Overexpression of Edg-2/vzg-1 Induces Apoptosis and Anoikis in Ovarian Cancer Cells in a Lysophosphatidic Acid-independent Manner

Tatsuro Furui, Ruth LaPushin, Muling Mao, Humera Khan, Steve R. Watt, Mary-Ann V. Watt, Yiling Lu, Xianjun Fang, Shinichi Tsutsui, Zahid H. Siddik, Robert C. Bast, Jr., and Gordon B. Mills

INTRODUCTION

Lysophosphatidic acid (LPA) is one of the major growth factors in ascites from ovarian cancer patients and appears to play an important role in proliferation, survival, and invasion of ovarian cancer cells. Recently, several groups have shown that Edg-2, which belongs to the G-protein coupled receptor family, is a functional LPA receptor. Northern blot analysis showed that most ovarian cancer cell lines express Edg-2. Edg-2 expression was especially high in the cisplatin-resistant and slowly proliferating A2780 cell line and was almost absent from the cisplatin-sensitive and rapidly proliferating A2780 cell line. We thus assessed whether Edg-2 could contribute to changes in cell viability, cell proliferation, or cisplatin resistance. Stable overexpression of Edg-2 in A2780 cells induced an exogenous LPA-independent decrease in proliferation but did not alter cisplatin sensitivity. The LPA-independent decrease in growth rate induced by overexpression of Edg-2 could be explained, at least in part, by Edg-2-induced apoptosis rather than by effects on cell cycle progression. In agreement with the results in stably transfected A2780 cells, transient expression of Edg-2 in Jurkat T cells also induced apoptosis. When cells were separated from the extracellular matrix, they underwent a specialized form of apoptosis called anoikis, which is particularly important in survival of cells in the circulation during metastasis. A2780 cells engineered to overexpress Edg-2 were particularly sensitive to anoikis. These observations suggest that Edg-2 may be a negative regulator for ovarian epithelial cell growth and metastasis.
MATERIALS AND METHODS

Cell Culture. RPMI 1640, DMEM with 4.5 μg/l of glucose, McCoy’s 5A medium, FCS, and Trypsin/EDTA were purchased from Life Technologies. Cell lines 2008 and 2008/c13*5.25 (26, 27) were kindly provided by Dr. Stephen B. Howell (Cancer Center, University of California, San Diego, La Jolla, CA), and A2780 and 2780cp (28, 29) were kindly provided by Dr. Thomas C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). HEY was obtained from Dr. Ron B. Buick (Ontario Cancer Institute, Toronto, Ontario, Canada; Ref. 30). T-antigen Jurkat cells were from Dr. Gerald R. Crabtree (Stanford University, Stanford, CA). A431 and T47D were provided by Dr. Mien Chi Hung (University of Texas M. D. Anderson Cancer Center, Houston, TX). 105E80 was from Wellie Auersterg (University of British Columbia, Vancouver, Canada). OCC1 was established in the laboratory of one of the authors (31). Other cell lines were obtained from the American Type Culture Collection (Rockville, MD), and all of them were maintained following the suppliers’ recommendations.

Preparation of Cisplatin. Cisplatin (Sigma Chemical Co.) was initially dissolved in PBS without Ca ++ or Mg ++ at 1.0 mM, and dilutions from this solution were made in media to obtain the desired drug treatment concentrations.

Cell Transfection. For transient transfection, Cos7 cells were transfected with the pcDNA3 expression vector alone (Invitrogen) or pcDNA3-Flag-hEdg-2 (provided by Drs. W. H. Moolenaar and G. C. M. Zondag, The Netherlands Cancer Institute, Amsterdam, the Netherlands) using FuGENE 6 reagent (Boehringer Mannheim, Inc.), according to manufacturer’s recommendations.

The plasmid pEGFP-F containing the farnesylated GFP was provided by Dr. Wei Jiang (Salk Institute, La Jolla, CA). pEF-BOS-GFP was made by removing the GFP insert from the original plasmid (pEGFP-F) digested with NheI/Sall and placing it into pEF-BOS expression vector (32) linearized with XbaI/Sall.

