Reexpression of the Tumor Suppressor Gene ARHI Induces Apoptosis in Ovarian and Breast Cancer Cells through a Caspase-independent Calpain-dependent Pathway


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ABSTRACT

ARHI, an imprinted putative tumor suppressor gene, encodes a M subunit 26,000 GTP-binding protein that is 60% homologous to ras and rap but has a dramatically different function. ARHI expression is down-regulated in a majority of breast and ovarian cancers. Using a dual adenovirus system, we have reexpressed ARHI in ovarian cancer and breast cancer cells that have lost ARHI expression. Reexpression of ARHI inhibited growth, decreased invasiveness, and induced apoptosis. At 5 days after infection with ARHI adenovirus, 30–45% of MDA-MB-231 breast cancer cells and 5–11% of SKOV3 ovarian cancer cells were apoptotic as judged by a terminal deoxynucleotidyl transferase-mediated nick end labeling assay and by Annexin V staining with flow cytometric analysis. Although poly(ADP-ribose) polymerase could be detected immunohistochemically in the nuclei of apoptotic cells, no activation of the effector caspases (caspase 3, 6, 7, or 12) or the initiator caspases (caspase 8 or 9) could be detected in cell lysates using Western blotting. When gene expression was analyzed on a custom cDNA array that contained 2304 known genes, infection with ARHI adenovirus up-regulated 15 genes relative to control cells infected with LacZ adenovirus. The greatest degree of mRNA up-regulation was observed in a Homo sapiens calpain-like protease. On Western blot analysis, calpain protein was increased 2–3-fold at 3–5 days after infection with ARHI adenovirus. No increase in calpain protein was observed after LacZ adenovirus infection. Calpain cleavage could be detected after ARHI reexpression, and inhibitors of calpain, but not inhibitors of caspase, partially prevented ARHI-induced apoptosis. Consequently, reexpression of ARHI in breast and ovarian cancer cells appears to induce apoptosis through a caspase-independent, calpain-dependent mechanism.

INTRODUCTION

The ARHI gene encodes a M subunit 26,000 GTPase with 50–60% amino acid homology to RAS and RAP. ARHI is a putative tumor suppressor gene whose expression is down-regulated in a majority of ovarian and breast cancers (1). Introduction of this gene into cultured ovarian and breast cancer cells has suppressed clonogenic growth associated with increased expression of p21, decreased cyclin D1 promoter activity, inhibition of signaling through RAS/MAP, and activation of JNK.3

The mechanism by which ARHI inhibits cell growth has not been examined previously.

Inhibition of signaling through RAS/MAP, and activation of JNK.3

Calpain-dependent Pathway 1

Ovarian and Breast Cancer Cells through a Caspase-independent

MATERIALS AND METHODS

Cell Culture. Two ovarian cancer cell lines [SKOV3 (3) and DOV13 (4)] and a breast cancer cell line [MDA-MB-231 (5)] were used in this study. The MCF-7 breast cancer cell line (6), HL-60 leukemia cell line (7), and Jurkat Burkitt’s lymphoma cell line (8) provided controls in caspase assays. Cells were maintained as monolayers in McCoy’s 5A medium (SKOV3), MEM (DOV13), or RPMI 1640 (MDA-MB-231, MCF-7, HL-60, and Jurkat) supplemented with 10% fetal bovine serum, 100 mL-g-glutamine, 100 mg/mL streptomycin, and 100 units/mL penicillin (culture medium). Cells were grown to near confluence at 37°C in an atmosphere of 95% humidified air and 5% CO2.

Binary Adenoviral Expression System. Initial attempts to package ARHI in 293 cells were not successful and yielded only low titers of virus, related in all probability to the toxic effects of ARHI expression on 293 packaging cells. Consequently, a binary adenoviral vector system was constructed that required infection with two different adenoviral vectors to achieve high levels of ARHI expression (9). Briefly, full-length ARHI cDNA was cloned into an adenoviral vector downstream of a GT (GALA/TATA) promoter that contained five GAL4-binding sites and a TATA box (Fig. 1). This Ad/GT-ARHI construct exhibited very low transcriptional activity in vitro and in vivo. However, ARHI expression could be induced by coinfecting cells with Ad/GT-ARHI and with the adenoviral vector Ad/PKG-GV16 that contained a promoter from the mouse housekeeping gene PGK and a GAL4-VP16 fusion protein that can transactivate the GT promoter. Controls were provided by coinfecting cells with Ad/PKG-GV16 and with Ad/GT-LacZ, an adenovirus that contained the Escherichia coli β-galactosidase gene under the control of a GT promoter.

