Apolipoprotein E Is Required for Cell Proliferation and Survival in Ovarian Cancer

Yu-Chi Chen,1 Gudrun Pohl,1 Tian-Li Wang,2 Patrice J. Morin,3 Björn Risberg,4 Gunnar B. Kristensen,3 Albert Yu,1 Ben Davidson,4 and Ie-Ming Shih1,2

Departments of Pathology, Gynecology and Oncology, Johns Hopkins University Medical Institutions and National Institute of Aging, Baltimore, Maryland and Departments of Pathology and Gynecologic Oncology, The Norwegian Radium Hospital, University of Oslo, Montebello, Oslo, Norway

Abstract

Apolipoprotein E (ApoE) has been recently identified as a potential tumor-associated marker in ovarian cancer by serial analysis of gene expression. ApoE has long been known to play a key role in lipid transport, and its specific isoforms may participate in atherosclerogenesis. However, its role in human cancer is not known. In this study, apoE expression was frequently detected in ovarian serous carcinomas, the most common and lethal type of ovarian cancer. It was not detected in serous borderline tumors and normal ovarian surface epithelium. Inhibition of apoE expression using an apoE-specific siRNA led to G2 cell cycle arrest and apoptosis in an apoE-expressing ovarian cancer cell line, OVCAR3, but not in apoE-negative cell lines. Furthermore, the phenotype of apoE siRNA–treated OVCAR3 cells was reversed by expressing engineered mutant apoE with introduced silent mutations in the siRNA target sequence. Expression of apoE in nuclei was significantly associated with a better survival in patients who presented peritoneal effusion at the time of diagnosis (5-year follow-up, P = 0.004). This study suggests a new role of apoE in cancer as apoE expression is important for the proliferation and survival in apoE-expressing ovarian cancer cells. (Cancer Res 2005; 65(1): 331-7)

Introduction

Identification of tumor-associated markers is critical not only in understanding tumorigenesis but also in providing new markers for diagnosis and prognosis (1, 2). Discovery of new markers seems more urgent for clinically aggressive cancers like ovarian carcinoma in which the current diagnostic and therapeutic approaches are limited (3). Recent advances in transcriptome-wide technologies have led to the discovery of a host of emergent tumor-associated markers (4–6). Comparing gene expression profiles in ovarian cancer to benign ovarian surface epithelial cells using serial analysis of gene expression (SAGE), Hough et al. (7) have identified a number of genes that are differentially expressed in ovarian cancer. We selected one of these genes, apolipoprotein E (ApoE), for further characterization because it is potentially involved in a number of signal transduction pathways that may be critical in regulating cancer cell proliferation and survival.

ApoE has long been known as an essential constituent of plasma lipoproteins responsible for cholesterol transport and metabolism. Plasma apoE is produced mainly in the liver but also in the brain, adrenal glands, kidney, and macrophages. It associates with cholesterol-rich proteins such as low-density lipoprotein (LDL) and enables these protein-lipid complexes to bind to the members of the LDL receptor family present in a variety of tissues (8). The binding of apoE and certain LDL receptor family members not only leads to endocytosis and subsequent importation of lipid-protein complex into the cells but also initiates signal transduction (9, 10). For example, one of the LDL receptor family members, gp330, has been known to mediate intracellular signaling through Src-homology recognition motifs for the PI3 kinase, protein kinase C, casein kinase II, and cyclic AMP–dependent protein kinase (11). The signaling pathways that are initiated by these receptor-ligand interactions on the cell membrane affect the proliferation of various normal cell types, including vascular smooth muscle (12–16), inhibit platelet aggregation (17), regulate inflammatory gene expression (18), and affect the metabolism and survival in neurons through the Akt pathway (19). In addition to the exogenous source of apoE, some cells produce endogenous apoE by transcriptional activation of the apoE gene. In contrast to exogenous apoE that binds to cell-surface LDL receptors and mediates endocytosis, endogenous apoE is mainly associated with Golgi compartments in the cytosol (20). The cellular functions of endogenous apoE are less well understood, but it may be secreted and act as an autocrine or paracrine growth factor, thereby modifying the microenvironment. For example, it has been shown that endogenous apoE stimulates serum-independent cell proliferation. In addition, endogenous apoE derived from macrophages plays an important role in the etiology of atherosclerosis (12).

