Enhancement of Bik Antitumor Effect by Bik Mutants

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Abstract

Bik was initially identified as a BH3-domain-only protein that interacts with E1B 19K. Although systemically administered wild-type Bik significantly inhibited tumor growth and metastasis in an orthotopic nude mouse model, the proapoptotic potency of Bik can be modulated by posttranslational phosphorylation. Here, we found that Bik mutants, in which threonine 33 and/or serine 35 were changed to aspartic acid to mimic the phosphorylation at these two residues, enhanced their binding affinity with the antiapoptotic proteins Bcl-XL and Bcl-2 and were more potent than wild-type Bik in inducing apoptosis and inhibiting cell proliferation in various human cancer cells. Bik mutants also suppressed tumorigenicity and tumor-taking rate in a mouse model. Moreover, Bik mutant-liposome complexes inhibited tumor growth and prolonged life span more effectively than the wild-type Bik-liposome complex in an in vivo orthotopic animal model. Thus, our results demonstrate that Bik mutant genes, more potent than wild-type Bik, induce cell death and suggest that their inhibition on the growth of various cancers should be explored further.

Introduction

Bik, also known as nbk, is a proapoptotic gene that contains only one of the Bcl-2 homology regions, the BH3 domain, and has been recognized recently as an essential initiator of apoptosis (1, 2). Bik-mediated apoptosis requires the BAX gene (3). In addition, Bik is implicated in the development of human breast and colorectal cancers by studies showing loss of informative alleles on chromosome 22q, where the Bik gene is located (4). The 18-kDa Bik protein interacts with E1B 19K and forms heterodimers with various antiapoptotic proteins, such as Bcl-2 and Bcl-XL, and thus inhibits their antiapoptotic function (1). Bik is also a downstream apoptotic effector of p53 in response to a physiological p53-mediated death stimulus provided by E1A (5, 6). Moreover, Bik can sensitize tumor cells to apoptosis mediated by certain chemotherapeutic agents (7), such as doxorubicin (8). All of these findings suggest that Bik is a potential therapeutic gene for human cancer. We demonstrated previously that the Bik gene, complexed with a nonviral gene delivery system when delivered with E1B 19K and forms heterodimers with various antiapoptotic proteins (1, 2). Bik was recognized recently as an essential initiator of apoptosis (1, 2). Bik is one of the BH3-only proteins, and many other BH3-only proteins, such as Bim, Puma, and Noxa, have been identified (9). Bik is one of the BH3-only proteins, and many other BH3-only proteins, such as Bim, Puma, and Noxa, have been identified (9).

Materials and Methods

Cell Lines. Human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-468, ovarian cancer cell SKOV-3, prostate carcinoma cell PC-3, non-small cell H1299, pancreatic cancer cell Panc1, and 293T cells were purchased from American Type Culture Collection (Rockville, MD) and cultured according to the vendor’s instructions.

Plasmid Construction and Site-directed Mutagenesis. Bik, Luc, and GFP-expressing plasmids were constructed by inserting the cDNAs of Bik, Luc, and GFP, respectively, into the pcDNA3 vector containing a CMV promoter. Site-directed mutagenesis was performed according to the manufacturer’s protocol (BD Biosciences, Palo Alto, CA). Threonine 33 and serine 35 of Bik were changed to aspartic acid by using the following primers: for T33D, 5’-GGTTTTGCGTACGACGTCTTGGAGAGGACC-3’ and 5’-CCTCTTCC-AGAGGACC-3’; for S35D, 5’-GCCATGACTGAGCCAGAAAGGCACGTG-3’ and 5’-CAGTGTCCTCTTGCGTACGTCAGTGCC-3’; and for T33DS35D (DD), 5’-GGTTTTGCGTACGACGTCTTGGAGAGGACC-3’ and 5’-GCCATGACTGAGCCAGAAAGGCACGTG-3’ and 5’-GCCATGACTGAGCCAGAAAGGCACGTG-3’. The sequences of the three Bik constructs were confirmed by automated sequencing.

Formulation. The nonviral gene delivery system, SN, was essentially a cationic liposome, which is formulated as described elsewhere (9).

Transfection. Cell transfections were performed as described previously (9).

Western Blot Analysis. Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na2PO4 (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na2VO4, 5 mM phenylmethylsulfonyl fluoride, 1% aprotonin, and 10 μg/ml leupeptin. The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with primary goat anti-Bik polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The immunoblots were visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ).

