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The Neutrophil Elastase Inhibitor Elafin Triggers Rb-Mediated Growth Arrest and Caspase-Dependent Apoptosis in Breast Cancer

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Abstract

Elafin, an endogenous inhibitor of neutrophil elastase, is expressed in human mammary epithelial cells but is transcriptionally downregulated in breast cancer cells. We hypothesized that elafin may exert a tumor-suppressive activity in the context of breast cancer. In this study, we show that the retinoblastoma (Rb) pathway governs the antitumor properties of elafin. In breast cancer cells with functional Rb, the expression of elafin triggered Rb-dependent cell cycle arrest. Elafin also exhibited suppressive activity in breast cancer cell lines lacking Rb, but this was associated with an induction of caspase-3–dependent, p53-independent apoptotic cell death. Normal mammary epithelial cells were not affected by elafin. Collectively, these results argue that elafin mediates tumor-suppressive effects that are cytostatic or cytotoxic depending on the Rb status. Our findings suggest that elafin could be engineered as a therapeutic modality to treat breast cancer without toxicity to normal proliferating cells. Cancer Res; 70(18): 7125–36. ©2010 AACR.

Introduction

The serine protease inhibitor elafin was originally purified from skin lesions associated with the inflammatory disease psoriasis. It was shown to be a specific inhibitor of human neutrophil elastase (HNE) and the highly related proteinase 3 (1). Elafin has a role in inflammation, in which it is hypothesized to act as part of the protective barrier erected by epithelial cells against tissue-degrading, leukocyte-secreted proteases (2, 3). Elafin may also have a role in cancer progression. Studies have shown that elafin is expressed in well-differentiated squamous cell carcinomas of the skin, head and neck, and esophagus and is absent in poorly differentiated tumors at these sites, suggesting that elafin downregulation has a role in the development of a poorly differentiated and aggressive tumor phenotype (4–7). The majority of breast tumor–derived cell lines show transcriptional downregulation of elafin as compared with human mammary epithelial cells (HMEC; ref. 8). CCAAT/enhancer binding protein β (C/EBPβ) transcriptional elements regulate elafin expression in HMECs, whereas frequent deregulation of C/EBPβ in breast cancer results in a transcriptional loss of elafin expression in tumor cells (9).

In the present study, we hypothesize that elafin possesses novel antitumor properties in breast cancer. Our analysis has defined an important role for the retinoblastoma protein (Rb) in the determination of cell fate following elafin expression. In normal HMECs with functional Rb, the overexpression of elafin has no effect; however, in tumor cell lines with functional Rb, elafin expression results in a G0–G1 cell cycle arrest. In both normal and tumor-derived breast cell lines with loss or inactivation of Rb, elafin expression results in caspase-3–dependent apoptotic cell death.

Approximately one quarter of all breast tumors exhibit a homologous loss of Rb (10, 11). Rb loss is frequently associated with aggressive breast cancer subtypes, including luminal B or basal-like (12, 13). These subtypes of breast cancer are resistant to currently available targeted therapies (i.e., antiepidermal growth factor receptor 2; refs. 14, 15). An effective targeted therapy capable of specifically targeting breast tumors with Rb loss may have significant clinical utility in highly aggressive breast cancer subtypes.

Materials and Methods

Cell lines and culture conditions

All cell lines were maintained in a humidified tissue culture incubator at 37°C and 6.5% CO2. All tumor-derived cell lines (MDA-MB-157, MDA-MB-231, MDA-MB-468, MCF-7, ZR75.1, and T47D) were obtained from the American Type Culture Collection. MDA-MB-468 cells were maintained in DMEM (HyClone) and all other tumor cell lines were maintained in α-MEM (HyClone) and all other tumor cell lines were maintained in α-MEM (HyClone), both supplemented with 10% FCS (Atlanta Biological). The immortalized HMECs 76NE6, 76NF2V, and 76NE7 were obtained from Dr. V. Band (Department of Genetics, Cell Biology and Anatomy, University
of Nebraska Medical Center, Omaha, NE) and were maintained in DCFI-1 medium (16, 17). The 76NE6 cell line lacks p53, the 76NE7 cell line lacks Rb and other pocket proteins, and the 76NF2V cell line expresses both p53 and Rb. The Rb and p53 status of all the cell lines used is indicated in Supplementary Table 1. All cells were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 6 weeks. There were enough frozen vials for each cell line to ensure that all cell-based experiments were performed on cells that had been tested and in culture for 6 weeks or less.

