Survivin and Granzyme B–induced apoptosis, a novel anticancer therapy

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Abstract

Survivin is an antiapoptotic protein highly expressed in malignant cells that confers resistance to cytotoxic therapy. Granzyme B is a potent cytotoxic protein that is released from mammalian natural killer cells and CTLs following noxious stimuli, including foreign invaders. Here, we took advantage of the properties of these two functionally divergent molecules to create a molecular agent that specifically activates Granzyme B within tumor cells. We designed Survivin and Granzyme B–induced apoptosis (SAGA), which consists of a fusion of the Survivin gene promoter to the coding sequence of active Granzyme B. In cultured human tumor cells transfected with SAGA DNA, Granzyme B is rapidly expressed and results in significant tumor cell death. In vivo, mice harboring human ovarian tumors had statistically significant clinical responses to SAGA treatment that were magnified following combination therapy with SAGA and paclitaxel. At the completion of a 3-week therapeutic trial, 3 of 15 animals were free of disease in the SAGA-treated group, and an additional eight animals had tumors that were nonpalpable and only detected on surgical resection. In contrast, 15 of 15 animals in the control and paclitaxel–only–treated groups had tumors at end of therapy. Treatment with SAGA with or without paclitaxel also prevented disease dissemination in 19 of 20 animals. These results strongly suggest that SAGA has the potential to be a potent agent for the treatment of primary and recurrent human ovarian carcinoma.

Moreover, we predict that SAGA will be useful therapeutically in any human cancer that expresses Survivin. [Mol Cancer Ther 2006;5(3):693–703]

Introduction

Ovarian carcinoma represents a significant women’s health concern, as it is the most common cause of death from gynecologic malignancy in the Western world (1). Within the spectrum of ovarian carcinomas, surface epithelial tumors represent 90% of all malignant ovarian neoplasms (2). Survival rates for surface epithelial ovarian carcinoma (30–40%) have remained relatively constant for the past 30 years (1). Despite the abundance of molecular studies in the field of cancer research, significant independent prognostic indicators used in treatment stratification of patients with ovarian tumors are primarily clinical. The expression of Survivin has recently been shown to be aberrantly elevated in >70% of epithelial ovarian tumors (3, 4). Research on the development and efficacy of novel therapeutics for ovarian carcinoma is critical to improve patient survival.

Granzyme B is a serine protease normally produced by natural killer cells and CTLs that is released from intracellular granules in response to stimuli that include viral or bacterial infection, abnormally proliferating cells, or foreign cell invasion (5, 6). This mechanism protects the host cell from destruction by intracellular pathogens, tumors, and foreign cells within the context of the normal immune system (5). Granzyme B is synthesized as a preproenzyme that is activated by two proteolytic cleavages that release an 18-amino-acid leader sequence coded by exon 1 and a dipeptide motif (Gly-Glu) at the NH2 terminus. These cleavages are required for full maturation of the enzyme and to allow it to fold into its catalytically active conformation. To gain entry into its target cell, Granzyme B relies predominantly on perforin, an auxiliary protein. Upon entry, active Granzyme B induces apoptosis through both mitochondrial-dependent and mitochondrial-independent mechanisms (5, 7–9). A decrease in mitochondrial membrane potential, direct cleavage of nuclear proteins leading to DNA fragmentation, and activation of the caspase-3 pathway are all known observed effects of Granzyme B activation (5, 8–10). These diverse mechanisms of Granzyme B–mediated programmed cell death ensure the successful progression of granule-mediated cell death even in target cells lacking functional caspase proteins, thus providing the host with overlapping safeguards against foreign invaders (10).

Here, we report the development of a novel hybrid vector designated Survivin and Granzyme B–induced apoptosis (SAGA). The vector is composed of the Survivin minimal promoter linked to the active Granzyme B cDNA and is...
designed for use in a gene therapy approach to cancer therapy. The Survivin promoter has been previously shown to be silenced in nonmalignant cells and tissues both in vitro and in vivo (11, 12). As the Survivin gene is only minimally expressed in nontransformed, differentiated cells (13) but is highly expressed in the majority of ovarian tumors, it is a logical choice for molecular therapeutic targeting in ovarian carcinoma. By expressing SAGA specifically in tumor cells, we emulate, in part, CTL activity whereby the native CTL-Granzyme B protein initiates programmed cell death through multimodal, nonoverlapping molecular pathways from within the cancer cell. In this work, we show that SAGA effectively reduces tumor growth in an i.p. xenograft model of a primary i.p. ovarian tumor, and that it seems to inhibit the development of metastatic disease in this model.

Materials and Methods

DNA Plasmids

Active human Granzyme B cDNA was amplified from an expressed sequence tag obtained from a human T-cell library with specific oligonucleotide primers. The cDNA fragment was ligated to pDRIVE Survivin (Invivogen, San Diego, CA). Potential clones were confirmed by automated sequencing. Endotoxin-free DNA from pDRIVE Survivin and SAGA were prepared using Qiagen EndoFree Maxiprep kit for all further experiments. DNA quality and concentration were estimated by spectrophotometric analysis.