T-antigen Jurkat cells were transiently cotransfected with pBOS-EGFP, which expresses a membrane anchored GFP, and pcDNA3 or pcDNA3-FLAG-Edg-2 by electroporation using GENE PULSER II (Bio-Rad), according to the manufacturer’s recommendations.

To establish stably Edg-2-expressing cell lines, A2780 cells were transfected with pcDNA3 or pcDNA3-Flag-hEdg-2 using FuGENE 6 reagent according to the manufacturer’s instructions. Cultures were maintained in medium containing 500 μg/ml G418 (Life Technologies, Inc.) for 14 days. Resistant clones were picked for expansion and characterization. Two separate clones transfected with pcDNA3 alone were selected and named E1 and E2 (Empty 1 and 2). Six clones were selected from the Edg-2 transfecants. Several clones expressed Edg-2 intracellularly without cell surface expression (not presented). Only clone 6 expressed significant cell surface levels of Edg-2. Clone 6 (transfected with pcDNA3-Flag-hEdg-2) was sorted by selecting the top 5% of cells reactive with anti-Flag M2 antibody (Sigma) and FITC goat antimouse antibody (Caltag). Cells were subcloned and 6.3 and H1 selected as high level expressors. All cells were used at early passage, and Edg-2 expression was confirmed by flow cytometry and Western blotting (see below).

Western Blotting. For the detection of Edg-2 exogenous expression, Western blotting was performed as described previously (33). Briefly, cells were lysed with lysis buffer containing 50 mM HEPES (pH 7.25), 150 mM NaCl, 50 μM ZnCl₂, 50 mM NaH₂PO₄, 50 μM NaF, 2 mM EDTA, 1 mM Na₃VO₄, 1% NP40, and 2 mM phenylmethylsulfonyl fluoride, and protein concentration was measured with BCA protein assay reagents (Pierce). Total cell lysates containing equal amounts of cellular protein were mixed with SDS sample buffer. After separation with 10% SDS-PAGE, proteins were electroblotted onto Immobilon-P (Millipore) and incubated with primary anti-Flag M2 antibodies. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Amersham) using horseradish peroxidase-conjugated secondary antibodies.

Flow Cytometric Analysis. A half million cells were harvested and washed with cold PBS twice. Cells were resuspended in 100 μl of PBS containing 3 μg of anti-Flag M2 antibody and 5% FBS. Cells were incubated with antibody for 45 min on ice, and then cells were washed with PBS containing 5% FBS twice. Pellets were resuspended in 100 μl of cold PBS containing 1 μg of FITC-conjugated goat antimouse IgG and placed on ice for 30 min. Cells were washed with PBS containing 5% FBS twice and then resuspended in PBS. Cells were assessed in a FACScan (Becton Dickinson) and analyzed with CellQuest (Becton Dickinson).

Receptor Binding Assay. Receptor binding assays were performed as described previously (23). Briefly, cells were maintained in RPMI 1640 containing 10% fetal bovine serum and washed three times with cold PBS before assessment of binding. Duplicate 0.2-ml aliquots of 2 × 10⁶ cells were incubated with 2 × 10⁵ cpm of [³H]LPA in 0.25% BSA-PBS binding buffer for 45 min at 0°C. The final concentration of [³H]LPA in the binding incubations was 10 nM. The binding cell suspensions were passed through GF/C filters that were washed with 12 ml of ice-cold PBS containing 0.05% Tween 20, air dried, and added to scintillation fluid for quantification of radioactivity bound to the cells. Total and nonspecific binding were evaluated in the absence and the presence of 10 μM nonradioactive LPA, respectively. Specific binding was calculated from the difference in cpm between total binding and nonspecific binding.

[³H]Thymidine Incorporation/DNA Synthesis. To measure proliferation, cells were incubated at 2500 cells/well in 100 μl of medium with 10% FCS in 96-well, flat-bottomed plates (Becton Dickinson) for 8 h. Cells were then starved by overnight incubation in serum-free complete medium, followed by incubation under the indicated conditions for an additional 18 h. Thymidine incorporation was determined by a 6-h pulse labeling with 1 μCi [³H]thymidine/well, followed by harvesting and scintillation counting.