**Generation of clonally derived cell lines**

MCF-7 cells stably expressing a shRNA vector targeting p53 were a generous gift from Dr. Xinbin Chen (Department of Surgical and Radiological Sciences, Schools of Medicine and Veterinary Medicine, University of California, Davis, CA). MDA-MB-231 and MCF-7 cells stably expressing shRNA directed against Rb were a generous gift from Dr. E. Knudsen (Department of Cancer Biology, Thomas Jefferson University, Philadelphia, PA). MCF-7 cells with reconstituted caspase-3 were a generous gift from Dr. B. Fang (Department of Thoracic and Cardiovascular Surgery, The University of Texas M.D. Anderson Cancer Center, Houston, TX). MCF-7 RBKD and MCF-7 shRNA control cell lines expressing caspase-3 or pcDNA3.1 empty vector were generated by transfection of the MCF-7 RBKD and MCF-7 shRNA control cell lines with 6 μg of pcDNA3.1/caspase-3 (also a gift from Dr. B. Fang) or 6 μg of pcDNA3.1 empty vector using Genejuce reagent (Novagen) according to the manufacturer’s instructions. Cells expressing these vectors were selected in α-MEM containing 0.5 mg/mL G418 (Mediatech) and 2 μg/mL puromycin (Sigma) for 4 weeks. Single-cell clones were selected and expanded in culture medium supplemented with 0.1 mg/mL G418 and 2 μg/mL puromycin (Sigma) and screened by Western blot analysis for the expression of Rb and caspase-3.

**Adenovirus**

Elafin and Firefly Luciferase transgenes were expressed downstream of a cytomegalovirus promoter in replication-incompetent adenovirus type 5 with deletions of the E1 and E3 genes. The elafin cDNA encodes the 57 COOH-terminal amino acids containing the elastase inhibitory domain (1). Viruses were amplified in the packaging cell line AD-293 (Stratagene) and purified in a CsCl gradient by centrifugation at 176,000 × g (Beckman ultracentrifuge). Multiplicity of infection (MOI) was calculated based on the number of plaque-forming units in AD-293 cells. For infections, 2 × 10^6 cells were infected at an MOI of 1 except for MDA-MB-231 cell lines, which, along with HMEC cell lines, were infected at an MOI of 2. Transgene expression was determined by Western blot analysis. Infection efficiency was examined using an identically titered adenovirus carrying green fluorescent protein (GFP); all cell lines were infected at >90% efficiency.

**Drug treatment**

The pan-caspase inhibitor zVad-fmk (Calbiochem) was solubilized in DMSO at 50 mol/mL and diluted in medium at a concentration of 50 μmol/L.

**Growth curves**

Five thousand tumor cells or 2,500 HMECs were plated in each well of a 24-well plate. After 24 hours, cells were treated with Ad-Elafin, Ad-luc, or PBS in triplicate. Cells were harvested every 24 hours for the indicated time periods. At each time point, cell number was determined using the trypan blue (Fluka) exclusion test.

**DNA content analysis**

After the indicated treatment, 2 × 10^6 cells were fixed by suspension in ice-cold 70% ethanol. Cells were stored at 4°C at least for 24 hours, washed with PBS, and stained in 0.5 mL of propidium iodide (PI) solution [10 μg/mL PI (Molecular Probes) + 20 μg/mL RNase A (Sigma)] in PBS containing 0.5% Tween 20 (Sigma) and 0.5% bovine serum albumin (Sigma) overnight at 4°C. The cells were incubated for 30 minutes at 37°C; PI fluorescence was measured using a BD FACS Calibur flow cytometer and analyzed based on DNA content using FloJo software.