Cell Culture

MCF10-A, a nontransformed breast cell line (American Type Culture Collection, Rockville, MD) was grown in mammary epithelial growth medium, serum free (Clonetics, Walkersville, MD) supplemented with 2 mL BPE, 0.5 mL human epidermal growth factor, 0.5 mL hydrocortisone, 0.5 mL GA-1000, 0.5 mL insulin, and 100 mg/mL cholera toxin (Sigma-Aldrich, St. Louis, MO) at 37°C, 5% CO2. HeLa, Daoy, MCF-7, MDA-MB231, HepG2, SW620, and CaCo2 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% fetal bovine serum at 37°C, 5% CO2. U2OS and Saos-2 were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum at 37°C, 5% CO2. Jurkat, 697Bcl2, HL60, KG1, SKOV-3, ES2, OVCA429, RH30, RH28, RD2, LN18, and A549 (American Type Culture Collection) were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C, 5% CO2. Transient transfections were done using Effectene transfection reagent (Qiagen, Chatsworth, CA) at a DNA/Effectene ratio of 1:10, as described (14, 15), except for leukemia cells where the DNA/Effectene ratio was 1:25, for 24, 48, or 72 hours. Vincristine sulfate was used at a concentration of 2 μM/L, and paclitaxel was used at a concentration of 10 μM/L in vitro.

Inhibition of Granzyme and Caspase Activities

To inhibit Granzyme B and general caspase activities, we used the cell-permeable inhibitors Ac-AVALLPAVLLA-LAPIEDT-CHO (Calbiochem, La Jolla, CA) and z-VAD-FMK (Calbiochem), respectively, at a final concentration of 20 μM/L.

Cell Viability

To determine cell viability, we did trypan blue exclusion assays. Experiments were done in quintuple.

Annexin V Assays

To analyze early apoptotic events, cells from the different experimental conditions were subjected to staining with Annexin V-FLUOS kit (Roche, Indianapolis, IN) as previously described (14, 15). Experiments were done in quintuple, and analysis was done by fluorescence-activated cell sorting.

Caspase-3 Assays

Two thousand cells from each experimental condition were assayed for caspase-3 activity using Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to manufacturer’s instructions. Caspase-3 activity was measured in a Victor-3 plate reader (Applied Biosystems, Foster City, CA) and expressed as relative luciferase units after background subtraction as previously described (15). Experiments were done in sextuple.

Promoter Activity Assays

Cultured cells were cotransfected with pDRIVE-Survivin and pRL-TK (transfection control) as described above. After 24 hours, cell lysates were prepared using Renilla lysis buffer (Promega). The lysates were used in both Renilla luciferase (Promega) and β-galactosidase assays (Promega), according to manufacturer’s instructions. β-Galactosidase activity was normalized against Renilla luciferase activity, and results were expressed as micro-units of β-galactosidase per milliliter of lysate. Experiments were done in triplicate. For the in vitro promoter experiments, three FVB mice were injected via lateral tail-vein with 10 mg of either pDRIVE-Survivin or CMV-β-galactosidase. Tissues were removed from injected animals (liver, lung, thymus, spleen, kidney, and muscle) at 48 hours after injection, and protein was isolated with Cell Lysis Buffer (Promega). β-Galactosidase activity was assayed using the β-galactosidase assay system (Promega) according to manufacturer’s instructions.

Animal Studies

Six- to 8-week old female nonobese diabetic/severe combined immunodeficient mice were injected with 2.5 × 106 proliferating SKOV-3 cells (prepared in PBS at a density of 1.25 × 107/mL) by i.p. injection. Palpable i.p. tumors developed within 3 weeks (21 days) in all mice injected (n = 50), representing a 100% take rate. In one experiment, mice were randomly assigned to two treatment groups (n = 10) consisting of control-treated (pDRIVE-Survivin, contains the lacZ gene under control of the Survivin promoter) or SAGA-treated mice. Treatment was administered i.p. twice weekly and consisted of 0.5 mg/kg of DNA complexed to in vitro JetPEI (Qiogene, Carlsbad, CA) at an N/P ratio of 10, for a total of 18 days. The injection area was massaged after removal of the needle to aid dispersal of the solution in the abdominal region. In another experiment, mice were randomly assigned to three treatment groups (n = 5) consisting of paclitaxel control, SAGA, or combination of SAGA and paclitaxel. SAGA treatment was administered as described above for
a period of 26 days after tumor establishment. Paclitaxel was administered i.p. (15 mg/kg in a total volume of 200 µL/injection) twice during the course of the treatment period (days 3 and 24). Survival time reflected the time required for the animals to reach any of the experimental end points, including tumor ulceration, weight loss exceeding 15% of body weight, weight gain exceeding 5 g, anorexia, diarrhea, and difficulties ambulating and/or feeding. The studies were done under approval of the Columbus Children’s Research Institute Animal Care Committee.

Biometric Analysis
Mice were weighed weekly after injection of tumor cells using a digital scale. Upon completion of the treatment period, necropsy was done, where the primary tumor, omentum, bladder, endometrium, ovaries, pancreas, spleen, and kidneys were dissected, measured, and weighed. The abdominal region was scored for metastatic foci by gross examination.

