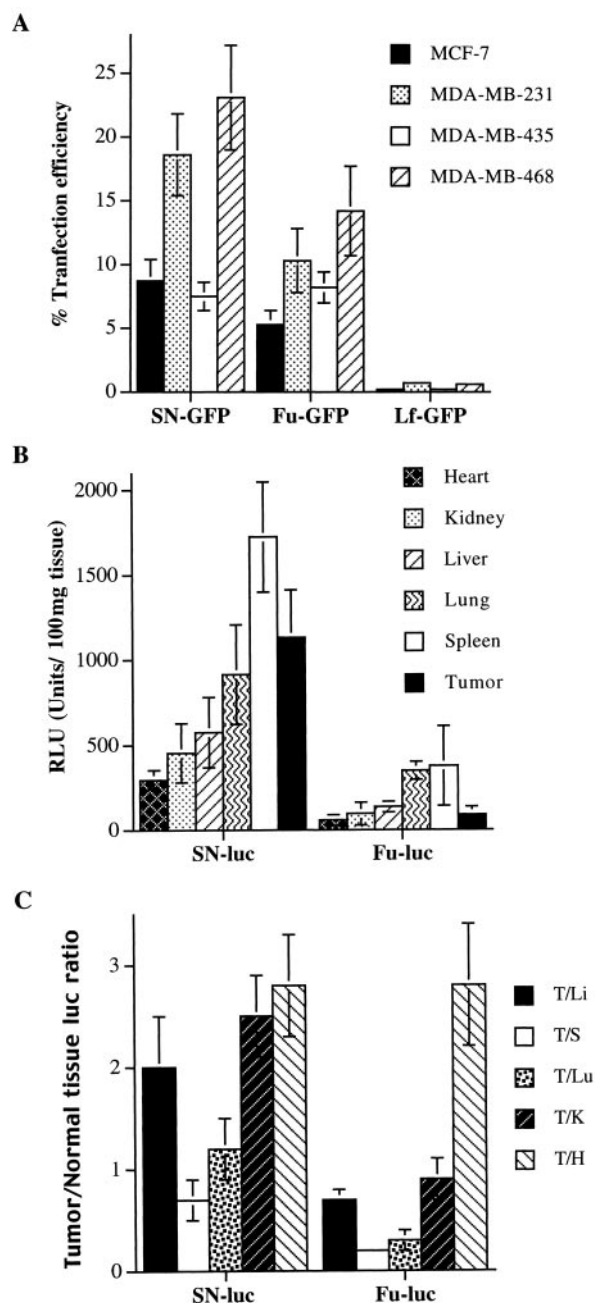


Fig. 1. SN is a better nonviral gene delivery system in serum-containing tissue culture. A, human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-435, or MDA-MB-468 ($4\text{--}5 \times 10^5$ cells/well in six-well plates) were transfected with SN-encapsulated *GFP* (SN-*GFP*), Fugene-6-*GFP* complex (Fu-*GFP*), or Lipofectamine-*GFP* complex (Lf-*GFP*), respectively, in DMEM/F12 medium supplemented with 10% FBS. The transfection condition was optimized for each formulation. The transfection efficiency was determined by counting the percentage of fluorescent cells under a fluorescence microscope. Each sample was randomly counted in six fields with >200 cells/field. The data are the means from three independent experiments; bars, SD. B, SN is efficient for systemic gene delivery to breast tumors *in vivo*. MDA-MB-231 cells (2×10^6 cells/mouse) were inoculated into the MFPs of nude mice. Six weeks later, mice bearing tumors received single i.v. injections of SN-entrapped luciferase plasmid (SN-*luc*). Twenty-four h after the injection, the *luc* activities in tumor and normal tissues as indicated were determined by a luminometer. The *luc* activity in 100 mg of wet tissue was used as RLU to measure gene expression and distribution in tissues. The commercial transfection kit Fugene-6 was used as a control. The data are the means from three mice of each group; bars, SD. The tumor:tissue expression ratios of luciferase are summarized in C. Bars, SD.

temic gene delivery than Fugene-6 (Fig. 1B). The luciferase expression levels in SN-*luc*-injected mice were 13-fold higher in tumors and 2–7-fold higher in normal organs compared with Fugene-6-*luc*-injected mice ($P < 0.001$). There was no detectable luciferase signal

in any organ and tumor in Lipofectamine-*luc*-injected mice (data not shown). By comparing the tumor *versus* normal tissue ratio of *luc* activity, we found that SN-mediated gene delivery was more tumor specific, and the tumor:normal tissue ratio of SN-*luc*-injected mice was 2–3-fold higher than that of Eugene-6-*luc*-injected mice (Fig. 1C). The luciferase expression level in the orthotopic tumors of SN-*luc*-injected mice was higher than in all other tissues except spleen. The RLUs in the tumors were 2.0-, 2.5-, and 3.8-fold higher than that in livers, kidneys, and hearts ($P < 0.01$, $n = 3$), respectively, and it was similar to that in lung (1132 to 915 RLU; $P > 0.01$). The higher gene expression in tumors implied the possibility that i.v. injection of SN encapsulating a therapeutic gene would kill breast tumor cells without life-threatening toxicity.

