AIM2 suppresses human breast cancer cell proliferation in vitro and mammary tumor growth in a mouse model

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Abstract

IFN-inducible proteins are known to mediate IFN-directed antitumor effects. The human IFN-inducible protein absent in melanoma 2 (AIM2) gene encodes a 39-kDa protein, which contains a 200-amino-acid repeat as a signature of HIN-200 family (hematopoietic IFN-inducible nuclear proteins). Although AIM2 is known to inhibit fibroblast cell growth in vitro, its antitumor activity has not been shown. Here, we showed that AIM2 expression suppressed the proliferation and tumorigenicity of human breast cancer cells, and that AIM2 gene therapy inhibited mammary tumor growth in an orthotopic tumor model. We further showed that AIM2 significantly increased sub-G1 phase cell population, indicating that AIM2 could induce tumor cell apoptosis. Moreover, AIM2 expression greatly suppressed nuclear factor-κB transcriptional activity and desensitized tumor necrosis factor-α-mediated nuclear factor-κB activation. Together, these results suggest that AIM2 associates with tumor suppression activity and may serve as a potential therapeutic gene for future development of AIM2-based gene therapy for human breast cancer. [Mol Cancer Ther 2006;5(1):1–7]

Introduction

IFNs play critical roles in host immune defense signaling pathways and have been successfully used for anticancer therapy (1–3). IFN-inducible proteins are thought to mediate the direct antitumor activity of IFNs (4) and have been used with great success in animal models by cancer gene therapy. The human IFN-inducible proteins IFI16, MNDA, AIM2, and IFIX, and the structurally related murine homologous genes p202, p203, p204, and p205 constitute the family as hematopoietic IFN-inducible nuclear proteins containing a 200-amino-acid repeat, HIN-200 (5). Many HIN-200 proteins have been shown to suppress cell growth (6). For example, murine protein p202 has been shown to suppress tumor growth and metastasis of human breast, pancreatic, and prostate cancer cells (7–10). The antigrowth activity of p202 is partly mediated by up-regulation of p21 and pRb and down-regulation of cyclin-dependent kinase 2 protein kinase activity (11). It has been shown that p202 interacts with several transcription factors, such as nuclear factor-κB (NF-κB), p50 and p65, activator protein, c-fos, c-jun, and E2F, and modulates their transcriptional activity (12, 13).

The human IFN-inducible protein IFIXα1 has been shown to reduce the anchorage-dependent and anchorage-independent growth and the tumorigenicity of breast cancer cells(14). Another human IFN-inducible protein that may associate with tumor suppression activity is AIM2. AIM2 gene was originally identified by subtractive cDNA selection for association with human melanoma tumorigenicity (15) and only shares 31% protein identity with IFIXα1 (14). The AIM2 gene contains a site of microsatellite instability that results in gene inactivation in 47.6% of colorectal tumors with high microsatellite instability (16). AIM2 gene mutation has also been found in association with gastric and endometrial cancers (17). These findings, in combination with the observation that AIM2 can be silenced by DNA methylation in its genome in immortalized cells (18), suggest that AIM2 is a tumor suppressor. However, suppression of cancer cell growth and tumorigenicity by AIM2 has not been shown (19).

In this study, we aim to determine whether AIM2 inhibits breast cancer cell growth in vitro and to explore the possibility of using AIM2 for breast cancer gene therapy. We showed that AIM2 DNA liposome treatment not only suppressed breast cancer cell growth in vitro but also inhibited mammary tumor growth in orthotopic tumor models. Our results indicate that AIM2 possesses tumor suppression activity, and that AIM2 transgene expression may be further explored as a novel gene therapy for patients with breast cancer.
Tumor Suppression by AIM2 in Breast Cancer