**Bromodeoxyuridine incorporation**

Cells were plated in a 100-mm^2 plate and treated 24 hours later with Ad-Elafin, Ad-luc, or PBS. Forty-eight hours after treatment, cells were pulsed for 1 hour with 10 μg/mL bromodeoxyuridine (BrdUrd; Invitrogen). Cells were prepared and stained with FITC-conjugated anti-BrdUrd monoclonal antibody (BD Biosciences) according to the manufacturer’s instruction. Cells were costained with PI solution. FITC and PI fluorescence was measured using a BD FACS Calibur flow cytometer and analyzed using FloJo software.

**Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling assay**

Both the floating and adherent cells were harvested 72 hours after treatment and subjected to terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (APO-BrdU TUNEL Assay Kit, Invitrogen). The assay was performed according to the manufacturer’s instructions. The Alexa-Fluor 488–conjugated anti-BrdUrd antibody was used in all cases except where GFP expression from stably transfected plasmids (RBKD and control cell line) interfered with detection of the Alexa-Fluor 488 dye; in this case, Alexa-Fluor 647–conjugated anti-BrdUrd antibody (BD) was substituted.

**Western blot analysis and immunoprecipitation/kinase assay**

Cell lysates were prepared and subjected to Western blot analysis as previously reported (18). For each Western blot, 50 μg were blotted onto a 7.5% (Rb, phospho-Rb, and PARP) or 13% (caspase-3, cleaved caspase-3, elafin, and actin)
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SDS-PAGE gel. The primary antibodies used were mouse monoclonal elafin (HM2063, HyCult Biotechnology), mouse monoclonal Rb 4H1 (Cell Signaling Technology), rabbit polyclonal phospho-Rb Ser807/811 (Cell Signaling Technology), rabbit polyclonal phospho-Rb Ser780 (Cell Signaling Technology), rabbit polyclonal PARP (Cell Signaling Technology), rabbit polyclonal caspase-3 (Cell Signaling Technology), rabbit polyclonal cleaved caspase-3 Asp175 (Cell Signaling Technology), and mouse monoclonal actin (Chemicon International, Inc.).

In vitro cyclin-dependent kinase 4 (CDK4) kinase assay was performed as previously described (19) using rabbit polyclonal CDK4 antibody (Santa Cruz Biotechnology) and GST-Rb 769 substrate (Santa Cruz).

Cell viability assay

Cell viability was measured by using an MTT assay in a 96-well plate format. Briefly, 5,000 cells were plated and treated as indicated, then incubated in 50 μL of 2.5 mg/mL MTT in serum-free medium for 4 hours, solubilized (20 mL 1 N HCl, 50 mL, 10% SDS, 430 mL isopropyl alcohol) for 1 hour on a horizontal shaker, and quantified using a spectrophotometer (Victor3, Perkin-Elmer) at a wavelength of 590 nm.

Statistical analysis

All pairwise comparisons were analyzed using Student’s t test. All error bars represent the SEM. Many of the error bars on the growth curves are obscured by the size of the symbol marking each data point; in these cases, the SEM associated with these measurement is negligible when compared with the large range on the y-axis; this is especially true for early time points when cell number is exceptionally low.