The Ad/PKG-GV16 and Ad/GT-LacZ adenoviral vectors were prepared by the M. D. Anderson Virus Vector Core Facility as described previously (2). The Ad/GT-ARHI adenoviral vector was prepared by cotransfecting 293 cells with pGT-ARHI and pJM17. Multiple recombinant virus clones were isolated from transfected 293 cells. Recombinant virus from a single plaque was identified by PCR, expanded in 293 cells, and purified twice by ultracentrifugation on a cesium chloride gradient. Virus titers were determined both by optical absorbance at A260 nm (1 A260 nm unit = 1012 particles/ml) and by plaque assay (2). Ad/GT-ARHI could be obtained with a titer as high as 3.8 × 1012

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3 The abbreviations used are: JNK, c-Jun-NH2-terminal kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling assay; PARP, poly(ADP-ribose) polymerase; vp, virus particle(s); pfu, plaque-forming unit(s); MOI, multiplicity of infection.

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Fig. 1. Ad/ARHI/GV16 binary adenoviral vector system for ARHI expression. The inducible adenovirus Ad/GT-ARHI (or Ad/GT-LacZ) is constructed by cloning ARHI or LacZ full-length cDNA downstream of a GT promoter. A second virus, Ad/PGK-GV16, expresses an inducer, the GAL4-VP16 fusion protein (GV16). Mixtures of Ad/GT-ARHI or Ad/GT-LacZ with Ad/PGK-GV16 are referred to as Ad/ARHI/GV16 or Ad/LacZ/GV16.

Ad/PGK-GV16

pGK

GV16

SV40 polyA

Ad/GT-ARHI or Ad/GT-LacZ

pGT

ARHI or LacZ

SV40 polyA

Fig. 1. Ad/ARHI/GV16 binary adenoviral vector system for ARHI expression. The inducible adenovirus Ad/GT-ARHI (or Ad/GT-LacZ) is constructed by cloning ARHI or LacZ full-length cDNA downstream of a GT promoter. A second virus, Ad/PGK-GV16, expresses an inducer, the GAL4-VP16 fusion protein (GV16). Mixtures of Ad/GT-ARHI or Ad/GT-LacZ with Ad/PGK-GV16 are referred to as Ad/ARHI/GV16 or Ad/LacZ/GV16.

Adenovirus Infection and Detection of ARHI Protein. Ovarian and breast cancer cells growing as monolayers were detached with 0.1% EDTA and 0.25% trypsin (Cellgro), washed twice in culture medium, and plated in 60-mm culture dishes with 4 ml of culture medium 24 h before infection with adenoviral vectors. Supernatant medium was aspirated, and fresh medium containing different combinations of adenoviral vectors were added to different plates. The Ad/GT-ARHI or Ad/GT-LacZ vector was mixed with the Ad/PGK-GV16 induction vector at a ratio of 2:1 unless otherwise indicated. This ratio was found to be optimal in preliminary experiments. Mixtures of the vectors are referred to as Ad/ARHI/GV16 and Ad/LacZ/GV16, respectively.

For detection of ARHI expression, cells were washed with PBS two times and lysed in radioimmunoprecipitation assay buffer [50 mM HEPES (pH 7.25), 150 mM NaCl, 50 mM KCl, 50 mM Na2HPO4, 50 mM NaF, 2 mM EDTA, 1% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM NaVO4, and 1 mM phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged at 10,000 × g for 15 min. The protein concentration of supernatant fluid was measured by the Bradford assay (Bio-Rad). Equal amounts of supernatant protein were separated by 12% SDS-PAGE and immunoblotted with anti-ARHI murine monoclonal antibody 15E11 (1). Horseradish peroxidase-conjugated goat antimouse IgG served as the secondary antibody for the enhanced chemiluminescence detection system (ECL; Amersham).