***bik* Transfection Induced Significant Apoptosis of Cancer Cells *in Vitro*.** Having shown the efficiency and specificity of the SN lipid in DNA delivery, we next tested the apoptosis induction of SN lipid combined with the *bik* gene. Breast cancer cell lines MCF-7, MDA-MB-435, MDA-MB-231, and MDA-MB-468 were transfected by either a *bik*- or a *luc*-expressing plasmid entrapped in the SN lipid (SN-*bik* or SN-*luc*) in serum-supplemented medium, and the levels of apoptotic cells were determined by flow cytometry (Fig. 2A). The results indicated that SN efficiently introduced the apoptotic gene into various cancer cell lines and resulted in a remarkable rate of apoptosis induction. The induction of apoptosis was independent of the status of the *p53* gene in the cancer cells because cell lines with mutated *p53* (MDA-MB-231 and MDA-MB-468) or wild-type *p53* (MCF-7 and A549) were similarly affected by the treatment. In contrast, SN-*p53* had only a minimal effect on A549 and MCF-7, which contain wild-type *p53* gene (7).

The SN-*bik*-induced apoptosis resulted in reduced potential for colony growth of cancer cells in soft agar. Compared with the non-treated control, colony formation of the SN-*bik*-treated MDA-MB-231 and MDA-MB-468 cells was reduced 86 and 95%, respectively, whereas only a minor effect was observed in the SN-vector-treated cells (Fig. 2B). These results suggest that SN-*bik* transfection can inhibit tumorigenicity of breast cancer cells.

SN-*bik* Inhibited Tumor Growth in Nude Mice. The antitumor activity of SN-*bik* was next tested in an *ex vivo* assay. The MDA-MB-231 and MDA-MB-468 cells were transfected in cell culture by SN-*bik* or SN-*luc*, and the transfected cells were then inoculated into the MFPs of nude mice. The growth of tumors was followed weekly (Fig. 3). SN-*bik* delayed tumor growth in mice by at least 3 weeks compared with the *luc* control. The tumor volume ratios of control *versus* treatment groups during weeks 2 to 9 ranged from 1.6 to 8.0 for MDA-MB-231 and from 1.6 to 6.9 for MDA-MB-468, suggesting a strong tumor suppression activity by SN-*bik* treatment *in vivo*.

We used an orthotopic breast cancer model to confirm the tumor suppression activity of SN-*bik*. MDA-MB-231 and MDA-MB-468 cells were inoculated into the MFPs of nude mice. Mice with established tumors were then treated with SN-*bik*, SN-*luc*, or PBS through tail vein injection. SN-*bik* injection significantly inhibited tumor growth in mice compared with the PBS- or SN-*luc*-treated mice. By week 5, the mean tumor volume of PBS- and SN-*luc*-treated mice was ~3-fold higher than that of SN-*bik*-treated mice in the MDA-MB-231 model ($P < 0.001$; Fig. 4A); in the MDA-MB-468 model, the mean tumor volume of control mice was 2-fold higher than that of SN-*bik*-treated mice ($P < 0.001$; Fig. 4B). The most significant tumor suppression effect could be observed by week 10, with an ~4-fold difference in tumor volumes between the control and treatment groups. In addition to decreasing tumor growth, SN-*bik* systemic treatment also strongly inhibited metastasis in nude mice as shown by a separate metastasis assay (Fig. 4C), suggesting that there are multiple mechanisms associated with the tumor suppression function of