Results

Exogenously expressed elafin induces apoptosis in HMECs lacking functional Rb

Elafin is cell cycle regulated, with the lowest relative expression occurring during the S phase of the cell cycle, indicating a possible role for elafin in cell cycle progression (8). Therefore, we first investigated if the exogenously expressed elafin can affect the growth of actively proliferating HMECs. For these experiments, we overexpressed elafin using an adenovirus system; subconfluent 76NE6, 76NF2V, and 76NE7 cells were treated with adenoviral elafin, adenoviral luciferase, or PBS. Lysates were subjected to Western blot analysis to show the expression of the elafin transgene in comparison with endogenous elafin expression in growth factor–deprived (the highest relative expression occurring in G0-G1)3 76NE6 cells (Fig. 1A, compare lanes C and E for each cell line). The overexpression of elafin caused no statistically significant change in the doubling times of Rb-proficient cell lines 76NE6 (PBS: 16 ± 1.1 hours, luciferase: 15 ± 0.5 hours, and elafin: 15 ± 0.3 hours) and 76NF2V (PBS: 18 ± 0.6 hours, luciferase: 18 ± 0.6 hours, and elafin: 20.3 ± 0.5 hours). However, overexpression of elafin in 76NE7 cells, which were devoid of Rb, resulted in a significant decrease in cell number by 96 hours after treatment (Fig. 1A). To determine if the decrease in cell number seen in 76NE7 cells was due to an Ad-elafin–induced block in proliferation or induction of apoptosis, we analyzed BrdUrd incorporation into DNA (Fig. 1B) and measured the percentage of TUNEL-positive cells (Fig. 1C). There was no statistically significant change in BrdUrd incorporation in any of the cell lines tested. 76NE6, 76NF2V, or 76NE7, on ectopic expression of elafin (Fig. 1B). However, when we measured DNA fragmentation using the TUNEL assay, we found that there was a significant increase in TUNEL-positive cells in Ad-elafin–treated 76NE7 cells compared with Ad-luciferase–treated 76NE7 or Ad-elafin–treated 76NE6 or 76NF2V cells (Fig. 1C). These results show that treatment of 76NE7 cells with Ad-elafin results in an increase in apoptosis leading to a significant reduction in cell number 96 hours after treatment. These results also suggest that elafin induces apoptosis preferentially in Rb-deficient cells.

Expression of elafin in Rb-negative breast cancer cells results in apoptosis

Based on the finding that elafin induces apoptosis in Rb-deficient HMECs, we next asked if Rb deficiency in breast cancer cells causes a similar susceptibility to elafin-induced apoptosis. For these studies, we examined the role of elafin in three breast cancer cell lines with functional or wild-type Rb (MCF-7, ZR75.1, and T47D) and three cell lines deficient in Rb (MDA-MB-157, MDA-MB-468, and MDA-MB-436). All six cell lines were treated with adenoviral elafin, adenoviral luciferase, or PBS and were examined for growth kinetics, BrdUrd incorporation, and TUNEL positivity (Fig. 2). Western blot analysis showed that adenoviral elafin resulted in 1- to 3-fold elafin expression compared with control (76NE6 cells growth factor deprived for 48 hours), suggesting that elafin expression in this experiment is at physiologically relevant levels (Fig. 2A). Treatment of Rb-positive breast cancer cell lines with Ad-elafin resulted in a modest growth inhibition, whereas treatment of Rb-negative cell lines resulted in a precipitous decline in cell number (Fig. 2B). All three of the Rb-expressing cell lines showed a significant reduction in BrdUrd incorporation, whereas the Rb-deficient cell lines showed no significant change in BrdUrd incorporation (Fig. 2C). However, examination of TUNEL positivity in these cells showed that whereas none of the Rb-positive cells succumbed to apoptosis, all three Rb-negative cells underwent apoptosis in response to elafin expression (Fig. 2D). The percent TUNEL positivity ranged between 22% and 30% of all Rb-negative cells treated with Ad-elafin, compared with 1% to 5% for those treated with Ad-luciferase (P = 0.0127–0.003). These findings suggest that elafin overexpression results in growth inhibition in Rb-positive cells and apoptosis in Rb-negative breast cancer cells.

Inhibition of caspase activity leads to attenuation of elafin-mediated apoptosis

We next examined if caspase activity is modulated by elafin in the Rb-negative cell lines by evaluating the ability
of Ad-elafin treatment to trigger the cleavage of caspase-3 and PARP, two indicators of caspase-mediated apoptosis (Fig. 3). The Rb-deficient cell line MDA-MB-468 was treated with PBS, Ad-luciferase, and Ad-elafin for the indicated time periods. Western blot analysis revealed cleavage of caspase-3 and PARP, suggesting the activation of caspases following the treatment of cells with Ad-elafin (Fig. 3A). We next investigated if inhibition of caspase activity could negate elafin-induced apoptosis. To this end, MDA-MB-468 and MDA-MB-157 cells were treated with Ad-elafin in the presence of the pan-caspase inhibitor zVad-fmk, DMSO, or PBS, and cell viability was examined over time. Both of these Rb-negative cell lines showed a statistically significant reduction in cell viability when compared with PBS or Ad-luciferase at 72, 96, and 120 hours. However, the addition of zVad-fmk was able to significantly inhibit Ad-elafin-induced apoptosis when compared with DMSO or PBS treatment (Fig. 3B). These results suggest that elafin induces caspase-dependent apoptosis in Rb-deficient breast cancer cells.