Cell Growth and Mitotic Index. Ovarian or breast cancer cells (3–5 × 104) were seeded in 100-mm culture dishes for 20 h before virus infection. Medium was aspirated from triplicate cultures and replaced with medium supplemented with diluent, Ad/ARHI/GV16, or Ad/LacZ/GV16. At different intervals, cells were trypsinized, and viable cells that excluded trypan blue dye were counted in a hemocytometer. The average numbers of cells per milliliter were calculated and plotted as a function of time.

To measure mitotic index, cells were plated in chamber slides. After 20 h, medium was replaced and supplemented with diluent, Ad/ARHI/GV16, or Ad/LacZ/GV16. At different intervals, cells were fixed in 4% paraformaldehyde for 10 min at room temperature and stained with H&E. The number of mitoses among 1000 cells was counted and presented as the mitotic index.

Clonogenic Growth. Ovarian or breast cancer cells (104) were incubated in 5-ml polypropylene tubes with diluent or infected with Ad/ARHI/GV16 or Ad/LacZ/GV16 for 3 h and serially diluted 5-fold. From each dilution, aliquots of 100 μl were pipetted into 6 wells of a 96-well culture plate (10). An additional 100 μl of media were added to each well, and cells were incubated for 21 days. Clonogenic growth of surviving cancer cells was evaluated by inverted phase microscopy, scoring the number of wells with at least one colony containing more than 30 cells. Estimates of the most probable number of residual clonogenic units were calculated by a modification of the method of Spearman and Karber (11). The number of clonogenic units was calculated as shown below.

\[ \text{Units} = \ln(0.10) + \ln(5/2) - \ln(5/6) r_i \]

where \( r_i \) = wells with cell growth. In all assays, clonogenic efficiency was estimated based on the growth of cells that had been treated with diluent and had not been infected with virus.

Growth of Heterografts in nu/nu Mice. The impact of ARHI expression on tumor growth in vivo was evaluated using heterografts of the human breast cancer MDA-MB-231 in immunosuppressed nu/nu mice. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23) and the institutional guidelines of The University of Texas M. D. Anderson Cancer Center. Human breast cancer xenografts were established in 6–8-week-old BALB/c nu/nu mice (Charles River). MDA-MB-231 cells (5 × 105) were infected s.c. into the mammary fat pad of each mouse. Tumors were measured twice a week, and tumor volumes were calculated as (a × b × c)1/3, where a, b, and c were three diameters. When palpable tumors had grown to a diameter of 0.5 cm, each mouse received three intratumoral injections of 50 μl of PBS containing 9 × 109 vp (1.5 × 109 pfu) of Ad/ARHI/GV16 or Ad/LacZ/GV16. Mice that received PBS or PBS containing 9 × 109 vp (1.5 × 109 pfu) of Ad/GT-ARHI only were used as controls. Animals were sacrificed when tumors reached a diameter of ~1.5 cm.

Cell Cycle Arrest and Apoptosis. Ovarian and breast cancer cells (3–5 × 105) were plated in 100-mm culture dishes for 20 h and incubated with diluent, Ad/ARHI/GV16, or Ad/LacZ/GV16. After infection, cells were trypsinized and collected in PBS at different intervals and fixed on ice with 1% paraformaldehyde and subsequently with 70% cold ethanol. After treatment with 10 μg/ml RNase, cells were stained with 50 μg/ml propidium iodide for cell cycle analysis using a FACScan (Becton Dickenson). Apoptotic cells were detected with a TUNEL assay, performed according to the manufacturer’s instructions.
instructions (BD Pharmingen). The fraction of apoptotic cells was confirmed in some assays with Annexin V. Cells were trypsinized and stained with Annexin V as described by the kit manufacturer (Roche Biochem).