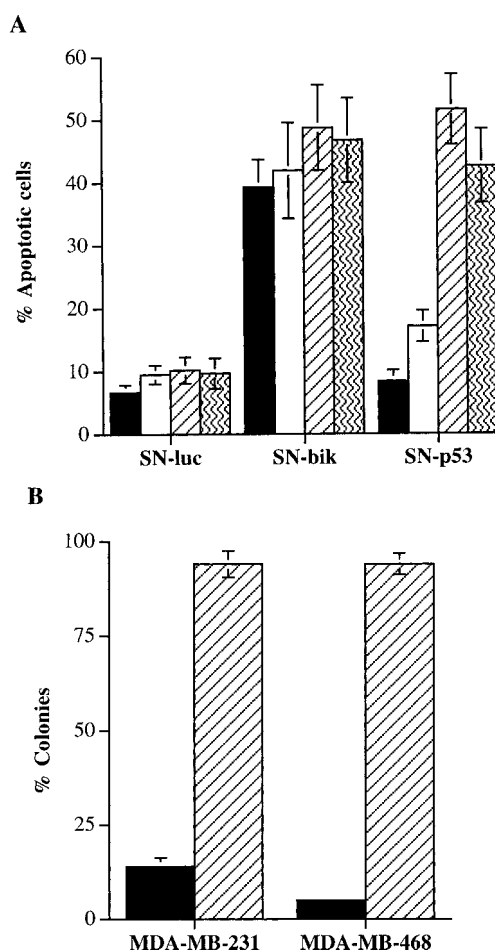


Fig. 2. Inhibition of cell proliferation by SN-delivered *bik*. A, the apoptotic potency of *bik* was compared with *p53* in the human lung cancer cell line A549 (wild-type *p53*) and human breast cancer cell lines MDA-MB-231 (mutant *p53*), MDA-MB-468 (mutant *p53*), and MCF-7 (wild-type *p53*). The cells were transfected with SN-*bik* with 2 μ g of DNA/ 10^6 cells in 10% serum containing medium. SN-*luc*-transfected cells were used as control. The apoptotic cells were determined with flow cytometry 24 h after transfection. ■, A549 (wt-*p53*); ▨, MDA-MB-231; □, MCF-7 (wt-*p53*); ▩, MDA-MB-468. B, MDA-MB-231 and MDA-MB-468 cells were transfected with SN-*bik* and seeded in 0.5% agar solution at 37°C in a six-well plate with 5×10^3 cells/well. The cells transfected with SN-entrapped vector (SN-vector) were used as controls. The number of cell colonies at least 100 μ m in diameter was counted 4 weeks later. The ratios of colony number of the treated cells to the colony number of control cells are shown. The data are the averages from three independent duplicate experiments; bars, SD. ■, SN-*bik*; ▨, SN-vector.

bik. The potential of clinical application of SN-*bik* was assessed in a gene therapy setting using an MDA-MB-468-derived orthotopic breast cancer model. Systemic treatment by SN-*bik* significantly increased the survival rate of the treated mice compared with the control groups treated with PBS or SN-*luc* ($P < 0.001$; Fig. 4D).

We have examined tumor tissues after i.v. SN-*bik* injection. A significant amount of apoptotic cells ($17\% \pm 3.5\%$) was detected in the orthotopic breast tumor tissue after single i.v. injection of SN-*bik*. In contrast, fewer apoptotic cells ($2.7\% \pm 0.8\%$) could be identified in the liver tissue of the same animal (Fig. 4, E and F). SN-*luc* injection did not induce a significant level of apoptosis in tumor or liver tissues. These results suggest that treatment by the particular liposome-*bik* formulation can lead to significant tumor cell death *in vivo* with low, nonspecific cytotoxicity in other normal tissues.

Discussion

We have shown that *bik* gene expression delivered by SN resulted in enhanced apoptosis and tumor suppression of breast cancer cells. In the soft agar and apoptosis assays, we observed more dramatic growth