Materials and Methods

Cell Lines and Plasmids

Human embryonic kidney cells (HEK-293T) and human breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453) were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM (HyClone Laboratories, Logan, UT) supplemented with 10% (v/v) fetal bovine serum and 250 μg/mL G418 (Invitrogen, Carlsbad, CA). For selection of double transfectants and induction of gene expression, the NH2-terminal FLAG tag peptide-fused AIM2 was cloned into pB1-EGFP Tet plasmid (Clontech), and the resulting vector was named pB1-EGFP-Tet-AIM2 (Fig. 1A, bottom). pB1-EGFP-Luc (Clontech) plasmid was used as a control. MCF-7 Tet-Off cells were transfected with pB1-EGFP-Tet-AIM2 or pB1-EGFP-Luc together with pcDNA6/c-myc/His (Invitrogen) using SN liposome (20). After 2 days, transfected cells were subjected to selection with 300 μg/mL blasticidin in the presence of 10% (v/v) fetal bovine serum and 250 μg/mL G418 (Invitrogen, Carlsbad, CA). For selection of double transfectants and induction of gene expression, the NH2-terminal FLAG tag peptide-fused AIM2 was cloned into pB1-EGFP Tet plasmid (Clontech), and the resulting vector was named pB1-EGFP-Tet-AIM2 (Fig. 1A, top) was constructed by inserting AIM2 cDNA into pCMV-Tag2C (Flag tag; Stratagene, La Jolla, CA).

Generation of Tetracycline-Inducible AIM2 Cell Lines

MCF-7 Tet-Off cells were purchased from Clontech (Palo Alto, CA) and maintained in DMEM/F-12 supplemented with 10% (v/v) fetal bovine serum. The AIM2 expression vector pCMV-Tag-AIM2 (Fig. 1A, top) was constructed by inserting AIM2 cDNA into pCMV-Tag2C (Flag tag; Stratagene, La Jolla, CA).

A

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Figure 1. Cloning and protein expression of AIM2. A, the coding sequence of AIM2 was inserted into pCMV-Tag2C and fused with FLAG tag at the NH2-terminal. pB1-EGFP-Tag-AIM2 was used for selection of Tet-Off gene expression stable transfectants. The tetracycline-responsive element (TRE) is upstream of the minimum CMV promoter (pminiCMV) and the AIM2 gene. SV40 polyA, SV40 polyadenylation signal. B, Western blotting was done with whole-cell lysates and using anti-Flag antibody. Left, transient expression of AIM2 in HEK-293T cells was achieved by transfecting cells with the AIM2-expressing vector pCMV-Tag-AIM2 or the control vector pCMV-Tag2C. Right, AIM2 expression in MCF-7 selected stable transfectants #13 and #25. The cells were tested under the culture condition with 2 μg/mL doxycycline (+) or removal of doxycycline (−). β-Actin was used as a loading control. NS, marks a nonspecific band detected by anti-flag antibody.
from Calbiochem (San Diego, CA) following the manufacturer's instructions. The apoptotic cells (bright green signaling) were counted under a microscope. 4',6-Diamidino-2-phenylindole was used for the visualization of both apoptotic and nonapoptotic cells. The apoptotic index was defined by the percentage of green signaling–positive cells among the total number of cells in each sample. Five fields with 100 cells per field were randomly counted for each sample. We counted a minimum of three samples for each group.

**Generation of AIM2-Inducible MCF-7 Tumor Xenografts**

AIM2-inducible MCF-7 cells (#13) were maintained in DMEM/F-12 medium supplemented with 10% (v/v) fetal bovine serum and 2 μg/mL doxycycline. After 24 hours, the cells were harvested in 0.2 mL of PBS and injected into one of the mammary fat pads within each mouse (107 per mouse). To support the growth of the estrogen-dependent MCF-7 tumors, a 0.72 mg 17β-estradiol 60-day release pellet (Innovative Research of America, Sarasota, FL) was implanted s.c. into the back of each mouse the day before tumor injection. During this experiment, the mice were fed sucrose water with or without doxycycline and were examined weekly to assess tumor growth.

**Colony-Forming Assay**

MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells were transfected with the AIM2 expression vector pCMV-Tag-AIM2 or the control vector pCMV-Tag2C using SN liposome and were selected in 500 μg/mL G418. After 3 weeks, the G418-resistant colonies were stained with crystal violet and counted as described previously (9). Experiments were repeated thrice.

**Cell Viability Assay**

MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells (2 × 105) were plated onto a six-well plate the day before transfection. Cells were transfected with 0.2 μg of CMV-Luc and different amounts of pCMV-Tag2C or pCMV-Tag-AIM2 by using SN as a gene delivery system. The total amount of DNA transfected at each AIM2 dose was kept constant (1.6 μg) by adding an appropriate amount of pCMV-Tag2C vector. At 36 hours after transfection, the cells were harvested, and luciferase activity was determined with a luminometer (Turner Designs Instruments, Sunnyvale, CA). Experiments were done thrice.