MTT Assay. To measure cell growth, MTT assays were carried out essentially as described (34). Briefly, cells were incubated in 100 μl of medium in 96-well plates with additions as indicated. After incubation, 20 μl of MTT solution [5 mg MTT (Sigma/ml in PBS)] was added and incubated at 37°C for 6 h. One hundred μl of lysing buffer [20% SDS in dimethyl formamide (Sigma), pH 4.7] was added to each well, followed
by overnight incubation at 37°C to dissolve the reduced MTT crystals; the relative cell viability was obtained by scanning with an ELISA reader with a 570-nm filter.

**Cell Number Counting.** To assess the growth rate of cell lines, the number of the viable cells were counted on a hemocytometer by microscopy. Briefly, $8 \times 10^4$ cells in complete culture media were plated in six-well plates. After 8 h, 3 wells were washed with PBS twice then harvested with 0.5 ml of Trypsin/EDTA. Cells were diluted with 1 ml of complete culture media and stained with trypan blue dye (Sigma).

**Preparation of Total RNA and Northern Blotting.** Total cellular RNA was extracted from cultured cells using the TRIZOL Reagent (Life Technologies, Inc.) according to manufacturer’s instructions. RNA samples (20 μg) were size fractionated by formaldehyde/agarose gel electrophoresis and capillary transferred to Hybond-N nylon membrane (Amersham). RNA was immobilized by UV cross-linking and then prehybridized and hybridized to $^{32}$P-labeled cDNA probes. Quality and comparable loading of RNA were confirmed by rehybridization of nylon membranes to the $^{32}$P-labeled cDNA of β-actin or 18S RNA.

**Apoptosis: Cell Cycle Analysis.** Progression of cells through the cell cycle was examined by flow cytometry. Cells were stained with anti-Flag M2 antibody to detect Flag expression and then fixed with 1% of paraformaldehyde and 70% ethanol and stored at $-20^\circ$C overnight. The cells were resuspended in 1 ml of cold PBS containing 20 μg/ml propidium iodide (Calbiochem), 0.5% Tween 20 (Sigma), and 10 μg of...
DNase-free RNase A (Sigma). Cells were incubated at room temperature for 30 min, and DNA content was determined using a FACScan. Cell sorting was performed using CellQuest. Cells were analyzed for DNA content using pulse processing. A dual-parameter dot plot of fluorescence width versus fluorescence area was displayed to allow the exclusion of cell aggregates from analysis. Sort regions were defined on a fluorescence area histogram with cells to the left of the G0-G1 peak (hypodiploid) identified as an apoptotic population.

Anoikis. Anoikis assays were carried out essentially as described (35). Briefly, 1.5 million cells were plated on 100-mm Petri dishes in 7 ml of culture medium and placed on a rocker in a humidified atmosphere containing 5% CO2 at 37°C. Twenty-four h (or indicated time) after plating, cells were collected, subjected to a brief trypsin treatment to dissociate spheroids, counted, and then fixd in 1% paraformaldehyde and stored in 70% ethanol at −20°C. Cells were then processed and analyzed for apoptosis using the APO-DIRECT kit (Becton Dickinson), FACScan, and CellQuest.

RESULTS

Expression of Edg-2/vzg-1 mRNA in Ovarian Cancer Lines. Edg-2 mRNA was detected in 3T3 mouse fibroblasts, cos7, NOE cells or Tag-immortalized NOE (IOSE80) and most of the ovarian cancer cell lines assessed with Northern blotting. In contrast, Edg-2/vzg-1 mRNA was not detected in Jurkat T-leukemia cells, SKBr3 breast cancer cells, or A2780 ovarian cancer cells (Fig. 1). The levels of Edg-2 did not demonstrate a consistent alteration with transformation, with some cancer cell lines having higher and some lower levels than NOE cells. 2780cp cells, which were derived from A2780 by selection in cisplatin (36, 37), express extremely high levels of Edg-2 mRNA.

Edg-2 Highly Expressing 2780cp Grows Slower than A2780. Prior studies demonstrated that 2780cp is highly resistant to cisplatin-induced cell death and grows slower than A2780 cells (36, 37). Drug sensitivity determined by MTT assay (Fig. 2) shows that the 2780cp is ~20-fold more resistant to cisplatin than A2780. The rate of growth of 2780cp cells was also markedly slower than that of A2780 cells in the presence of 10% FCS (Fig. 3).