Histologic Examination
Tissues isolated after dissection were fixed in 10% neutral-buffered formalin for 16 hours at 4°C and processed for paraffin embedding. Sections were taken at 5 µm in a Leica microtome. Tumors were characterized by staining with H&E and immunohistochemistry for vimentin, pan-keratin, leukocyte common antigen, and epithelial membrane antigen using a Ventana automated stainer. Procedure periodic acid-Schiff staining for cytoplasmic glycogen content was done according to standard protocols to confirm the clear cell adenocarcinoma diagnosis. Tissue sections pretreated with 1% diastase for 1 hour served as a negative control (procedure periodic acid-Schiff-D). To characterize response to therapy histologically, we did in situ immunohistochemical staining with antibodies for Ki-67, human Survivin (B, and Survivin (16). In situ terminal deoxynucleotidyl transferase labeling (also referred to as TUNEL assays) was done with the TdT-FragEL DNA Fragmentation detection kit (Calbiochem) according to manufacturer’s instructions. Collagen-Masson’s trichrome staining was done according to standard protocol. Quantification of staining was done on multiple high-powered fields (10–20) representative of the tissue and confirmed by a certified pathologist (S.H.).

Microscopy
Living cells in 12-well plates were photographed using phase contrast with a Leica inverted microscope at a ×100 magnification. Tissue sections were photographed using phase contrast with a Leica upright microscope at magnifications from ×50 to ×400. Quantification of staining was done on 10 to 20 high-powered fields.

Toxicology
Before euthanasia, mice were anesthetized, and blood was collected via cardiac puncture using a 20-gauge needle. Metabolic assays, including alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine, and electrolyte panel, were done at the Department of Laboratory Medicine of the Columbus Children’s Hospital, according to standard operating procedures.

Biostatistical Analysis
The growth of cells in vitro was modeled with a polynomial curve (cubic), and the rates of growth were compared with a Wilcoxon rank sums test. Differences in tumor weight, size, and number of tumor nodules were analyzed with a two-sample t test with equal variance and two-sided significance. Differences in the incidence of metastasis and palpable tumors after treatment were analyzed by a two-way Fisher’s exact and χ² tests. Kaplan-Meier curves were obtained using the start of treatment as day 1. A log-rank test for equality of survivor functions was used. To analyze differences among mitotic cells, proliferation, and cell death, a simple linear regression model was fitted using animal ID as a cluster variable. To evaluate the differences in the levels of Survivin expression, we used a nonparametric, two-sample Wilcoxon rank sum (Mann-Whitney) test. An ANOVA with repeated measurements was used to analyze body weight changes. In all cases, P ≤ 0.05 was considered significant. All statistical tests were two sided, done, and analyzed with STATA statistical software.

Results
SAGA Rationale and Design
Taking advantage of the tumor specificity of the Survivin promoter (11, 12), we used it to drive the expression of Granzyme B in a genetic approach to cancer therapy. Our novel hybrid construct consisted of a fusion of 268 bp of the human Survivin promoter, with the coding sequence of the active form of human Granzyme B (Fig. 1A). The sequence of Granzyme B used in the construct does not necessitate activation by proteolytic cleavage, as we have fused an artificial start codon to the active form of the enzyme that lacks the first 20 amino acids of the zymogen. Once translated, this sequence encodes the active form of Granzyme B, containing the serine protease and chymotrypsin domains essential for its protease activity. Included within the 268 bp of the Survivin promoter are critical sequences responsible for Survivin’s natural transcriptional regulation, such as the cell cycle–dependent element/cell cycle gene homology region sites and E2F-like, TP53, and Sp1 binding sites (17–20). DNA was transfected into target cells, and Survivin B was expressed through activation of the Survivin promoter. This designed mechanism of Granzyme B expression and activation is perforin independent. The complete construct was designated SAGA.

Specificity of Tumor Cell Growth Inhibition Induced by SAGA
To evaluate the cell growth inhibitory effects of SAGA in vitro, we used multiple transformed cell lines. These included leukemias (T-acute lymphoblastic leukemia, B-acute lymphoblastic leukemia, and acute myelogenous leukemia), central nervous system tumors (medulloblastoma, glioblastoma), soft tissue sarcomas, osteosarcomas (containing wild-type or mutated TP53), hepatocellular carcinoma, colorectal tumors, lung, breast, cervical, and ovarian carcinomas. SAGA efficiently inhibited cell growth.
in all tumor cell lines tested (Fig. 1B), as shown by the reduced growth of treated cells over a 48-hour period compared with control-transfected cells of the same type (Fig. 1B). When used in combination with the chemotherapeutic agents vincristine or taxol in leukemia, medulloblastoma, glioblastoma, rhabdomyosarcoma, breast, and cervical carcinoma cells, a synergistic effect on cell growth inhibition was observed. This was particularly evident in Figure 1.
leukemia, glioblastoma, and breast carcinoma (Fig. 1B, dashed line with open squares). Variable responses to treatment with SAGA were observed, and these largely correlated with the efficiency of DNA delivery observed for the different cell lines (Supplementary Table S1; Fig. 1B). Glioblastoma and the breast carcinoma cell line MDA-MB231 had the lowest transfection efficiency (10–15%), and their growth was less affected, whereas HeLa and Daoy had the highest transfection efficiencies (80–90%), and consequently, a greater impairment on cell growth was observed. Response did not correlate with the level of activation of the Survivin promoter, as shown by promoter reporter experiments in a selection of the cell lines tested (Fig. 1C).