Besides its well-recognized role in lipid metabolism and atherosclerogenesis, apoE has been shown to involve in several pathophysiologic processes not directly related to lipid transport. Specific isoforms of apoE participate in the development of Alzheimer’s disease, immunoregulation, and neurite outgrowth (20–23), suggesting that apoE may play a more general role than simply lipid transport. In order to explore the new roles of apoE in human cancer, we evaluated apoE expression in tumor tissues and established a cell culture model to study its cellular function by knocking down apoE expression in apoE overexpressing ovarian cancer cells. Besides, the prognostic relevance of apoE expression was determined to show its potential application as a marker to predict clinical outcome in ovarian cancer patients.

Materials and Methods

Tissue Samples. The acquisition of paraffin tissues and frozen specimens was approved by the Institutional Review Boards at Johns Hopkins University and the informed consent was obtained from the Norwegian Radium Hospital. A total of 194 ovarian carcinomas from the ovary, 119

Note: Yu-Chi Chen and Gudrun Pohl contributed equally.

Requests for reprints: Ie-Ming Shih, Johns Hopkins Medical Institutions, 1503 East Jefferson Street, Room B-315, Baltimore, MD 21231. Phone: 410-502-7774; Fax: 410-502-7943; E-mail: isshih@jhmi.edu.

1,2005 American Association for Cancer Research.
metastatic serous carcinomas, 148 specimens of effusion containing serous carcinomas, 10 ovarian serous borderline tumors, 10 ovarian serous cysts, and 10 normal ovarian surface epithelium were retrieved from the department of Pathology at the Johns Hopkins Hospital and the Norwegian Radium Hospital. More than 95% of ovarian carcinoma was of serous type and of high grade except 20 low-grade serous carcinomas. Formalin-fixed paraffin-embedded tissue blocks from the above cases were obtained from archival material. We also included eight frozen tissue samples of high-grade ovarian serous carcinoma and seven primary cultures of ovarian surface epithelium for Western blot analysis. The diagnoses of the paraffin and fresh tissue specimens were confirmed by microscopic examination on the tissue sections before analysis. All the eight fresh ovarian cancer tissues had corresponding paraffin tissue for immunohistochemistry. The diagnosis of effusion samples was established by evaluation of smears and cell block sections from formalin-fixed paraffin-embedded pellets by experienced cytopathologists (B.R. and B.D.) and were then further characterized using immunocytochemistry with broad antibody panels against carcinoma, mesothelial, and leukocyte epitopes, as previously described (24, 25). The procedures for fixation and block preparation for effusion specimens were identical to those employed for tissue samples.

**Immunohistochemistry and Western Blot Analysis.** Expression of apoE was studied by immunohistochemistry and Western blot analysis. The antibody used for immunohistochemistry was an apoE-specific monoclonal antibody (Transduction Lab, Lexington, KY) at a dilution of 1:600 followed by the EnVision + System using the peroxidase method (DAKO, Carpinteria, CA). The frequency of positive cells was estimated by randomly counting ~500 tumor cells from three different high-power fields (×40). For the negative control, an isotype (IgG1)-matched antibody, MN-4, was used in parallel (26). A positive reaction was defined as discrete localization of the brown chromogen in the cytoplasm or nucleus. A specimen was scored in a blinded fashion by observers according to the following criteria: negative, 1% to 5%, 6% to 50%, 51% to 100% of immunoreactive neoplastic cells with cytoplasmic/nuclear staining. Any discrepancy between the two observers was resolved through rescoring by another investigator.

Western blot analysis was done using the same antibody (1:1,000) on seven specimens of ovarian surface epithelial cell cultures, eight high-grade serous carcinoma tissues and four ovarian cancer cell lines, including OVCAR3, SKOV-3, OV-90, and CAOV3. Similar amounts of total protein from each lysate were loaded and separated on 12% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and electrophoretically transferred to a nitrocellulose membrane. Western blot was developed with a detection reagent (Promega, Madison, WI) as described above. The blots were visualized with chemiluminescence (Pierce, Rockford, IL) and were developed by chemiluminescence (Pierce, Rockford, IL). The sequences for the control siRNA were 5′-aagtggtgccacggctgag-3′ (siRNA-1) and 5′-ggatgctgaagctcataaa-3′ (siRNA-2). The sequences for the control siRNA were 5′-aactggcactctgcttt-3′ (siRNA-3) and 5′-aaggtgctgaagctcataaa-3′ (siRNA-4). The sequences of target and control siRNA were derived from the apoE coding region, but the control siRNA did not have an effect on apoE expression. Blast analysis (http://www.ncbi.nlm.nih.gov/BLAST/) did not reveal overlapping regions between both target sequences and other human genes. Both adherent and floating cells were harvested for Western blot, cell cycle analysis, and apoptosis detection at different time points.