Edg-2/vzg-1 Overexpression Does Not Significantly Alter Cisplatin Sensitivity. To determine whether the overexpression of Edg-2/vzg-1 in 2780cp contributed to the drug resistance of 2780cp, A2780 cells were stably transfected with pcDNA3 or pcDNA3-Flag-hEdg-2. The expression of Flag-hEdg-2 was confirmed by Western blotting using anti-FLAG antibody (M2; Fig. 4A), flow cytometric analysis using the same antibody, and FITC-conjugated goat antimouse IgG as secondary antibody (Fig. 4B) and LPA binding (Fig. 4C). Clone 6, clone 6.3, and H1 express Flag-tagged hEdg-2, as determined by Western blotting and flow cytometry, whereas E1 and E2 derived from cells transfected with an empty vector did not. Furthermore, 6.3 cells and H1 cells that were engineered to overexpress Edg-2 showed 7–10-fold more specific LPA binding than E1 and E2 cells. Intriguingly, 2780cp only demonstrated 2-fold higher LPA binding that A2780, despite the marked increase in mRNA levels (Fig. 1). Dose-response curves for cisplatin sensitivity showed that clone 6 was slightly more resistant to cisplatin than E1 or E2. Conversely, clone 6.3 and H1, which had been
Fig. 4 Expression of Flag-hEdg-2 in stable transfected cell lines. A2780 cells were stably transfected with pcDNA3 (E1 and E2) or pcDNA3Flag-hEdg-2 (6, 6.3, and H1) and expression of FLAG-hEdg-2 checked by Flag expression. A. Western blotting with anti-Flag M2 antibody. Lysates of Cos7 cells transiently transfected with pcDNA3 or pcDNA3Flag-hEdg-2 were used for negative and positive controls. Twenty μg of each cell lysate were separated through 10% SDS-PAGE and electroblotted to Immobilon-P. Membranes were probed with anti-Flag M2 antibody. To confirm that equal amounts of protein were loaded in each lane, membranes were reprobed with anti-β-actin antibody. Arrows, molecular weights (Rainbow Marker; Bio-Rad). B. Flag-hEdg-2 expression on the cell surface detected by flow cytometry. Unfixed cells were incubated with anti-Flag M2 antibody and FITC conjugated secondary antibody. FL1-H, intensity of FITC fluorescence; count, the number of cells. M1, cells expressing elevated levels of Edg-2 on their surface. C, receptor binding assay. Two million cells in cold PBS containing 0.25% BSA were incubated with 2 × 10^5 cpm of [3H]LPA for 45 min. The binding cell suspensions were passed through GF/C filters that were washed with 12 ml of ice-cold PBS containing 0.05% Tween 20, air dried, and added to scintillation fluid for quantification of radioactivity bound to the cells. Total and nonspecific binding were evaluated in the absence and the presence of 10 μM nonradioactive LPA, respectively. The data represent one of two independent experiments. The average was calculated for groups of three; bars, SE. Data shown in the graph is percentage of control.
Edg-2/vzg-1 Overexpressing Cell Lines Show Slower Growth Rates. Potential differences in growth rates of the stably transfected A2780 lines were assessed by counting trypan blue-stained cells and by MTT dye conversion (Fig. 6A). Independent of exogenous LPA, but in the presence of fetal bovine serum which contains LPA (1), Edg-2-expressing clone 6.3 and H1 cell lines grew more slowly than did parental cells or cells transfected with empty vector. Doubling time of the E1 cell line was ~17.9 h, whereas that of clone 6.3 cells was 22.2 h. Cell cycle transit, determined by DNA synthesis of E1 cells, was consistently higher than that of Edg-2-expressing 6.3 and H1 (Fig. 6B). However, the kinetics of thymidine incorporation paralleled the growth rate (compare Fig. 6, A and B). Thus, the differences in thymidine incorporation likely reflect the differences in the number of cells present during the thymidine pulse. To determine whether the effect of Edg-2 expression on cell proliferation might be attributable to low levels of LPA present in serum (1), thymidine incorporation was examined in the presence of various concentrations of LPA (Fig. 6C). Edg-2 overexpression in A2780 did not influence the proliferative response to LPA. Thus, the Edg-2 receptor does not appear to link LPA to proliferation or account for the effects of LPA on proliferation in ovarian cancer cells (3, 9), at least as represented by A2780.