Figure 1. Exogenously expressed elafin induces apoptosis in HMECs lacking functional Rb. 76NE6, 76NF2V, and 76NE7 cells were treated with PBS (P), adenoviral luciferase (L), or adenoviral elafin (E). A, cells were harvested 48 h after treatment and subjected to Western blot analysis with the indicated antibodies. 76NE6 cells cultured in growth factor-depleted DCFI-1 medium were prepared as a control (C) for elafin expression. Relative elafin levels were calculated by densitometry (top). B, growth was monitored by trypan blue exclusion test every 24 h for 96 h. 76NE7 cells overexpressing elafin showed a statistically significant difference (t test) in cell number at 72 h (P = 0.0122, versus PBS; P = 0.0125, versus Ad-luc) and 96 h (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc). C, proliferation was assessed 48 h after treatment by measuring BrdUrd incorporation. D, apoptosis was assessed 72 h after treatment by a TUNEL assay. All experiments were repeated in triplicate.
Figure 2. Expression of elafin in Rb-negative breast cancer cells results in apoptosis. MCF-7, ZR75.1, T47D, MDA-MB-157, MDA-MB-436, and MDA-MB-468 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. A, cells were harvested 48 h after treatment and subjected to Western blot analysis with the indicated antibodies. B, elafin expression caused a statistically significant difference in cell number at 96 h in MCF-7 (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc), ZR75.1 (P = 0.0199, versus PBS; P = 0.0027, versus Ad-luc), T47D (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc), MDA-MB-157 (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc), MDA-MB-436 (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc), and MDA-MB-468 (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc), and at 72 h in MDA-MB-157 (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc), MDA-MB-436 (P = 0.0075, versus PBS; P = 0.0152, versus Ad-luc), and MDA-MB-468 (P < 0.0001, versus PBS; P < 0.0001, versus Ad-luc). C, proliferation was assessed 48 h after treatment by measuring BrdUrd incorporation. D, apoptosis was assessed 72 h after treatment by a TUNEL assay. All experiments were repeated in triplicate.
Knockdown of Rb in MDA-MB-231 cells increases sensitivity to elafin-induced apoptosis

Next, we asked if there is a cause-and-effect relationship between Rb and elafin by evaluating the ability of Rb down-regulation to sensitize MDA-MB-231 cells to apoptosis when challenged with elafin. MDA-MB-231 cells expressing Rb shRNA (RBKD) cells were evaluated by Western blot analysis, showing efficient knockdown of Rb and expression of the elafin transgene (Fig. 4A). Treatment of the RBKD cells with Ad-elafin resulted in a significant reduction in cell number 72 and 96 hours after treatment when compared with MDA-MB-231 parental or shRNA clones (Fig. 4A and B). TUNEL staining showed a significant increase in the percentage of apoptotic cells in RBKD cells when compared with control cell lines (Fig. 4C). These results illustrate a direct role for Rb deficiency in sensitizing breast cancer cells to elafin-induced apoptosis.