To show the tumor specificity of SAGA, we also transfected the nontransformed human breast epithelial cell line MCF10-A (21, 22) with the SAGA plasmid. Both control- and SAGA-transfected cells grew with an approximate doubling time of 48 hours (Fig. 1B, bottom). SAGA did not inhibit cell growth or induce programmed cell death in these cells due to the absence of Survivin expression in this representative nonmalignant cell type and hence the inability to activate granzyme B. As it has been previously shown that the human Survivin promoter contains conserved elements with the murine Survivin promoter such that it can be activated in mouse tumor cells (23), we injected non–tumor-bearing mice with DNA plasmids encoding the β-galactosidase gene under the control of a cytomegalovirus or the human Survivin promoter to again show the tumor-specific activity of SAGA. We did an analysis of β-galactosidase activity in tissue lysates collected from the injected mice using the β-galactosidase enzyme assay system. The Survivin promoter was silent in all the tissues analyzed (Fig. 1D), suggesting that even if Survivin was expressed at low levels in normal mouse tissues, these levels were not sufficient to activate SAGA. These two types of experiments support the tumor specificity of the SAGA construct.

**SAGA Induces Apoptosis in Ovarian Carcinoma Cells**

**In vitro**

To study the in vivo effects of SAGA on an ovarian cancer xenograft model, we chose the SKOV-3 cell line. SKOV-3 is an epithelial ovarian adenocarcinoma cell line, originally isolated from the ascitic fluid of a patient with metastatic ovarian cancer. It has a known high resistance to cell death of several cytotoxic drugs (24). SKOV-3 cells also express Her2/neu at high levels, a finding clinically synonymous with poor prognosis (25, 26). We transfected SKOV-3 cells with SAGA or pDRIVE-Survivin and assessed the growth of the cells over a period of 72 hours. Additional controls included SAGA-transfected cells that were treated with either a cell permeable Granzyme B inhibitor (Ac-AAVALLAPIEDT-CHO) or a broad caspase inhibitor (z-VAD-FMK). The rates of growth in control and SAGA-treated cells with and without inhibitors were compared at 24, 48, and 72 hours (Fig. 2A). All points were significantly different between groups ($P = 0.0008$), showing that SAGA had a strong growth inhibitory effect on SKOV-3 cell growth.

![Figure 2.](http://mct.aacrjournals.org/) SAGA inhibits tumor growth and induces apoptosis in ovarian carcinoma cells. SKOV-3 cells were transfected with SAGA or pDRIVE-Survivin DNA as described in Materials and Methods. **A**, growth of control (○), SAGA (□), SAGA plus z-VAD-FMK (△), and SAGA plus Granzyme B inhibitor (×) treated cells was followed for 72 h. **B**, control- and SAGA-treated cells were stained with Annexin V/FITC and analyzed by flow cytometry. Single-variable analysis (FITC) showed a higher number of cells staining positive for Annexin V in the SAGA-treated group (open area) relative to the control treated group (shaded area). **C**, one thousand cells from each experimental condition were subjected to caspase-3/7-GLO assay to determine the levels of activation of caspase-3. SAGA-treated cells show elevations in caspase-3 activity, in contrast with near background activation of control-treated cells.

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5 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
in vitro. These results further support the specificity of cell death from SAGA, as its effects were reversed by a potent Granzyme B–specific inhibitor and strongly inhibited by a broad caspase inhibitor.

The molecular effects of SAGA on apoptosis were assessed using two different assays: Annexin V staining and caspase-3 activation. Annexin V is a calcium-dependent, phospholipid-binding protein with high affinity for phosphatidylserine (27). This protein is a sensitive probe for phosphatidylserine exposure to the outer leaflet of the cell membrane and therefore effectively detects early apoptotic events (27). We observed a mean Annexin V staining in 36.7% of SAGA-treated cells compared with <1% in control cells at 48 hours after treatment (Fig. 2B). Caspase-3, a direct cleavage target of Granzyme B, was also used to assess SAGA’s role in apoptosis. Caspase-3 activation was observed in SAGA-treated cells at levels 77 times higher than control cells at 48 hours after transfection (Fig. 2C). The rates of cell death were statistically different between groups for both Annexin V staining and caspase-3 activation, suggesting that SAGA effectively induces caspase-3–mediated programmed cell death in these cells.