Calpain Expression and Lack of Caspase Activation. Ovarian or breast cancer cells (3–5 x 10^5) were plated in 100-mm culture dishes and incubated with diluent or infected with Ad/ARHI/GV16 or Ad/LacZ/GV16. DOV13 cells and MDA-MB-231 cells were incubated with 4000 vp (67 pfu)/cell, and SKOV3 cells were incubated with 6000 vp (100 pfu)/cell. At different intervals, cells were lysed, and proteins were analyzed by Western blot for caspase and calpain. Antibodies reactive with caspase 3, 6, 7, 8, 9, or 12 (Cell Signaling) and calpain (Chemicon) were incubated with immunoblotted protein. Goat antimouse or antirabbit IgG were used to develop the reactions. Enhanced chemiluminescence (Amersham) was used to detect proteins recognized by the immunological reagents. For analysis of PARP binding to cleaved DNA, cells were stained with a rabbit polyclonal anti-PARP antibody (Roche Biochem), and antibody binding was detected with fluoresceinated sheep antirabbit IgG. After washing, cells were examined using a fluorescence microscope.

Inhibition of Calpain Activity. DOV13 and MDA-MB-231 cells (3–5 x 10^5) were plated in 100-mm culture dishes and incubated overnight to allow the formation of an adherent monolayer. Cancer cells were preincubated with calpain inhibitor I (5 µM), PD150606 (5 µM), or PD151746 (5 µM). Negative controls were provided by incubating cells for 2 h with diluent alone, with the inactive calpain inhibitor analogue PD145305 (5 µM), or the pan-
caspase inhibitor Z-VAD-fmk (5 μM). After 2 h, medium containing the inhibitors or diluent was removed, and cells were incubated with fresh medium alone, Ad/ARHI/GV16, or Ad/LacZ/GV16 at 4 × 10^3 vp/cell. At different intervals, cells in G0-G1 phase (G0/G1), S phase (S), and G2-M phase (G2/M) were measured by flow cytometry (10^5 cells/sample). In vitro assay differences of 1.4% are statistically different (P < 0.05) within an experiment. Replicate experiments are shown in A and B. In A, the fraction of cells in G0-G1 after infection with Ad/ARHI/GV16 differed from control (P < 0.05) and from Ad/LacZ/GV16 (P < 0.0001) at 48–66 h. In B, the fraction of cells in G0-G1 after infection with Ad/ARHI/GV16 differed from control (P < 0.05) at 60–66 h and from Ad/LacZ/GV16 (P < 0.0001) at 54–66 h. The fraction of cells in S phase after infection with Ad/ARHI/GV16 differed from control (P < 0.05) and from Ad/LacZ/GV16 (P < 0.05) at 48–66 h in A and at 60–66 h in B.

RESULTS

Coinfection with Binary Adenoviral Vectors Induces ARHI Reexpression. To test the potential of ARHI for gene therapy, we attempted to develop high titers of a single adenoviral vector that could express ARHI linked to a strong promoter. Multiple constructs proved toxic for packaging cells, prompting the use of a binary adenoviral vector system that has circumvented similar problems in preparing vectors that contained other growth-regulatory genes (9). In one adenoviral vector, ARHI was placed downstream of a promoter with five GAL4-binding sites that permitted very little ARHI expression in vivo, was not toxic for packaging cells, and could be obtained at high titer. A second adenoviral vector was constructed that expressed a GAL4-VP16 fusion protein. Initial experiments were designed to test whether coinfection of ovarian or breast cancer cells with both vectors would induce substantial expression of ARHI protein.

![Fig. 6](cancerres.aacrjournals.org) Effect of reexpression of ARHI on cell cycle distribution of MDA-MB-231 breast cancer cells. Cells were treated with Ad/ARHI/GV16 or Ad/LacZ/GV16 at 4 × 10^3 vp/cell. At different intervals, cells in G0-G1 phase (G0/G1), S phase (S), and G2-M phase (G2/M) were measured by flow cytometry (10^5 cells/sample). In vitro assay differences of 1.4% are statistically different (P < 0.05) within an experiment. Replicate experiments are shown in A and B. In A, the fraction of cells in G0-G1 after infection with Ad/ARHI/GV16 differed from control (P < 0.05) and from Ad/LacZ/GV16 (P < 0.0001) at 48–66 h. In B, the fraction of cells in G0-G1 after infection with Ad/ARHI/GV16 differed from control (P < 0.05) at 60–66 h and from Ad/LacZ/GV16 (P < 0.0001) at 54–66 h. The fraction of cells in S phase after infection with Ad/ARHI/GV16 differed from control (P < 0.05) and from Ad/LacZ/GV16 (P < 0.05) at 48–66 h in A and at 60–66 h in B.