Edg-2/vzg-1 Overexpression Induces Cell Death. Because changes in the rate of cell cycle transit did not appear to account for the differences in proliferation or doubling time between parental and Edg-2-expressing A2780 cells, we assessed whether expression of Edg-2 might alter rates of apoptosis. The total cell population of clones E1 and E2, which do not express Edg-2, were analyzed. 6.3 and H1 were stained for Edg-2 expression with anti-Flag M2 antibody, and only Flag (Edg-2)-positive cells were examined. Apoptotic cells were detected as a hypodiploid peak on propidium iodide staining. E1 showed 1.19% apoptotic cells, E2 showed 1.1% apoptotic cells (not presented), 6.3 showed 6.90% apoptotic cells, and H1 showed 7.06% apoptotic cells (Fig. 7A). This degree of apoptosis could account, at least in part, for the decreases in proliferation observed with Edg-2 overexpressing cells (Fig. 6).

When cells are detached from their extracellular matrix, they undergo a form of apoptosis designated anoikis (38, 39). Anoikis is a critical determinant of whether a cell will survive in the circulation during metastases (40). Clones E1, E2, 6.3, and H1 were cultured under conditions that prevent attachment to plates for 24 h and were analyzed for apoptosis using a terminal deoxynucleotidyl transferase-mediated nick end labeling assay and flow cytometry (Fig. 7B). Under anoikis conditions, E2 showed a low level of cell death (0.09%), whereas 6.3 showed marked death (63%). In similar experiments, H1 demonstrated a 46-fold increase in anoikis as compared with E2. E1 and E2 were not substantially different in multiple experiments (not presented). Thus, expression of Edg-2 renders A2780 cells sensitive to apoptosis and particularly susceptible to anoikis.

2780cp Cells Are More Sensitive to Anoikis. Because overexpression of Edg-2 sensitized A2780 cells to anoikis, we assessed whether 2780cp cells, which have high levels of Edg-2, may also be more sensitive to anoikis. In the presence of 10% fetal bovine serum, there was no difference in anoikis in A2780 and 2780cp cells (Fig. 8, 0.01% versus 0.06%). This suggested that 2780cp may have adapted to survival in the presence of serum over the multiple cell passages (40 passages) that occurred since the initial derivation of 2780cp. Indeed, when the cells were cultured in the presence of limiting concentrations of serum, 2780cp were markedly more sensitive to anoikis than were A2780 (Fig. 8, 87% versus 52%). The overexpression of Edg-2 in 2780cp may thus contribute to an increased sensitivity to anoikis.

Expression of Edg-2 Induces Apoptosis in T-Antigen Jurkat Cells. We tested the relationship between cell death and Edg-2 expression in Jurkat T cells to determine whether Edg-2 could induce LPA-independent cell death in multiple cell lineages. We carried out flow cytometric analysis of T-antigen immortalized Jurkat cells transiently transfected with Edg-2 by electroporation. Cells were cotransfected with pEF-BOS-GFP (to allow identification of transfected cells) and pcDNA3 or pcDNA3FLAGhEdg-2. After 24 h, ~6% of the cells expressed GFP (Fig. 9). 5.04% of the GFP-positive cells in the pcDNA3 cotransfected cells and 13.92% of the GFP-positive cells in the pcDNA3FLAGhEdg-2 cotransfected cells underwent apoptosis (Fig. 9), as indicated by the presence of a hypodiploid peak. Thus, Edg-2 induces a LPA-independent increase in apoptosis in multiple cell lineages.
DISCUSSION

Several studies have shown that Edg-2/vzg-1 is a receptor for LPA (22–25, 41). LPA appears to play an important role in the genesis of ovarian cancer (3, 4, 9, 42), increasing cellular activation (3, 9), cellular proliferation (3, 9), drug resistance (18), i.p. growth (4, 42), protease production, and LPA production. Furthermore, LPA levels are markedly elevated in ascites (19) and plasma (20) from ovarian cancer patients, implicating it in ovarian tumorigenesis. We thus assessed which, if any, of the effects of LPA are mediated by Edg-2 in ovarian cancer cells.