**SAGA Reduces Growth of Ovarian Tumors In vivo**

To characterize the clinical and histologic features of a human ovarian carcinoma xenograft model in vivo, we injected $2.5 \times 10^6$ SKOV-3 cells into nonobese diabetic/severe combined immunodeficient mice i.p. All mice developed palpable i.p. tumors within 3 weeks. Tumors became visible as a protrusion through the abdominal wall as early as 4 weeks after injection (Supplementary Fig. SIA). At necropsy, multiple tumor nodules were frequently observed surrounding the primary tumor. Metastatic foci were also observed in other abdominal regions (9 of 10 animals). The larger primary tumors were adherent to the fat in the pelvic region and/or in the peritoneum. Metastatic nodules were found in these regions, in the omentum, mesentery, and throughout the abdomen (data not shown). Histologically, the tumors expressed the mesenchymal cytokeratin protein vimentin and the epithelial markers epithelial membrane antigen and keratin (Supplementary Fig. S1B). High cytoplasmic glycogen content was also detected by procedure periodic acid-Schiff staining in the majority of tumor cells (Supplementary Fig. S1B), a characteristic finding in human clear cell adenocarcinomas.

To evaluate the efficacy of SAGA in the treatment of ovarian carcinoma, 6- to 8-week-old female nonobese diabetic/severe combined immunodeficient mice were injected i.p. with $2.5 \times 10^6$ SKOV-3 cells ($n = 20$). Mice were weighed weekly and examined for tumor engraftment. By 3 weeks, all mice had palpable tumors and were randomly assigned to one of two treatment groups, control or SAGA treated ($n = 10$). SAGA-treated mice received i.p. injections of 0.5 mg/kg of SAGA DNA coupled to linearized polyethyleneimine twice weekly for a total of 18 days, whereas control mice received a similar injection of control DNA (pDRIVE-Survivin) coupled to linearized polyethyleneimine, on the same schedule. The size of treated tumors was dramatically reduced in SAGA-treated animals as noted on physical examination of the animals as early as 1 week after administration of the first course of treatment. Only 2 of 10 animals had palpable tumors at the end of the experimental period compared with 10 of 10 control animals ($P = 0.001$; Table 1). Tumors from control animals were also visible externally at the end of the treatment period. At necropsy, the primary tumors were easily detected in all control-treated animals. The mean number of primary tumor nodules isolated in each control-treated animal was 9 compared with only 1.2 in SAGA-treated animals (Table 1). The difference in the number of tumor nodules between the two groups was statistically significant (7.8; 95% confidence interval, 4.6–11.0; $P = 0.0001$). Only 8 of 10 animals treated with SAGA had visible tumors at necropsy, indicating that two animals had achieved a complete clinical remission. Of the remaining eight animals with tumors, two animals had tumors <1 mm in diameter. The overall mean diameter of SAGA-treated tumors was 2.8 mm compared with a mean diameter of 11.4 mm for control tumors. The difference in tumor size between groups was highly significant (8.5 mm; 95% confidence interval, 6.3–10.7 mm; $P < 0.001$). Consistent with the findings of a dramatic reduction of tumor size, the mean tumor weight for SAGA-treated animals was 22 mg compared with a mean weight of 146 mg in control-treated tumors. This represents a 6.5-fold difference in tumor weight that was also highly significant (123 mg; 95% confidence interval, 83–164 mg; $P < 0.001$).

**SAGA Prevents Metastatic Spread and Increases Overall Survival**

Metastatic nodules, presenting as tumor masses distant from the site of injection, were observed in 9 of 10 control-treated animals. In contrast, 0 of 10 SAGA-treated animals developed metastases ($P < 0.001$; Table 1). Metastatic nodules in control-treated tumors were found within the abdominal cavity distinct from but within the vicinity of the primary tumor as well as in other regions, including the mesentery and lesser omentum, attached to the fat surrounding the pancreas, spleen, and endometrium, or within the bladder and the ovaries. Metastatic foci within the ovaries themselves were detected in 3 of 10 control-treated

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**Table 1. Biometric analysis of control- and SAGA-treated tumors**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animals with tumors at completion of therapy</th>
<th>Disease dissemination</th>
<th>Ovarian metastases</th>
<th>Survival at endpoint</th>
<th>Tumor weight (mg)</th>
<th>Tumor size (mm)</th>
<th>No. nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>9/10</td>
<td>3/10</td>
<td>3/10</td>
<td>146.0 ± 56.4</td>
<td>11.4 ± 2.2</td>
<td>9.0 ± 4.7</td>
</tr>
<tr>
<td>SAGA</td>
<td>8/10</td>
<td>0/10</td>
<td>0/10</td>
<td>9/10</td>
<td>22.0 ± 22.4</td>
<td>2.8 ± 2.4</td>
<td>1.2 ± 0.8</td>
</tr>
</tbody>
</table>

*P* value from Student’s *t* test.
animals (Table 1). These presented unilaterally as one or two small nodules within the stromal region of the ovary. Invasion of the lymphatic system, a known mechanism of metastatic spread in human ovarian tumors, was clearly visible in control-treated tumors (data not shown).