**AIM2 Gene Therapy**

Six-week-old female nude mice (10 mice per group; Charles River Laboratories, Wilmington, MA) were injected with 2 × 106 MDA-MB-435 cells under the second or third left mammary fat pad. After the tumors had grown to 0.5 cm in diameter about 7 days after tumor inoculation, the mice were treated with SN2 liposome (20, 22) with 20 μg of either pCMV-Tag-AIM2 or pCMV-Tag2C twice a week by i.t. injection for 2 weeks. Tumor size was measured in two dimensions with a caliper twice a week and calculated as S2L/2, where S is the shortest diameter of the tumor in millimeters and L is the longest diameter of the tumor in millimeters (Student's t test, day 21, P < 0.01). Animal care and experimental procedures were conducted in compliance with the Institute's Animal Care and Use Committee regulations following NIH guidelines.

**Immunostaining**

Inducible AIM2 clone (#13) of MCF-7 cells was cultured in a four-well glass chamber overnight. The enhanced GFP (EGFP) expression vector served as a control. Three days after induction, the cells were washed with PBS, fixed with 3% parafomaldehyde in PBS for 30 minutes at room temperature, and washed again with PBS. The primary antibody, anti-Flag antibody (1:100), was incubated with the cells at room temperature for 1 hour. The cells were then washed with PBS and incubated with the secondary antibody conjugated with green fluorescence (FITC) at room temperature for 1 hour. After incubation, the cells were washed with PBS, air-dried, and incubated with the fluorescent dye 4',6-diamidino-2-phenylindole (1:100 in 50% glycerol and PBS). A coverslip was placed on top of the slide for visualization by immunofluorescence microscopy.

**Transfection and Luciferase Assay**

MDA-MB-435 and MDA-MB-453 cells were transfected with 0.5 μg of kB-Luc construct, an IκB promoter-driven luciferase gene (9) and 0.1 μg of the internal transfection control, pRL-TK (Promega). NF-κB (p65) expression vector has been described previously (23). Forty-eight hours after transfection, the cells were harvested, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the protocol supplied by the manufacturer. The relative activities were calculated by setting the luciferase activities obtained from transfections without pCMV-Tag-AIM2 at 100%. We did three independent experiments of this assay. Human tumor necrosis factor-α (20 ng/mL; R&D Systems, Inc., Minneapolis, MN) was used to stimulate the NF-κB transcriptional activity.

**Results and Discussion**

**AIM2 Constructs and Generation of Tetracycline-Inducible AIM2 Cell Lines**

To investigate whether AIM2 has tumor-suppressing activity, we first generated an AIM2 expression vector. AIM2 cDNA was cloned into transient expression vector pCMV-Tag2C, and the construct was designated pCMV-Tag-AIM2 (Fig. 1A, top). AIM2 protein was readily detected in lysates from HEK-293T cells transiently transfected with AIM2, via Western blot analysis using anti-Flag antibody (Fig. 1B, left). The observed molecular weight of AIM2 was ~39 kDa, which was consistent with the predicted size.

We then established MCF-7 transfectants that stably expressed AIM2 under the control of a tetracycline-repressible promoter (Fig. 1A, bottom). Two independent AIM2 expression cell lines (#13 and #25) were selected.
and expressed high levels of AIM2 after withdrawal of doxycycline (Fig. 1B, right), indicating success of the Tet-off–mediated inducibility. The higher molecular weight bands indicated flag-tagged AIM2, whereas the lower molecular weight bands represent the nonspecific proteins detected by anti-flag antibody.