It has been shown recently that most human and mouse tissues express Edg-2/vzg-1, including the ovary (23, 25).

A2780 ovarian cancer cells, which are widely used to represent cisplatin-sensitive ovarian cancer cells (36, 37), did not express detectable levels of Edg-2/vzg-1, but 2780cp cells, which are highly resistant to cisplatin (36, 37) and were derived from A2780 cells, expressed high levels of Edg-2/vzg-1 (Fig. 1). This suggests that overexpression of Edg-2/vzg-1 could potentially explain the resistance of 2780cp to cisplatin. This would be compatible with the ability of LPA to inhibit cisplatin-induced apoptosis in HEY ovarian cancer cells (18). However, overexpression of Edg-2 failed to transmit drug resistance to A2780, indicating that Edg-2 was unlikely to mediate drug resistance directly.

2780cp cells have been reported to grow more slowly than A2780 cells (37), perhaps accounting in part for their resistance to cisplatin, which is a cell cycle active agent (43). The data suggest that Edg-2 may contribute to the slower growth rate of 2780cp because Edg-2-transfected A2780
grew slower than control cells (Fig. 6), similar to the observation that 2780cp cells, which express higher levels of Edg-2, grow slower than A2780 cells, which do not express Edg-2 (Fig. 3; Ref. 37). Strikingly, Edg-2 overexpressing A2780 cells did not exhibit changes in rates of cell cycle progression but rather exhibited increased rates of apoptosis when compared with A2780 cells, which do not express Edg-2. The differences in the growth rate between Edg-2-expressing cells and nonexpressing cells could possibly result from Edg-2-expressing cells dying through apoptosis (Fig. 7A). The effect of Edg-2 expression on apoptosis was particularly apparent when cells were grown under conditions that contribute to anoikis (Fig. 7B). This suggests that Edg-2 may be a negative regulator of cell survival and may play a role in regulating the metastatic cascade of which bypassing anoikis is an integral component (40).

The effect of Edg-2 on apoptosis in ovarian cancer cells and Jurkat T cells was independent of exogenous LPA. Furthermore, expression of Edg-2 in A2780 cells did not alter the ability of LPA to induce proliferation. Ovarian cancer cell lines can produce LPA (44), potentially resulting in autocrine activation of the receptor. Furthermore, apoptosis was seen in the presence of 10% FCS, a rich source of LPA (3, 19, 45, 46), which could lead to activation of Edg-2. In addition, FCS may contain additional, as yet unidentified, factors that can activate Edg-2. The effects of overexpression of Edg-2 do not appear to be attributable to sequestration of LPA because the addition of up to 20 μM LPA did not reverse the effects of overexpression of Edg-2 (not presented). Alternatively, high-level expression of Edg-2 may be sufficient for ligand-independent activation of the receptor. In support of this contention, overexpression of Edg-2 in baculovirus leads to LPA-independent coupling to Gi1, Goa, and G11 (47).

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Fig. 7  Edg-2 overexpression induces apoptosis and anoikis. A, DNA content was assessed with flow cytometry. The hypodiploid peak (apoptotic cells) is indicated with M1. E1 cells showed 1.19% of apoptotic cells; 6.3 and H1 showed 6.9 and 7.06% of apoptotic cells, respectively. In similar experiments, E2 showed 1.1% apoptotic cells (not presented). This experiment was repeated five times. B, cells were cultured on a rocking platform to prevent adherence for 24 h. DNA of cells was double stained with propidium iodide and FITC-dUTP using the APODIRECT kit. R2, cells that are undergoing anoikis. The population of cells undergoing anoikis in E2 and 6.3 were 0.09 and 62.92%, respectively, a 600-fold increase. In similar experiments, H1 demonstrated a 46-fold increase in apoptosis as compared with E2. E1 and E2 were not substantially different in multiple experiments. This experiment was repeated four times.
receptors that contribute to cell proliferation. This is similar to studies with other families of GPCR, where both positive and negative receptors exist, and suggests that selective agonists for Edg-2 may decrease the growth of ovarian cancer cells and potentially tumor cells from other lineages.

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