**Cell Cycle Analysis and Apoptosis Detection.** Both attached and floating cells were harvested for study. For DAPI staining, 3 × 10^5^ cells were resuspended in 50 μL of PBS and 350 μL of staining solution containing 0.6% NP40, 3% paraformaldehyde, and 10 μg/mL DAPI. DAPI-stained cells were subject to cell cycle analysis using a BD-LSR flow cytometer (Becton Dickinson, Mountain View, CA). The sub-G1 population in the cell cycle analysis was defined as the fraction of apoptotic cells. To confirm the apoptotic cells, DAPI- or annexin V-stained cells were also examined under a fluorescence microscope. The staining of annexin V has been described in our previous report (28).

Briefly, 10^5^ cells were suspended in 100 mL of annexin-binding buffer and were then incubated with 5 μL of Alexa568-bound annexin V (Molecular Probes, Eugene, OR) at room temperature for 15 minutes. Apoptotic cells were defined as those cells that were fluorescent after annexin V staining. The percentage of annexin V-positive cells was measured by the flow cytometer.

**Rescue Assay by Expression of Engineered ApoE with Silent Mutations.** In order to show the specificity of apoE siRNA in targeting apoE, we did a rescue assay by expressing engineered mutant apoE that was not sensitive for apoE siRNA-1 silencing in OVCAR3 cells. To achieve this, we introduced silent mutations into apoE cDNA, encoding the targeted gene that were thought to destroy complementarity with the siRNA, whereas maintaining the wild-type amino acid sequence. The cDNA of apoE was prepared from OVCAR3 cells, PCR amplified, and cloned to a mammalian expression vector, pcDNA6 (Invitrogen) using the following primers: 5′-acatgtaagttctgtgggctg-3′ (forward primer) and 5′-gttgaggctgctgccaca-3′ (reverse primer). Site-directed mutagenesis was used to generate mutant apoE with silent mutations using the template of wild-type pcDNA6/apoE. The silent mutations were designed to be located in the apoE siRNA-1 targeting sequence. Two primers were designed to generate two mutated nucleotide residues. To generate the mutation close to the 3′ end, we use the reverse primer, 5′-cagggaaagctgagctgagagccagga-3′ and the forward primer, 5′-gttgaggctgctgccaca-3′, which in combination with the reverse primer designed above, created a 74-bp fragment. To generate another mutation close to the 5′ end, we designed the forward primer 5′-Ggaggtggagctgccagaagggaga-3′, which in combination with the reverse primer used above resulted in an 886-bp fragment. Another PCR was done with both of the fragments to create a fusion PCR product containing the mutations using the PCR condition as follows: denaturation for 2 minutes at 94°C followed by three cycles of denaturation at 94°C for 10 seconds, annealing at 67°C for 30 seconds and extension at 68°C followed by three cycles of denaturation at 94°C for 10 seconds, annealing at 47°C for 30 seconds and extension at 68°C followed by three cycles of denaturation at 94°C for 10 seconds, annealing at 47°C for 30 seconds and extension at 68°C followed by 27 cycles of denaturation at 94°C for 10 seconds, annealing at 56°C for 30 seconds and extension at 68°C. The PCR product was cloned to pcDNA6 to generate pcDNA/mApoE and it was sequenced to validate the mutations.