Table 1 Mitotic index of MDA-MB-231 breast cancer cells infected with adenovirus AD/ARHI/GV16 and Ad/LacZ/GV16 (4000 VP/cell)

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.0 ± 6.3^a</td>
<td>30.3 ± 13.6</td>
<td>32.7 ± 2.9</td>
<td>41.3 ± 16.0</td>
</tr>
<tr>
<td>Ad/LacZ + GV16</td>
<td>9.7 ± 1.9</td>
<td>20.0 ± 7.9</td>
<td>30.0 ± 11.2</td>
<td>40.0 ± 17.4</td>
</tr>
<tr>
<td>Ad/ARHI + GV16</td>
<td>7.0 ± 3.5</td>
<td>8.3 ± 1.2</td>
<td>13.7 ± 5.7^b</td>
<td>18.3 ± 11.8^b</td>
</tr>
</tbody>
</table>

^a Mean number of mitoses/1000 cells ± SE for three experiments on different days.
^b Differs from control, P < 0.05 (t test).
Coinfection of SKOv3 ovarian cancer cells with Ad/LacZ/GV16 failed to induce detectable ARHI on Western blot analysis (Fig. 2). Infection with Ad/ARHI/GV16 produced a time- and dose-dependent expression of ARHI (Fig. 2). Neither Ad/GT-ARHI nor Ad/PGK-GV16 alone produced detectable ARHI expression (data not shown). ARHI protein was strongly expressed at 24 h (Fig. 2A) and could be detected as early as 12 h after infection. ARHI expression could be readily detected for 5 days (Fig. 2A).

ARHI expression was measured 3 days after infection of SKOv3 cells with different numbers of Ad/GT-ARHI vp. As few as 500 vp (8 pfu)/cell could induce detectable ARHI expression (Fig. 2B). Increasing numbers of particles produced more intense ARHI expression with a maximum at 8000 particles (133 pfu)/cell (Fig. 2B; additional data not shown). Using the same dual vector system, similar levels of ARHI could be reexpressed in MDA-MB-231 breast cancer cells and DOV13 ovarian cancer cells.

The relative efficiency of adenovirus-mediated gene transfer was also assessed as the percentage of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-stained cells after infection with adenovirus Ad/LacZ/GV16 containing bacterial LacZ gene. Differences were detected between the cell lines tested. More than 90% of MDA-MB-231 breast cancer cells or DOV13 ovarian cancer cells were LacZ positive with a MOI of 4000 vp (67 pfu)/cell. A higher MOI of 6000 vp (100 pfu)/cell was needed to obtain the same level of transduction in SKOv3 ovarian cancer cells, correlating with the number of vp required for optimal ARHI expression. Consequently, different MOIs were used in additional studies of growth inhibition and apoptosis with each cell line.

ARHI Reexpression Inhibits Growth of Ovarian and Breast Cancer Cells. The dual adenovirus system was used to determine the impact of ARHI reexpression on the growth of ovarian and breast cancer cell lines. Infection with Ad/ARHI/GV16 (4000 vp, 67 pfu) markedly inhibited anchorage-dependent growth of DOV13 ovarian cancer cells (Fig. 3A), MDA-MB-231 breast cancer cells (Fig. 3B), and SKOv3 ovarian cancer cells (Fig. 3C) between 3 and 5 days after infection. The number of uninfected DOV13 cells increased 347% over 5 days, whereas the number of DOV13 cells infected with Ad/ARHI/GV16 increased 30% (Fig. 3A). Coinfection with Ad/LacZ/GV16 produced slight but significant inhibition, seen most clearly on days 4 and 5. Consequently, adenoviral infection or overexpression of the bacterial galactosidase protein may also partially inhibit growth of ovarian and breast cancer cell lines in culture.