In Vitro Direct Protein-binding Assay. For fusion protein-binding assays, GST-Bcl-2 and GST-Bcl-XL plasmid DNAs were transformed into BL21 DE3 cells and induced with 0.5 mM isopropyl-β-D-thiogalactoside. A 10-ml aliquot of culture was analyzed by SDS-PAGE to evaluate fusion protein induction. The remaining culture was sonicated on ice with 10 short bursts at 10 s each and clarified by centrifugation, and the supernatant was resuspended.

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in 50% (vol/vol) glutathione-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). An aliquot of the protein-bound beads was analyzed by SDS-PAGE to ensure that equal amounts of pure fusion protein were present.

In vitro binding assays were performed by incubating equal amounts of GST, GST-Bcl-2, and GST-Bcl-X<sub>L</sub> immobilized onto glutathione-Sepharose beads, with in vitro-translated Bik wt or mutant (T33S35D), which was labeled with [35S]methionine using the TNT coupled reticulocyte lysate system (Promega Corp., Madison, WI). The mixture, which was diluted in 0.5 ml of the RIPA-B cell lysis buffer, was incubated for 3 h at 4°C under gentle agitation and washed five times with lysis buffer, and the binding complexes were dissolved in SDS-PAGE buffer. All samples were boiled for 5 min and were resolved by 15% SDS-PAGE. The gels were dried and exposed to X-ray films.

Luc Assay. To determine the Bik dose effect on cell proliferation, different cancer cells were cotransfected with 50 ng of CMV-Luc and increasing amounts (0, 0.5, or 2 μg) of CMV-Bik. The total amount of DNA transfected at each dose was kept constant (2.05 μg) by adding an appropriate amount of pCDNA3 vector. Forty-eight h after transfection, cells were harvested, and Luc activity was measured by using the Luc assay system (Promega) according to the manufacturer’s protocol. The relative activities were calculated by setting the Luc activities obtained from transfections without CMV-Bik (0 μg) at 100%. The data represent mean ± SD of three independent experiments.

Apoptosis Assay. For in vitro studies, standard fluorescence-activated cell sorter analysis was used to identify apoptotic cells. Briefly, cells were transfected with SN-Bik or other agents. Twelve or 24 h after transfection, the apoptotic cells were assessed by flow cytometric detection of sub-G<sub>1</sub> DNA content after being stained with propidium iodide.

Ex Vivo Tumor Inhibition Assay. An ex vivo tumor inhibition assay was performed as described elsewhere (9). Female nude mice were inoculated with 4 × 10<sup>6</sup> breast cancer MCF-7 cells into the MFP, and male nude mice were inoculated with 1 × 10<sup>6</sup> prostate cancer PC-3 cells into s.c. connective tissue.

Antitumor Activity Assay. The antitumor activity assay was done as described previously to study tumor growth inhibition by BikDD (9).

Statistical Analysis. All data analyses used two-sided log-rank statistical tests.

Results and Discussion

To search for forms of Bik that would be more potent than wt Bik in cancer gene therapy, three Bik mutants were constructed by site-directed mutagenesis. We replaced Bik residues threonine 33, serine 35, or both with aspartic acid (T33D, S35D, and T33S35D) to mimic constitutive phosphorylation. Expression efficiencies of these mutants were similar to that of wt Bik (Fig. 1A). Because the BH3-domain-only proapoptotic protein Bik can bind to the antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub> and enhanced the cell-killing effect, compared with wt Bik in two different human cancer cell lines, A. Western blot analysis of wt and mutant Bik protein expression after transient transfection with wt or mutant Bik in 293T cells. Actin was used as an equal loading control. B. in vitro binding assay showed that the Bik mutant T33S35D (BikDD) exhibited stronger in vitro binding affinity with the antiapoptotic proteins Bcl-2 and Bcl-X<sub>L</sub>. The GST-Bcl-2 and GST-Bcl-X<sub>L</sub> fusion proteins were incubated with an in vitro-translated wt Bik and double-mutant BikDD labeled with [35S]-methionine in immunoprecipitation buffer, then recovered on glutathione beads, and analyzed by SDS-PAGE. The intensity of double mutant, relative to the wt is shown. C, the human breast cancer cell line MCF-7 and the prostate cancer cell line PC-3 were cotransfected with 100 ng of CMV-GFP and 2 μg of CMV-Bik wt or mutants. pcDNA3-transfected cells were used as a control. Twelve or twenty-four h after transfection, the apoptotic cells were harvested and assessed by flow cytometric detection of sub-G<sub>1</sub> DNA content after being stained with propidium iodide. The data represent means of three independent experiments; bars, SD. *, significant difference compared with wt (P < 0.05).