Survival within the two treatment groups, as defined by the time required for the animals to reach any of the preestablished experimental end points (see Animal Studies) throughout the treatment period was calculated by Kaplan-Meier analysis. The median survival time for control-treated animals was 13 days from the start of treatment (25th percentile = 9 days), whereas 100% of SAGA-treated animals survived the entire length of the follow-up period (18 days). At the end of the experimental period, there were 10 survivors in the SAGA-treated group (100%) compared with only three in the control-treated group (30%). The survival differences between control and SAGA-treated animals was significantly different by log-rank test ($P = 0.0014$; Fig. 3).

**Paclitaxel Increases the Efficacy of SAGA Treatment in Ovarian Carcinoma**

To assess potential synergistic effects on the inhibition of tumor growth in vivo, we used a combination of SAGA and paclitaxel. Tumors were established in nonobese diabetic/severe combined immunodeficient mice as described above ($n = 15$). By 3 weeks, all mice had palpable tumors and were randomly assigned to one of three treatment groups: paclitaxel, SAGA, or SAGA plus paclitaxel ($n = 5$). Animals were treated for a period of 26 days with paclitaxel (15 mg/kg, two injections at 3-week intervals), SAGA (0.5 mg/kg of SAGA DNA coupled to linearized polyethyleneimine twice weekly), or the combination of SAGA and paclitaxel (0.5 mg/kg of SAGA DNA coupled to linearized polyethyleneimine twice weekly and 15 mg/kg, two injections at 3-week intervals, administered 48 hours after SAGA injection). All treatments were administered i.p. Route. A decrease in tumor size by physical examination was evident in the SAGA and in the combination therapy groups as early as 1 week after the first treatment course. The decrease in tumor sizes was more significant in animals undergoing combination therapy (Table 2). At the end of the 26-day treatment period, 5 of 5 (100%) of the paclitaxel-treated animals still had tumors compared with 4 of 5 (80%) and 3 of 5 (60%) animals in the SAGA and combination therapy groups, respectively. This represents an increased response rate in the combination arm compared with that observed with SAGA treatment alone.

At the completion of the experimental time course, the primary tumor nodules were resected, weighed, and measured. Tumors isolated from paclitaxel-treated animals had a mean tumor weight of 236 mg compared with 46.8 mg in the SAGA-treated and 11.2 mg in animals treated with combination therapy (Table 2). The differences in tumor weight were significant between paclitaxel and SAGA ($P = 0.004$), paclitaxel and combination ($P = 0.0001$), but also between SAGA and combination ($P = 0.008$). Similarly, mean tumor size in paclitaxel-treated animals was 10.4 mm compared with 3.5 mm in SAGA-treated and 1.4 mm in combination treated animals. These differences were also significant between paclitaxel and SAGA ($P = 0.0003$), paclitaxel and combination ($P < 0.0001$), and SAGA and combination ($P = 0.038$). Taken together, these findings suggest that although the use of paclitaxel does not have a significant therapeutic effect on ovarian tumors when used as a single agent with the dosing schedule used in this tumor model, its use in combination with SAGA significantly enhances SAGA’s efficacy to inhibit tumor growth.

**Molecular Analysis of SAGA-Treated Tumors**

Evaluation of the expression of the proliferation marker Ki-67 and the cell death marker TUNEL were done to determine the effects of SAGA treatment on inhibiting cell growth and inducing programmed cell death in the ovarian tumors in vivo. SAGA-treated tumors had a lower number of mitotic figures than control-treated tumors ($P < 0.001, R^2 = 0.78$; Fig. 4A). Although viable tumor cells were observed in SAGA-treated animals at the completion of therapy, a 60% decrease in the number of proliferating cells was observed by Ki-67 staining ($P < 0.001, R^2 = 0.72$; Fig. 4B). This was accompanied by an increased number of cells undergoing programmed cell death assessed by TUNEL assay (5.6 times increase compared with control-treated tumors; $P < 0.001, R^2 = 0.77$; Fig. 4C).

To molecularly link the mechanism of SAGA-induced cell death in vivo to Survivin and Granzyme B, we determined the levels of Survivin and of Granzyme B in the treated tumors. The number of Survivin-expressing cells was decreased by 60% in SAGA-treated tumors ($P = 0.0039$), showing the specific targeting and elimination of Survivin-expressing tumor cells by SAGA (Fig. 4D). Granzyme B was also observed in the predicted cytoplasmic pattern in tumors treated with SAGA but not in any control-treated tumors ($P < 0.0001$; Fig. 4E). SAGA-treated tumors displayed areas of karyopyknosis and karyorrhexis by histology, corresponding to apoptotic cells as identified by in situ TUNEL. These large patches of TUNEL-positive cells were associated with reactive fibrosis, which enveloped the involved regions of the tumor in a thick layer of fibroblastic

![Figure 3](image-url)
tissue, as visualized by Masson’s trichrome staining (Supplementary Fig. S2). By contrast, large control-treated tumors lacked the organized regions of fibrosis observed in SAGA-treated tumors.