Inhibition of cancer cell growth was also assessed using a 21-day clonogenic assay. Tumor cells were infected with Ad/ARHI/GV16 or Ad/LacZ/GV16 before plating in a limiting dilution clonogenic assay. Surviving clonogenic units were enumerated by measuring colony formation after 3 weeks of incubation. Infection with increasing numbers of vp of Ad/ARHI/GV16 [1000–4000 vp (16–67 pfu)/cell] produced progressively greater inhibition of clonogenic growth of MDA-MB-231 (Fig. 4). At an optimal dose of 4000 vp (67 pfu)/cell, reexpression of ARHI inhibited clonogenic growth by about 2 log units (99%), whereas incubation with Ad/LacZ/GV16 inhibited clonogenic growth by <0.3 log units (50%). Reexpression of ARHI exerted quantitatively different effects in other cancer cell lines. Coinfection with Ad/ARHI/GV16 inhibited clonogenic growth of SKOv3 cells by 1.5 log units (data not shown).
ARHI Reexpression Suppresses Growth of Human Tumor Heterografts. To assess the antitumor activity of ARHI gene expression against human cancer heterografts, BALB/c nu/nu mice were injected with MDA-MB-231 human breast cancer cells. When tumors became palpable, mice were treated with three intratumoral injections of adenovirus or diluent at 3-day intervals. Intratumoral injection of Ad/ARHI/GV16 induced a partial regression of the established tumor and completely suppressed tumor growth relative to tumor-bearing mice treated with diluent alone (Fig. 5). Less marked inhibition of tumor growth was observed with the Ad/GT-ARHI vector alone or with Ad/LacZ/GV16. Significantly greater inhibition was observed after treatment with Ad/ARHI/GV16 than after treatment with Ad/GT-ARHI alone or with Ad/LacZ/GV16.

Reexpression of ARHI Causes Cell Cycle Arrest and Induces Apoptosis. Inhibition of cancer cell growth by ARHI reexpression might result from cell cycle arrest, apoptosis, or both mechanisms. To evaluate the possible role of cell cycle arrest and apoptosis in ARHI-induced growth inhibition, ovarian and breast cancer cells were infected with Ad/ARHI/GV16 or Ad/LacZ/GV16. At different intervals, cell cycle distribution was evaluated by flow cytometric analysis after staining of cellular DNA with propidium iodide. Apoptotic cells were quantitated with a TUNEL assay. Infection of MDA-MB-231 cells with Ad/ARHI/GV16 increased the fraction of cells in G0-G1 phase and decreased the fraction of cells in S phase after 60–66 h when compared with untreated cells (P < 0.05) or with cells infected with Ad/LacZ/GV16 (P < 0.0001) in replicate experiments (Fig. 6). No G0-G1 arrest was produced by infection with Ad/LacZ/GV16 or Ad/ARHI/GV16 when compared with mock-infected controls, consistent with an effect of adenovirus infection. When a mitotic index was calculated, ARHI expression reduced mitotic cells by 44–72% between 1 and 5 days...
Table 2. T-scores for differential expression of genes in SKOv3 ovarian cancer cells infected with different doses of Ad/ARHI/GV16 compared with cells infected with the same dose of Ad/LacZ/GV16.

<table>
<thead>
<tr>
<th>Adenovirus 100 pfu/cell</th>
<th>Adenovirus 50 pfu/cell</th>
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<th>Name</th>
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<tr>
<td>5.877</td>
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<td>I-FN-γ up-regulated I-5111 protein precursor</td>
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<td>5.06</td>
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<td>3.679</td>
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After infection (Table 1). During the first 2 days, infection with Ad/LacZ/GV16 also decreased the mitotic index, but this nonspecific effect of viral infection or overexpression of foreign protein was not observed on days 3–5. Taken together, these data indicate that reexpression of ARHI induces G0 arrest in MDA-MB-231 cells for at least 64 h and decreases the mitotic index.