We then tested the ability of the Bik mutants to inhibit the growth of human cancer cell lines in vitro. Using a luciferase assay as an index for cell survival, the Bik mutants were shown to be more potent than wt Bik in multiple cancer cell lines including lines from cancers of breast, ovary, prostate, lung, and pancreas (Fig. 2). In general, all three Bik mutants appeared to exert stronger growth-inhibitory effects than wt Bik; the BikDD seemed to be more potent. During site-directed mutagenesis, an unexpected triple mutant T33DS35DD102E(DDE) formed, in which Asp-102 was changed to Glu, in addition to the double mutation of Thr-33 and Ser-35. This BikDD activity was found to be similar to that of BikDD and stronger than the wt Bik in multiple cancer cell lines (data not shown), suggesting that the Asp to Glu mutation does not alter its activity and further supports that the double mutations in Thr-33 and Ser-35 result in higher activity.

To test whether the Bik mutants also had greater antitumor activity than wt Bik, we first performed an ex vivo tumorigenicity assay in nude mice. The human breast cancer cell line MCF-7 and the prostate...
human breast cancer cell line MCF-7 (ex vivo suppressive effects in an prostate cancer cell line PC-3 were cotransfected with 50 ng of CMV-Luc, the small lung cancer cell line H1299, the pancreatic cancer cell line PANC1, and the breast cancer cell lines MDA-MB-231 and MCF-7, the ovarian cancer cell line SKOV-amounts (0, 0.5, or 2 g) of CMV-Luc were transfected with either CMV-Bik, wt or mutants, delivered by SN liposome in culture plates. Mice inoculated with Bik-mutant transfectants. Tumor growth from Bik mutants’ transfectants in nude mice was delayed by at least 3 weeks compared with that from the pcDNA3 control transfectants. The tumor volume ratios of wt Bik mice and Bik mutant mice during week 7 ranged from 2.1 to 3.7 for MCF-7 cells and from 2.2 to 4.1 for PC-3 cells. The average tumor weight in mice treated with wt Bik was about 2- to 4-fold greater than that of mice treated with Bik mutant (Fig. 3B).

These results show that the three Bik mutants inhibited tumor cell growth better than wt Bik in vitro and ex vivo and, of the three Bik mutants, BikDD appeared to be the most potent one. To further compare the antitumor activities in a gene therapy setting, we tested the BikDD and wt Bik genes in a previously established animal tumor model (9). Mice with established tumors were treated with wt Bik, BikDD, or pcDNA3 control by using the SN nonviral delivery system. Treatment with BikDD inhibited tumor growth in mice to a significantly greater extent than that of wt Bik or pcDNA. By weeks 3–5, the mean tumor volume of wt Bik-treated mice was larger than that of BikDD-treated mice in both models. The most significant tumor suppression effect was observed by weeks 8 and 9, with a 2–3-fold difference in tumor volumes between the wt and mutant treatment groups (P<0.05; Fig. 4A). Treatment with BikDD yielded a significantly higher survival rate among the treated mice than treatment with wt Bik (P<0.05; Fig. 4B). These median survival times were 175 days for BikDD-treated mice and 112 days for wt Bik-treated mice with MCF-7 tumors; the median survival times were 140 days for MCF-7 or s.c. connective tissue of male nude mice (for PC-3). We found that the Bik mutant transfectants had less tumor growth potential ex vivo (Fig. 3A) and lower tumor-taking rates (Fig. 3B) than wt Bik transfectants. Tumor growth from Bik mutants’ transfectants in nude mice was delayed by at least 3 weeks compared with that from the pcDNA3 control transfectants. The tumor volume ratios of wt Bik mice and Bik mutant mice during week 7 ranged from 2.1 to 3.7 for MCF-7 cells and from 2.2 to 4.1 for PC-3 cells. The average tumor weight in mice treated with wt Bik was about 2- to 4-fold greater than that of mice treated with Bik mutant (Fig. 3B).

cancer cell line PC-3 were transfected with either CMV-Bik wt or the Bik mutants in culture plates. Cells transfected with pcDNA3 vector were used as a control. Twenty-four h later, the treated cells were harvested and then inoculated into the MFP of female nude mice (for MCF-7) or s.c. connective tissue of male nude mice (for PC-3). We found that the Bik mutant transfectants had less tumor growth potential ex vivo (Fig. 3A) and lower tumor-taking rates (Fig. 3B) than wt Bik transfectants. Tumor growth from Bik mutants’ transfectants in nude mice was delayed by at least 3 weeks compared with that from the pcDNA3 control transfectants. The tumor volume ratios of wt Bik mice and Bik mutant mice during week 7 ranged from 2.1 to 3.7 for MCF-7 cells and from 2.2 to 4.1 for PC-3 cells. The average tumor weight in mice treated with wt Bik was about 2- to 4-fold greater than that of mice treated with Bik mutant (Fig. 3B).