**Lack of Toxicity Associated with SAGA Therapy**

In addition to the antitumorigenic potential of SAGA, we also investigated the safety of SAGA using our *in vivo* model. Metabolic assays were done on untreated, control-treated, and SAGA-treated mice following the completion of our therapeutic trial to assess liver and kidney functions. These chemical analyses included assays for alanine aminotransferase, alkaline phosphatase, blood urea nitrogen, creatinine and serum sodium, potassium, chlorine, and bicarbonate. We observed no significant differences from published normal values (28–31) for any variable tested. No significant differences were observed among control, SAGA-treated, and untreated animals, suggesting that neither our delivery vehicle nor our therapeutic agent were toxic at the concentrations used in our studies (Supplementary Table S2).

Weekly weight measurements of animals from both groups showed that there were no significant variations in weight between the control-treated and SAGA-treated groups (*P* = 0.86; data not shown).

We further analyzed the gross pathology of treated mice (data not shown). There were no signs of necrosis in any of the abdominal organs analyzed (ovaries, endometrium, bladder, spleen, pancreas, liver, stomach, kidneys, and intestine). Additionally, there were no signs of necrosis in other organs outside the abdominal cavity (heart, lungs, and brain). The *Survivin* promoter has been previously reported to be silent in healthy, nontransformed tissues (11). Nonetheless, because Granzyme B is a highly cytotoxic protein, we assayed a multitude of tissues from SAGA-treated mice for the expression of Granzyme B by immunohistochemistry. We did not detect Granzyme B expression in any of the tissues analyzed (ovaries, endometrium, spleen, pancreas, kidneys, adrenal glands, liver, and stomach). Further analysis was done on splenocytes and bone marrow extracted from treated and untreated animals. We assayed for apoptosis and necrosis by Annexin V/propidium iodide staining and observed no differences between treated and untreated animals, thus suggesting no untoward effects of the treatment with SAGA (data not shown).

**Discussion**

Ovarian carcinoma is the fifth leading cause of death from cancer among women in the United States and the fourth among women over 40 years of age, resulting in an estimated 14,000 deaths per year (1). Although treatment of early-stage ovarian cancer yields 5-year survival rates close to 90% (32), ~25% to 40% of patients (especially those with unfavorable prognostic indicators) are likely to relapse. Patients who clinically relapse <6 months after chemotherapy have very limited treatment options, often with low response rates to standard chemotherapeutic agents and a poor median survival (11 months; ref. 33). For this reason, there is a pressing need for the development of novel therapies that will effectively treat advanced and recurrent ovarian carcinoma. The SKOV-3 i.p. model we employed in this study proved to be an ideal animal study model for biotherapy research in ovarian carcinoma, as it simulates the i.p. disseminating behavior of human ovarian carcinoma. The inhibitor of apoptosis gene, *Survivin*, is expressed at high levels in 70% of human ovarian tumors (3, 4). A hallmark feature of this gene is that it is up-regulated in the majority of malignant cancers; however, it is expressed at either low or undetectable levels in most normal tissues (11, 12). The regulation of *Survivin* expression in tumor cells occurs predominantly through engagement of specific elements within its promoter by key proteins involved in cancer initiation and progression, including the tumor suppressor proteins TP53 and RB (17–20). In this work, we found that by manipulating the human *Survivin* promoter to drive the expression of the highly cytotoxic active human Granzyme B protein in human tumor cells, we could effectively inhibit i.p. ovarian tumor growth and, strikingly, diminish intra-abdominal metastatic dissemination.

The SAGA approach provides a fusion between suicide gene therapy and immunotherapy, both of which are currently used individually in biological therapies for cancer. CTL-mediated immunity is an important natural response to tumor cell growth (5, 34). It is also an important therapeutic avenue that has been explored in clinical trials to reduce tumor cell proliferation (35–37). Most immunotherapy studies targeted to cancer cells rely on the use of activated T lymphocytes to perform this action. These studies can be hampered by the lack of antigen-presenting tumor cells within the patient. Suicide gene approaches are currently used by a number of different groups to treat cancer (38, 39). We believe the SAGA approach may have several advantages over these other methods. First, the *Survivin* promoter is either silenced in healthy tissues or is not sufficiently activated to potentiate cell death in the few tissues that express it at low levels. This is an attractive feature, as it would be expected to confer minimal toxic side effects. *Survivin* is

Table 2. Biometric analysis of i.p. ovarian tumors treated in combination with paclitaxel and SAGA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animals with tumors at completion of therapy</th>
<th>Disease dissemination</th>
<th>Tumor weight (mg)</th>
<th>Tumor size (mm)</th>
<th>No. nodules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paclitaxel</td>
<td>5/5</td>
<td>4/5</td>
<td>236.0 ± 79.8</td>
<td>10.4 ± 1.3</td>
<td>6.2 ± 3.9</td>
</tr>
<tr>
<td>SAGA</td>
<td>4/5</td>
<td>1/5</td>
<td>46.8 ± 46.6</td>
<td>3.5 ± 2.6</td>
<td>2.2 ± 0.8</td>
</tr>
<tr>
<td>Paclitaxel + SAGA</td>
<td>3/5</td>
<td>0/5</td>
<td>11.2 ± 13.5</td>
<td>1.4 ± 1.5</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