Apoptosis of ovarian and breast cancer cells was detected by flow cytometric analysis using Apo-bromodeoxyuridine or Annexin V. Increasing numbers of apoptotic MDA-MB-231 cells were observed between 2 and 5 days after infection with Ad/ARHI/GV16 (Fig. 7B). At 72 h after infection, about 30% of ARHI-infected MDA-MB-231 cells had undergone apoptosis, and this percentage increased to more than 45% by day 5 after infection. An excess of apoptotic cells was not observed after mock infection or after infection with Ad/LacZ/GV16. ARHI reexpression induced apoptosis in other cancer cell lines, albeit at a different level. In SKOv3, a maximum of 11% apoptotic cells was observed after Ad/ARHI/GV16 infection, with baseline rates of apoptosis of around 2% for Ad/LacZ/GV16 and mock-infected cells (Fig. 7A). Consequently, growth inhibition may relate to both cell cycle arrest and apoptosis, depending on the interval after infection and on the cell line.

ARHI-induced Apoptosis Occurs through a Caspase-independent, Calpain-dependent Pathway. Because apoptosis is most frequently mediated by activated caspases that lead to PARP binding to fragmented DNA, we have attempted to detect PARP binding immunohistochemically and to detect caspase activation by Western blots analysis in cancer cells undergoing apoptosis after infection with Ad/ARHI/GV16. Although PARP could be detected immunohistochemically in the nuclei of apoptotic cells (Fig. 8), active fragments of caspase 3 (Fig. 9A), 6 (data not shown), 7 (Fig. 9B), 8 (Fig. 9C), 9 (data not shown), and 12 (Fig. 9D) were not found on Western analysis studied at multiple intervals after infection.

A possible factor that might mediate apoptosis was identified on expression array analysis. Total RNA was extracted from SKOv3 cancer cells infected with Ad/ARHI/GV16 or Ad/LacZ/GV16. cDNA was prepared from these samples, and analysis was performed on a CGH4 array that contained 2304 genes of known and unknown function. Overexpression of ARHI was associated with the up-regulation of 15 genes and down-regulation of 6 genes. The most...
markedly up-regulated gene was the *Homo sapiens* calpain-like protease (Table 2) with a T-score of $-8.452$ after infection with 50 pfu and $-12.568$ after infection with 100 pfu. ARHI-induced up-regulation of calpain was confirmed by Western blot analysis (Fig. 9E).

To determine whether blocking calpain activity could inhibit ARHI adenovirus-induced apoptosis, the effect of calpain inhibitor I, a selective active site inhibitor of calpain, was tested. Baseline levels of apoptosis (2–3%) were induced by mock infection or by infection with LacZ adenovirus. Infection with Ad/ARHI/GV16 increased apoptosis to 10%. The addition of calpain inhibitor I significantly reduced the percentage of apoptotic cells induced by Ad/ARHI/GV16 but did not affect apoptosis after mock infection or infection with Ad/LacZ/GV16 (Fig. 10A).

Although calpain inhibitor I blocks the enzyme’s active site and is one of the most specific calpain inhibitors, it can also inhibit certain other papain-like cysteine proteases. To further implicate calpain in ARHI-induced apoptosis, two novel calpain inhibitors (PD150606 and PD151746) that block Ca$^{2+}$-binding sites of calpain were also tested. An inactive analogue (PD145305) was used as a control. PD150606 (data not shown) and PD151746 (Fig. 10B) both inhibited ARHI-induced apoptosis, whereas the inactive analogue PD145305 did not (Fig. 10C). Caspase inhibitor Z-VAD-fmk failed to inhibit ARHI-induced apoptosis (Fig. 10D). Taken together, these data suggest that reexpression of ARHI induces caspase-independent, calpain-dependent apoptosis.