Rb is required for elafin-induced G0-G1 arrest in MCF-7 cells

We have shown that treatment of Rb-positive breast cancer cell lines with elafin causes them to undergo proliferative arrest, not apoptosis (Fig. 2). We next set out to decipher the
role of elafin in mediating cell cycle arrest in Rb-positive breast cancer cell lines by modulating the expression of both elafin and Rb and assessing the downstream consequences on cell proliferation and apoptosis (Figs. 5 and 6). When we treated MCF-7 (Rb positive) cells with either Ad-luciferase or Ad-elafin and subjected them to cell cycle analysis, we found that overexpression of elafin causes cells to accumulate in the G1-G0 phase of the cell cycle and nearly eliminates the S-phase population by 72 hours (Fig. 5A). We next examined if elafin overexpression modulates the phosphorylation of Rb and the kinase activity of CDK4 in the Rb-positive MCF-7 breast cancer cells. For these experiments, MCF-7 cells were treated with PBS, Ad-luciferase, or Ad-elafin for the indicated time periods and subjected to Western blot and kinase assays. Elafin treatment resulted in a decrease in the phosphorylation of Rb using phospho-specific antibodies directed against Ser807/811, Ser780, and total phosphorylated Rb (Fig. 5B). The CDK4 kinase activity was measured using GST-Rb as a substrate and showed a profound decrease in the kinase activity of CDK4 following overexpression of elafin at 24, 48, and 72 hours (Fig. 5C). These results suggest that the expression of elafin in MCF-7 cells causes a G1-G0 arrest characterized by a decrease in Rb phosphorylation due, in part, to attenuation of CDK4 kinase activity.

Next, we set out to examine if Rb downregulation in MCF-7 cells is sufficient to convert cell fate from growth arrest to apoptosis following elafin expression (as was the case in the Rb-negative MDA-MB-231 cells; Fig. 4). To accomplish this, we treated MCF-7 cells stably expressing an shRNA vector targeting Rb with PBS, Ad-luciferase, and Ad-elafin and followed cell proliferation over 96 hours. Efficient Rb knockdown and consistent expression of the elafin transgene were confirmed by Western blot analysis (Fig. 6A). MCF-7 shRNA control cells treated with Ad-elafin showed a moderate reduction in cell number at 96 hours when compared with controls (Fig. 6A), similar to the pattern seen in the MCF-7 parental cell line (Fig. 5A). However, MCF-7 RBKD cells showed no significant change in growth kinetics following treatment with Ad-elafin (Fig. 6A). In fact,
comparison of the parental, shRNA control, and RBKD cell lines illustrates a significant reduction in the sensitivity of RBKD cells to elafin-induced cell cycle arrest (Fig. 6B). This indicates that knockdown of Rb in MCF-7 cells does not sensitize cells to apoptosis but is sufficient to attenuate cell cycle arrest. Consequently, MCF-7 RBKD cells expressing elafin have a significantly reduced BrdUrd incorporation as compared with shRNA controls and parental MCF-7 cells (Fig. 6C).

Collectively, these results show that elafin-induced growth arrest in breast cancer cells is Rb dependent. We next explored a role for p53 in elafin-mediated growth arrest. Using MCF-7 cells stably expressing a vector generating short oligonucleotides (siRNA) against p53 (Fig. 6D), we showed that overexpression of elafin caused a significant reduction in cell number at 96 hours, identical to the control cell line (Fig. 6D) and the MCF-7 parental cell line (Fig. 4A). BrdUrd incorporation following Ad-elafin treatment showed no statistically significant difference in the elafin-mediated inhibition of BrdUrd incorporation when compared with control cell lines (Fig. 6E). These results indicate that elafin overexpression causes Rb-dependent cell cycle arrest in MCF-7 cells independently of p53 activity.

**Overexpression of elafin causes apoptosis in MCF-7 cells only after knockdown of Rb and restoration of caspase-3**

Downregulation of Rb was shown to sensitize MDA-MB-231 cells to elafin-induced apoptosis (Fig. 4); however, downregulation of Rb in MCF-7 cells was unable to replicate these results (Fig. 6). MCF-7 cells lack endogenously expressed caspase-3, which is required for elafin-induced apoptosis (Fig. 3). This led to the hypothesis that loss of Rb and presence of caspase-3 are both required for elafin-induced apoptosis in MCF-7 cells. To address this hypothesis, we first confirmed that elafin expression fails to induce apoptosis following knockdown of Rb alone or reconstitution of caspase-3 alone. MCF-7 RBKD cells, described in Fig. 6, and MCF-7 cells with reconstituted caspase-3 were treated with PBS, Ad-luciferase, and Ad-elafin, and apoptosis was measured by TUNEL assay, which revealed no significant increase in apoptotic cell death under either condition (Fig. 7A).