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also expressed at very high levels in the majority of human malignancies, representing an additional attraction for Survivin-mediated therapies. In this study, we showed the effectiveness of SAGA in a wide variety of tumor cells in vitro. A critical feature of our proposed methodology is the use of the active form of human Granzyme B and not the inactive zymogen. This presents a major advantage over some gene therapy approaches, as it is a human protein and it eliminates the need for treatment with a prodrug, a required component in many suicide gene approaches currently available (38, 39). Intraperitoneal delivery of SAGA complexed to linearized polyethyleneimine results in Survivin-specific expression of human Granzyme B exclusively within the tumor, making it a powerful, tumor-specific agent. SAGA’s molecular mechanism of action is based on that of Granzyme B activity, but by using this method, we bypass a number of obstacles encountered with current CTL-mediated therapy, avoiding most tumor evasion mechanisms.

It is well established that Granzyme B can promote activation of several members of the caspase family of cysteine proteases through proteolytic processing of these proteins. Granzyme B can also promote caspase activation indirectly through proteolysis of the Bcl-2 family protein Bid (40). Proteolysis of Bid by Granzyme B results in the release of mitochondrial cytochrome c into the cytosol (41). Cytochrome c efflux from mitochondria then leads to the engagement of the apoptosome pathway and ultimately to programmed cell death (42). Studies using purified Granzyme B suggest that nanomolar amounts of this enzyme are sufficient to engage the target cell death machinery (43). Ultimately, Granzyme B triggers a two-tiered apoptotic cascade involving at least seven caspases with caspase-3 playing a major role (7). By unleashing Granzyme B in Survivin-expressing tumor cells with SAGA, we effectively activate multiple pathways of apoptosis within the tumor, a unique aspect of this single-agent therapy. As a consequence of SAGA treatment, Survivin-expressing cells are specifically targeted, leading to Survivin-specific downstream effects, including a decrease in cell proliferation and an increase in programmed cell death. These effects are specific to Survivin activation and Granzyme B activity, as they can be inhibited by Z-VAD-FMK and by a cell permeable Granzyme B inhibitor, as shown. The combined molecular effects of SAGA alone resulted in a significant decrease in tumor size, tumor weight, and number of tumor nodules, as well as a complete clinical remission in 3 of 15 (20%) treated animals. Strikingly, 95% of tumors isolated from SAGA-treated mice were localized to the injection site, suggesting that treatment with SAGA greatly diminishes i.p. dissemination of the disease. These results support a potential role for SAGA therapy in the treatment of early as well as late-stage ovarian disease.

Results from our study also show a synergistic cell growth inhibitory effect of SAGA and paclitaxel in ovarian tumors in vivo. From a molecular standpoint, paclitaxel acts by inducing a G2-M block in tumor cells through promotion and stabilization of microtubule assembly (44, 45). Accumulation of tumor cells in G2-M induces an increase in Survivin expression at the transcriptional level via the cell cycle–dependent element/cell cycle gene homology region domains in its promoter. An additional mechanism has also been observed in which the Survivin promoter activity is increased by paclitaxel immediately before an arrest in G2-M (46). Paclitaxel is therefore a logical choice for combination therapy with SAGA. We observed a significant increase in complete clinical remission and decrease in tumor growth following treatment in vivo with paclitaxel and SAGA in combination.

Finally, we also showed that the use of SAGA in an ovarian carcinoma xenograft model has minimal or no toxic side effects on kidney, liver, and bone marrow functions, thus conferring SAGA an attractive toxicologic profile. The use of the Survivin promoter to specifically activate gene expression of a proapoptotic molecule is a novel concept. Furthermore, the use of the active form of Granzyme B to

Figure 4. Molecular effects of SAGA treatment in vivo. Tumors isolated from control- and SAGA-treated animals were fixed, processed, and paraffin embedded. Sections were collected at 5-μm thickness and stained with H&E, immunostained with Ki-67, labeled with terminal deoxynucleotide in situ, and stained with a polyclonal anti-Survivin antibody and a polyclonal antibody to human Granzyme B. Quantification of staining was done in 0.1 mm² areas by counting the number of positive cells in control-treated (black columns) and SAGA-treated (white columns) tumors. Quantification of staining for mitotic index (A), proliferation (B), apoptosis (C), Survivin (D), and Granzyme B (E).
encode a proapoptotic agent for tumor-targeted therapy has not been previously described. Selection of active Granzyme B, a molecule natively employed by the immune system to target tumor cells, ensures that treated cells will undergo cell death through multimodal, nonoverlapping pathways. This mechanism overrides multiple pathways that are commonly mutated in cancer cells to prevent activation of the programmed cell death pathway. It is not dependent on engagement of common tumor suppressor genes, such as TP53 or RB, which are often mutated or deleted in cancer cells.

In conclusion, our study shows that SAGA is a potent and specific mediator of cell death in an i.p. ovarian carcinoma tumor model. The antitumorigenic and anti-metastatic properties of this molecule observed here support its future preclinical development with potential clinical applications in the treatment of multiple human malignancies that express Survivin at high levels.

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