**DISCUSSION**

Using a binary adenoviral vector system, we have achieved high levels of ARHI expression in human ovarian and breast cancer cells. Reexpression of ARHI inhibited cancer cell proliferation in monolayer cultures, decreased clonogenicity, and inhibited growth of heterografts in immunosuppressed mice. Maximal antitumor activity was produced with constructs that contained ARHI, but some inhibition of cancer cell and heterograft growth was observed after expression of LacZ mediated by the binary adenoviral system. Consequently, inhibition of cancer cell growth may relate to the action of virus or to the overexpression of protein, in addition to any specific effects of ARHI. Moreover, in the heterograft model, it is not possible to assess the potential toxicity of the dual vector system because adenovirus cannot infect normal murine cells. Should aden-o-ARHI prove toxic in the cotton rat or in clinical trials, vectors can be sought with more specific promoters such as the telomerase promoter that might drive expression of ARHI more selectively in ovarian cancer tissue. Possibly of greater concern is a failure to produce complete regression of tumor heterografts. At present, we are exploring combinations of the dual vector ARHI expression system with chemotherapeutic agents. Combinations of ARHI gene therapy with paclitaxel have proven more toxic for cancer cells in culture than either treatment alone.

Inhibition of tumor growth could be related to at least two mechanisms. Reexpression of ARHI protein caused cell cycle arrest and apoptosis in MDA-MB-231 breast cancer cells. Similar results were observed in ovarian cancer cell lines. Apoptosis often involves activation of caspases (12). A careful examination of caspase expression and cleavage failed to reveal activation of caspase 3, 6, 7, 8, 9, or 12 after ARHI reexpression. By contrast, calpain was overexpressed and cleaved in cancer cells after expression of ARHI. Several calpain inhibitors prevented ARHI-induced apoptosis, but the pan-caspase inhibitor Z-VAD-fmk did not.

Calpains are a family of calcium-activated cysteine proteases that include several tissue-specific isoforms (n-calpain) and two ubiquitous isoforms (μ-calpain and m-calpain; Ref. 12). Calpains are distributed widely throughout the cytosol of many cell types. Activation of calpain is an early event in the cellular response to a variety of stressful and potentially pathogenic conditions. Prolonged activation of calpains can lead to cell death (13).

Calpains are regulated by Ca$^{2+}$ and target cellular proteins that are instrumental for maintaining the integrity of the cytoskeleton, making them attractive candidates for mediating certain events during apoptosis. Calpain I may be activated in the cytosol or when bound to the cell membrane, whereas calpain II activation occurs primarily at membrane sites. Our results showed ARHI stimulated only calpain I. Of interest are recent reports that inhibitors of JNK activation block calpain and caspase-induced apoptosis in neurons (14). Reexpression of ARHI has been shown to induce JNK activation, and this may be an important mechanism for activating calpains.

Calpains have been implicated in apoptosis based on two types of observations: (a) the activation of calpains during cell death; and (b) the inhibition of apoptotic execution by various calpain inhibitors. In this study, we provide evidence that calpain is involved in ARHI-induced apoptosis, demonstrating that ARHI-infected ovarian and breast cancer cells exhibit increased amounts of calpain, calpain is cleaved, and inhibition of calpain activity reduces apoptosis. Currently available calpain inhibitors include modified peptides that compete for the active site of the protease (15, 16) and inhibitors that do not bind to the active site, whose selectivity comes from their unique interaction with the calcium-binding domains of calpain, a feature not found in other protease inhibitors. In our experiments, both classes of calpain inhibitor resulted in significant reduction of apoptosis induced by ARHI infection. Known calpain substrates include cytoskeletal associated proteins, kinases and phosphatases, membrane receptors, transporters, and oncogenes.

Recently, Gil-Parrado *et al.* (17) reported that calpains may cleave BCL-2 and permit the translocation of Bax to the mitochondrion. Bid may also be cleaved and presumably translocated to the mitochondria, amplifying the apoptotic signaling pathway. In the case of ARHI-induced apoptosis, these mechanisms may not be relevant because caspase activation was not observed.

Taken together, our data indicate that reexpression of ARHI in cells that have lost expression of this gene can produce a modest G0-G1 cell cycle arrest and more marked induction of apoptosis. Inhibition of tumor cell growth appears to be related predominantly to caspase-independent, calpain-dependent apoptotic events.

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