To directly examine if Rb downregulation and presence of caspase-3 are required for apoptosis in MCF-7 cells following elafin expression, we established clones of both the MCF-7 RBKD and MCF-7 shRNA control cell lines expressing either the pcDNA3.1 backbone vector or the pcDNA3.1-caspase-3 vector. Figure 5. Expression of elafin in MCF-7 cells causes a G0-G1 arrest. A, MCF-7 cells were infected with adenoviral luciferase or adenoviral elafin. DNA content was analyzed by PI staining; cell cycle distribution was calculated using the Dean-Jett-Fox model. B and C, MCF-7 cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. B, lysates were subjected to Western blot analysis and probed with the indicated antibodies. C, CDK4 was immunoprecipitated from 250 μg of protein lysates and then the immunocomplex was subjected to in vitro lysates using GST-Rb as a substrate.
vector. Knockdown of Rb, stable expression of caspase-3, and expression of the elafin transgene were verified by Western blot analysis (Fig. 7B). Treatment of MCF-7 RBKD cells with Ad-elafin caused a severe reduction in cell viability following reconstituted caspase-3 (Fig. 7C). The same experiment was performed in MCF-7 shRNA control cell lines; however, in these cells, reconstitution of caspase-3 did not lead to a significant decrease in viability compared with controls (Fig. 7D).

Figure 6. Rb is required for elafin-induced G0-G1 arrest in MCF-7 cells. MCF-7 shRNA control and RBKD cell lines were treated with PBS, adenoviral luciferase, or adenoviral elafin. A, lysates were collected 48 h after treatment and analyzed by Western blot. Growth was monitored by trypan blue exclusion test every 24 h for 120 h. Elafin expression caused a statistically significant difference in cell number at 96 h only in MCF-7 shRNA control cells ($P < 0.0001$, versus PBS; $P = 0.0001$, versus Ad-luc). B, cell number at 96 h was compared for MCF-7 parental (refer to Fig. 4A), shRNA control, and RBKD cell lines as a percentage of the PBS control. C, proliferation was assessed 48 h after treatment by measuring BrdUrd incorporation. D, MCF-7 p53KD and siRNA controls were treated with PBS, adenoviral luciferase, or adenoviral elafin. Lysates were collected at 48 h and analyzed by Western blot. Elafin overexpression caused a statistically significant difference in cell number at 96 h in both MCF-7 p53KD ($P < 0.0001$, versus PBS; $P < 0.0001$, versus Ad-luc) and MCF-7 siRNA control cells ($P < 0.0001$, versus PBS; $P < 0.0001$, versus Ad-luc). E, proliferation was assessed 48 h after treatment by measuring BrdUrd incorporation. All experiments were repeated in triplicate.
Figure 7. Overexpression of elafin causes apoptosis in MCF-7 cells only after knockdown of Rb and restoration of caspase-3. A, MCF-7 RBKD, shRNA control, MCF-7 pcDNA3.1 empty vector, and pcDNA3.1-caspase-3 expressing cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. Lysates from MCF-7 pcDNA3.1 empty vector and pcDNA3.1-caspase-3 expressing cells were subjected to Western blot analysis 48 h after treatment (right). Apoptosis was assessed by a TUNEL assay (left). B, stable clones and a stable pool were generated on the MCF-7 RBKD background expressing either pcDNA3.1 empty vector or pcDNA3.1-caspase-3. Cells were treated with PBS, adenoviral luciferase, or adenoviral elafin. Lysates were collected from each cell line and subjected to Western blot analysis with the indicated antibodies. PBS-treated cells were used to assess the levels of Rb and caspase-3, and MDA-MB-231 lysate was used as a control for caspase-3 expression. C, viability was measured by an MTT assay every 24 h for 120 h and calculated at each time point by normalizing the values from luciferase- and elafin-treated cells to PBS control and then plotting the difference between the viability of elafin- and luciferase-treated cells (i.e., elafin effect − viral effect). D and E, stable clones and a stable pool were also generated on the MCF-7 control shRNA background expressing either pcDNA3.1 empty vector or pcDNA3.1-caspase-3. These cells were assayed in the same manner as in B and C. F, the viabilities measured at 120 h in the cell lines generated from the MCF-7 RBKD and MCF-7 control shRNA as well as the parental cell lines were pooled and statistically compared. G, apoptosis was measured by a TUNEL assay.
and E). Comparison of cell viability at 120 hours after treatment showed that elafin-mediated loss of cell viability in MCF-7 RBKD clones expressing caspase-3 was significantly different from that observed in all other MCF-7 cell lines examined (Fig. 7F, compare RBKD CASP3 with the other cell populations). To determine if this reduced viability occurred as a result of increased apoptosis, TUNEL assay was performed on elafin-expressing MCF-7 RBKD cells stably expressing either caspase-3 or the pcDNA3.1 backbone (Fig. 7G). These results revealed a significant increase in the percentage of cells undergoing apoptosis in MCF-7 RBKD cells expressing caspase-3 versus control cells (PCDNA + Ad-luc, PCDNA + Ad-elafin, or CASP3 + Ad-luc). These results indicate that loss of Rb and the reconstitution of caspase-3 are both required for elafin-induced apoptosis in the MCF-7 cell line. T47D cells are Rb positive and endogenously express caspase-3; therefore, we tested the validity of our observation in these cells by knockdown of Rb using siRNA and subsequent treatment with PBS, Ad-luciferase, and Ad-elafin. The analyses reveal that knockdown of Rb is sufficient to sensitize a caspase-3-positive cell line to elafin-mediated apoptosis (Supplementary Fig. 1).