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![Fig. 2. The expression of the Bik mutant exhibited stronger growth-inhibitory effects than wt Bik in different human cancer cell lines. A comparison of the growth-inhibitory effects between the Bik wt and BikDD mutant in different human cancer cell lines. Human breast cancer cell lines MDA-MB-231 and MCF-7, the ovarian cancer cell line SKOV-1, the small lung cancer cell line H1299, the pancreatic cancer cell line PANC1, and the prostate cancer cell line PC-3 were cotransfected with 50 ng of CMV-Luc and increasing amounts (0, 0.5, or 2 g) of CMV-Bik, wt or mutants. The relative activities were calculated by setting the Luc activities obtained from transfections without CMV-Bik (0 g) at 100%. The data represent means of three independent experiments; bars, SD. *p<0.05, significant difference compared with wt (P<0.05).

![Fig. 3. The Bik mutants exhibited stronger suppressive effects in an ex vivo assay than wt Bik. The human breast cancer cell line MCF-7 (A) and the prostate cancer cell line PC-3 (B) were transfected with CMV-Bik, wt or mutants, delivered by SN liposome in culture plates. Mice inoculated with MCF-7 cells were implanted with a 0.72-mg 17β-estradiol pellet 2 weeks before inoculation. pcDNA3-transfected cells were used as a control. Twenty-four h later, the treated cells were harvested and inoculated in MFP for MCF-7 or s.c. connective tissue of nude mice, with eight mice in each group. Tumor sizes were measured weekly, as shown (A). After 7 weeks, the mice were sacrificed, and the tumor weight and tumor-forming rate were measured as shown (B). Bars, SD. *p<0.05, mutant tumors whose sizes were significantly different than wt tumors (P<0.05); **, wt Bik whose sizes were significantly different than pcDNA3 control tumors (P<0.05).

![Fig. 4. The expression of the Bik mutant exhibited stronger growth-inhibitory effects than wt Bik in different human cancer cell lines. A comparison of the growth-inhibitory effects between the Bik wt and BikDD mutant in different human cancer cell lines. Human breast cancer cell lines MDA-MB-231 and MCF-7, the ovarian cancer cell line SKOV-1, the small lung cancer cell line H1299, the pancreatic cancer cell line PANC1, and the prostate cancer cell line PC-3 were cotransfected with 50 ng of CMV-Luc and increasing amounts (0, 0.5, or 2 g) of CMV-Bik, wt or mutants. The relative activities were calculated by setting the Luc activities obtained from transfections without CMV-Bik (0 g) at 100%. The data represent means of three independent experiments; bars, SD. *p<0.05, significant difference compared with wt (P<0.05).
Bik MUTANT GENE THERAPY

Fig. 4. The BikDD mutant gene delivered by SN significantly inhibited the growth of human tumors in mice. A. models used were orthotopic human breast cancer MCF-7 cells (injected into the MFP, 4 x 10^6 cells/mouse), where each mouse was implanted s.c. with a 0.72-mg 17-β-estradiol pellet 2 weeks before inoculation, and the model of ectopic prostate cancer PC-3 cells (injected s.c., 1 x 10^6 cells/mouse) in mice. Two weeks later, the mice bearing tumors were randomly divided into three groups with five mice in each. One group received multiple injections of SN-BikDD DNA (15 μg of DNA/mouse), three times a week for a total of 12 treatments, orthotopic breast cancer by i.v. injection, and ectopic prostate cancer by intratumor injection; the other group received the same three times a week for a total of 12 treatments, orthotopic breast cancer by i.v. injection, and ectopic prostate cancer by intratumor injection; the other group received the same

BikDD-treated mice and 105 days for wt Bik-treated mice with PC-3 tumors. The results indicate that BikDD inhibited ~50% of the tumor growth and significantly increased survival rates, compared with wt Bik.

Taken together, we found that the BikDD mutant enhanced the binding affinity with antiapoptotic proteins Bcl-XL and Bcl-2 and is more potent than wt Bik in inducing apoptosis and inhibiting cell proliferation. We also demonstrated that the three Bik mutants are more potent than wt Bik in both i.v. (MCF-7, mammary tumor model) and intratumor (PC-3, s.c. tumor model) treatments. The BikDD mutant was further shown to be more effective than wt Bik in inhibiting tumor development and prolonging survival in a gene therapy setting. Thus, the BikDD mutant may serve as a more effective agent than wt Bik for cancer gene therapy.

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References

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