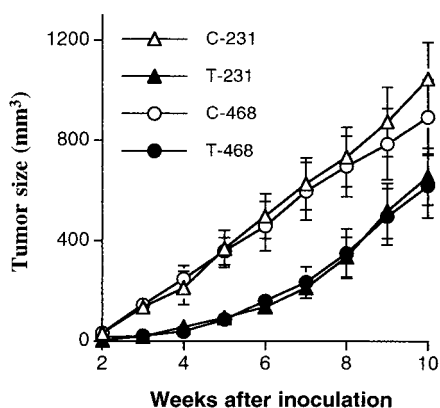
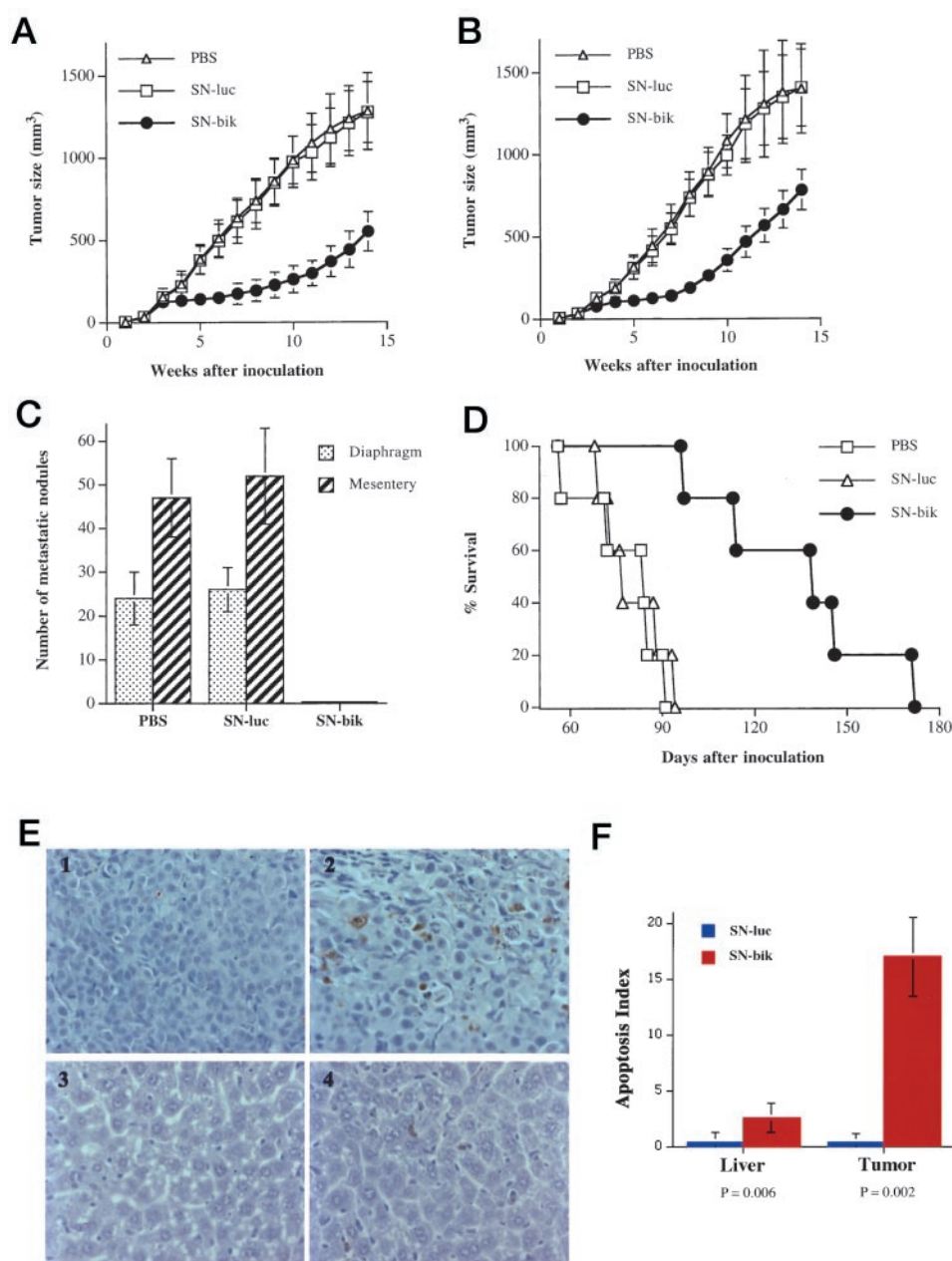


Fig. 3. *Ex vivo* assay of SN-*bik* tumor suppression function. Cells (2×10^6) from the MDA-MB-231 and MDA-MB-468 lines were transfected with SN-*bik* (T) or SN-*luc* (C) in culture plates. Twenty-four h later, treated cells were inoculated in the MFPS of nude mice, with 5 mice in each group. Tumor size was measured weekly (triangles, MDA-MB-232; circles, MDA-MB-468).

inhibition effects than would be expected based on the data of transfection efficiency. A possible explanation is that the expression of apoptotic genes, such as *bik*, can result in significant bystander effects, as described previously by others (8–10). In addition, the nature of low sensitivity of *GFP* scoring, which was used to determine the transfection efficiency of the SN lipid, may have underestimated the transfection efficiency.