**Discussion**

Elafin is expressed in HMECs, but it is transcriptionally downregulated in tumor-derived cell lines (9). In this study, we show that expression of elafin causes cell cycle arrest or apoptosis in breast tumor-derived cell lines depending on the Rb status but does not affect normal HMECs with an intact G1-S checkpoint. This suggests that elafin has novel antitumor properties in breast cancer and represents a candidate therapeutic capable of specifically targeting tumor cells with disruption of the G1-S checkpoint with no toxicity in normally dividing cells.

We initially observed that expression of elafin in HMECs lacking Rb (76NE7) caused these cells to undergo apoptotic cell death. Such a Rb-dependent apoptotic effect led us to hypothesize that tumor cells with a deregulated Rb pathway could also be forced to undergo apoptosis following elafin expression. Indeed, we observed that the expression of elafin can induce apoptosis in Rb-negative cells but requires caspase-3 activation. Breast cancer cells, which are Rb negative but do not express caspase-3, are resistant to apoptosis. We also found that Rb-positive breast cancer cells are growth inhibited in response to elafin expression.

The loss of elafin in breast cancer cell lines suggests a tumor-suppressive role for elafin in the mammary gland. There is precedent for the existence of a tumor suppressor serine protease inhibitor that is downregulated in breast cancer cells. Maspin, a member of the serpin family of serine protease inhibitors, is differentially expressed in normal mammary epithelial cells and breast tumor cells by epigenetic processes (20). Since its identification, maspin has emerged as a tumor suppressor in breast, prostate, and ovarian cancers with diverse roles in angiogenesis, tumor invasion, apoptosis, and metastasis (21–23). Dissimilar from elafin, a protease target has not been identified for maspin. Elafin is a potent inhibitor of the serine protease HNE, which has an established role during tumorigenesis in the breast and other tissue types (24–27). HNE is an independent prognostic marker in breast cancer and has been correlated with poor prognosis, increased metastasis, and resistance to chemotherapy (24, 25, 28–31). In experimental models, pharmacologic HNE inhibitors were able to attenuate the development of skin tumors, reduce growth and metastasis in a lung xenograft model, and inhibit the proliferation/chemotaxis of pancreatic cells (32–35). Several HNE substrates with relevance to tumorigenesis have been described, including insulin receptor substrate 1, cyclin E, and cut-like homeobox 1 (36–40). Given the intracellular functions of HNE in promoting tumorigenesis, elafin may be a critical component of the intracellular control of HNE in mammary epithelial cells. Our experimental reintroduction of elafin into breast tumor cells suggests that the pathways deregulated by HNE may be critical to proliferation and survival. In particular, elafin toxicity in tumor cells lacking Rb suggests that HNE is required to modulate the apoptotic effect of E2F transcription factors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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