**AIM2 Protein Suppressed Breast Cancer Cell Proliferation and Induced Cell Apoptosis**

Next, we aimed to determine whether expression of AIM2 could suppress breast cancer cell proliferation and tumor formation using MCF-7 Tet-Off stable transfectants in the cell growth assay. As shown in Fig. 2A, expression of AIM2
significantly hampered growth of MCF-7 tTA-Tag-AIM2 cells. Suppression of AIM2 expression by doxycycline greatly increased proliferation of MCF-7 tTA-Tag-AIM2 cells in two independent MCF-7 stable lines #13 and #25 (Fig. 2A). These results suggest that AIM2 expression could suppress MCF-7 breast cancer cell growth. As expected, doxycycline has no significant effect on cell growth for the vector control cell line MCF-7 tTA-Luc (Fig. 2A). To elucidate the mechanism by which AIM2 suppressed breast cancer cell proliferation, we did cell cytometry flow analysis to determine the cell population distribution. The results showed that expression of AIM2 by withdrawal of doxycycline increased the sub-G1 cell population, suggesting that AIM2-expressing cells underwent apoptosis (data not shown). We further confirmed the AIM2-induced cell apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling assay (Fig. 2B, left). As expected, expression of AIM2 by withdrawal of doxycycline significantly increased the percentage of apoptotic cells by 5-fold (Fig. 2B, right). The control group of MCF-7 tTA-Luc showed no significant change of apoptotic cell population with or without doxycycline.

AIM2 Protein Suppressed Breast Cancer Cell Tumorigenicity

To further test whether AIM2 could suppress mammary tumor formation in vivo, the tetracycline-inducible MCF-7 stable transfectant was implanted into nude mice. AIM2 expression completely suppressed tumor formation (Fig. 2C). Following statistical analysis by Student’s t test, we found a statistically significant difference in MCF-7 tTA-AIM2 tumor growth between DOX+ and DOX− groups ($P = 0.025$). Furthermore, no substantial difference was observed in mice with MCF-7 tTA-Luc fed with and without doxycycline (Fig. 2C, inset). It is noticed that only 4 of 10 mice in MCF-7 tTA-AIM2 cell injected and DOX+ group had tumor growth, suggesting that some antitumor effects were present, and the expression of AIM2 gene was not suppressed completely. As shown in Fig. 1B (left), there is a minor expression of AIM2 in MCF-7 tTA-AIM2 cell lines in the presence of doxycycline at 7 days. The results from Fig. 1B indeed suggested that there is a leaking expression of AIM2 in these stable lines. The incomplete suppression may contribute to the low tumor incidence of MCF-7 tTA-AIM2-bearing mice with doxycycline sucrose water feeding. Taken together, these observations strongly suggest that AIM2 expression inhibits breast cancer cell growth in vitro and mammary tumor formation in vivo.

We next examined whether AIM2 also targets other breast cancer cells. We first did a relative cell viability assay using pCMV-Luc cotransfection. Luciferase activity was used as an indication of living cells. As expected, AIM2 expression inhibited the viability of MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453 breast cancer cells in a dose-dependent manner (Fig. 3A). Consistently, AIM2 inhibited the colony-forming ability of these breast cancer cells (Fig. 3B). AIM2 expression vector pCMV-Tag-AIM2 or the control vector pCMV-Tag2C were transfected into the four human breast cancer cell lines and selected with G418. At 3 weeks, the number of G418-resistant colonies in all four cell lines was at least 85% lower in AIM2-transfected cells than in control vector-transfected cells (Fig. 3B).
AIM2 concentrations were 0.5 and 1.5 in independent experiments; n activated desensitizes TNF-α cotransfected with cells. Inhibits NF-κB means of three independent experiments; Columns, AIM2 suppresses NF-κB transcriptional activity. Figure 4.

We further examined whether the human AIM2 gene can be used as a therapeutic gene in a gene therapy setting using a previously described mammary tumor model (24). Compared with pCMV-Tag2C (control), AIM2 DNA-SN liposome treatment significantly reduced the growth of MDA-MB-435 and MDA-MB-453 cells. n-B-Luc activity without AIM2 suppression was set at 100%. Columns, means of three independent experiments; bars, SD. B, AIM2 desensitizes TNF-α-activated NF-κB transcriptional activity. TNF-α-activated n-B-Luc activity without AIM2 suppression was set at 100%. AIM2 concentrations were 0.5 and 1.5 μg. Columns, means of three independent experiments; bars, SD.

In summary, the findings reported in this study show a potent antitumor activity of AIM2 and thus reveal the feasibility of using AIM2 in gene therapy to treat human breast cancer. Furthermore, AIM2 protein’s antitumor activity correlates with its ability to induce apoptosis by antagonizing NF-κB activity. Taken together, our findings provide a mechanism by which AIM2 suppressed tumor growth and the rationale for future potential use of AIM2-based gene therapy in treating breast cancer.

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References


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