The *bik* gene is a potent inducer for apoptosis. Apoptosis triggered by *bik* does not require the function of *p53* (4). This was also shown in this study in which *bik* induced apoptosis in a panel of cancer cell lines, regardless the status of the *p53* gene (Fig. 2, A and B). This property makes *bik* a potentially useful anticancer agent against cancers that do not respond to *p53* or *p53*-dependent gene therapy. As potent as it is, the effectiveness of *bik* depends on its systemic targeting to cancer cells, which requires a delivery system that can withstand i.v. conditions and carry the therapeutic gene to the targeted cells.

Fig. 4. The *bik* gene delivered by SN significantly inhibited growth of orthotopic breast tumors in mice. A and B, human breast cancer cell lines MDA-MB-231 (A) and MDA-MB-468 (B) were inoculated into the MFPS of nude mice at 2×10^6 cells/tumor. After 2 weeks, the mice bearing tumors were randomly divided into three groups with 5 mice in each. One group received six i.v. injections of SN-*bik* (15 μ g DNA), one group received the same volume of PBS, all with a 3-day interval. C, i.v. injection of SN-*bik* inhibited metastasis of orthotopic breast cancer in mice. MDA-MB-468 cells were inoculated into the MFPS of nude mice, and the mice bearing tumors were treated with SN-*bik*, SN-*luc*, or the same volume of PBS as described in A and B. The treatment started on day 21 after inoculation, and the mice were sacrificed on day 60. Metastatic tumor nodules with diameter >1 mm in the peritoneal cavity were counted. D, the *bik* gene delivered by SN significantly prolonged the life of mice with orthotopic breast cancer. MDA-MB-468 cells (3×10^6 cells/site) were inoculated into the MFPS of nude mice. After 3 weeks, the tumor-bearing mice were randomly divided into three groups with 5 mice in each and treated as described in A and B. E, systemic i.v. SN-*bik* treatment induced a high level of apoptotic cells in the tumor tissue but not in normal organs. The tumor (1 and 2) and liver (3 and 4) tissue samples from SN-*bik* (2 and 4) or SN-*luc* (1 and 3) injected mice were used for *in vivo* apoptosis assay. Apoptotic cells (brown staining) were determined based on terminal deoxynucleotidyl transferase-mediated nick end labeling assay as described in "Materials and Methods." F, the numbers of apoptotic cells in liver and tumor tissues were counted and compared between SN-*bik* and SN-*luc* treatments. Bars, SD.



Nonviral gene delivery by liposomes is a promising strategy because the liposome vehicle has very low immunogenicity and toxicity. The clinical application of liposomal treatment is, however, haunted by the low stability and the low DNA transduction efficiency in the presence of serum, a condition encountered with systemic treatment. The main reason is that, structurally, these formulations are simply formed by complexing the liposome particles with DNA by the opposite static charge. Thus, DNA, liposome, and the liposome-DNA complexes are all exposed to neutralizing serum components and the RES directly, without protection. Once any one of these elements in the formulation is destroyed or inactivated, gene delivery is aborted. That is why liposome- and peptide-DNA complexes, such as Lipofectamine and Fugene-6, are inefficient *in vivo*, and most of the complexed particles quickly disappear from blood within several minutes after i.v. injection (11–13). In this study, we used a modified cationic liposome formulation (SN) containing a surface-protection polymer to stabilize the liposome-DNA particles for i.v. injection. This formulation entraps condensed DNA into the internal aqueous phase of the liposomes, and the positively charged liposome surface is coated by the polymers to protect the liposome from the attack of serum components, *e.g.*, high-density lipoprotein and the RES. The particle size of SN-DNA is also very small, ranging from 60 to 170 nm. Liposome stabilization by entrapment and surface protection make the SN formulation a more efficient i.v. DNA delivery system than other common nonviral gene delivery systems, such as Fugene-6 and Lipofectamine. An assay of the organ distribution showed higher tumor *versus* normal tissue ratios after i.v. injection of SN-*luc* than of Fugene-6-*luc* (Fig. 1, *B* and *C*), suggesting that the RES, which typically clears such particles from the circulation, engulfed SN-DNA particles at a slower rate than the other nonviral gene delivery systems tested.

Our results showed that the SN formulation significantly enhanced the expression index of the targeting gene in the tumor tissue. More importantly, the SN system supported efficient i.v. delivery of the therapeutic gene. This characteristic makes the SN formulation an attractive system to target metastatic disease systemically. A combination of SN and a therapeutic gene such as the proapoptotic *bik* gene, under the control of a tissue/tumor-specific promoter, would further

increase the tumor targeting index. Once that is accomplished, titration of doses and administration schedules to achieve the best therapeutic window would be warranted.

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