For an efficient transfection, OVCAR3 and 293 cells were transfected with the pcDNA/mApoE or vector (pcDNA) by electroporation using a Nucleofector II (Amaxa, Köln, Germany). The cells were cultured in 24-well plates for 18 hours. The cells were transfected with apoE siRNAs or control siRNAs. Western blot analysis was done in 293 cells to assess whether mutant apoE expression was not sensitive for apoE siRNA silencing. OVCAR3 cells were analyzed for cell cycle progression and apoptosis. We determined if pcDNA/mApoE-transfected cells could rescue the apoE siRNA-1–induced phenotypes.
ApoE Expression Analysis Using the SAGE Database. To further extend our findings of apoE expression in ovarian serous carcinomas, we compared the gene expression levels in the SAGE libraries of carcinomas that are deposited in the public database (http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd=tagsearch). A total of 34 libraries were retrieved and they included carcinoma libraries and normal counterparts from pancreas, prostate, breast, stomach, and colon in addition to ovary. The selection of libraries for analysis was based on their availability in the SAGE database (till January 2004). The tag number from each library was retrieved by requesting the apoE tag sequence (CGACCCCACG) from the SAGE database. They were expressed and normalized as the number of tags of apoE per one million of total tags in a given SAGE library.

Statistical Analysis. Statistical analysis was done by applying the SPSS-PC package (version 10.1, SPSS, Chicago, IL, 2001). P < 0.05 was considered statistically significant. Clinical and pathologic data were available for the majority of patients. Studies of the association between apoE expression in effusions and solid tumors and clinicopathologic parameters were undertaken using the two-sided \( \chi^2 \) test. Univariate survival analyses were done using the Kaplan-Meier method and log-rank test. Expression categories in the clinical sample survival analysis were clustered as described in figure legend so as to allow for a sufficient number of cases to be included in each category. Student’s \( t \) test was used to evaluate the difference in cell number in culture.

Results

ApoE Expression in Ovarian Cancer Tissues. To assess the biological roles of apoE in tumor progression, we first did immunohistochemistry on surgically removed ovarian tumors and their benign counterparts. ApoE immunoreactivity was present in 85% of primary high-grade serous carcinomas, 90% of solid metastatic high-grade serous carcinomas, and 64% of effusion specimens containing high-grade serous carcinomas. In contrast, apoE staining was not detectable in benign counterparts, including 10 normal ovarian surface epithelium, 10 serous cystadenomas, and 10 serous borderline tumors (Fig. 1). Cytoplasmic staining was shown in 81% of cases and nuclear immunoreactivity was observed in 19% of cases. Low-grade serous carcinoma, a less common type of serous tumors, was positive for apoE in only 40% of cases and the staining pattern was always focal. The staining intensity was always strong in positive cells and therefore percentage scoring was used in this study. The specificity of apoE immunoreactivity in tissues was validated by Western blot analysis. As shown in Fig. 1, a predominant ~34-kDa band corresponding to apoE protein was present in immunostaining-positive samples (CA1 and CA3-7) but not in immunostaining-negative specimens.
(CA2 and CA8). ApoE protein was also present in the cytoplasm of the OVCAR3 ovarian cancer cell line but not in the SKOV3, CAOV3, and OV90 ovarian cancer cell lines. In addition, apoE immunoreactivity was not detected in any of the normal ovarian surface epithelial cultures (Fig. 1). To determine whether OVCAR3 cells secreted apoE, we did Western blot on the conditioned serum-free medium of OVCAR3 cells. There was no detectable apoE protein found in the conditioned medium up to 96 hours after incubation (data not shown).

The Phenotypes after apoE Knockdown. In order to characterize the functional role of apoE in ovarian carcinogenesis, we did an apoE knockdown assay using apoE specific siRNAs to reduce its expression in the OVCAR3 cell line that expresses apoE and in a control cell line, SKOV3 that does not express apoE based on Western blotting (Fig. 1). We designed a total of four sets of siRNA that targeted different regions of the apoE open reading frame. Based on Western blot analysis, two siRNAs (siRNA-1 and siRNA-2) showed a knockdown effect and the other two (siRNA-3 and control siRNA-4) did not. Therefore, siRNA-3 and siRNA-4 were used as control siRNAs. Both apoE siRNAs (siRNA-1 and siRNA-2) were used in the biological assays. As compared with apoE siRNA-2, apoE siRNA-1 was more effective in silencing apoE expression. As shown in Fig. 2A, apoE expression was significantly reduced 24 hours after treatment of apoE siRNAs in OVCAR3 cells but not in the same cells treated with the control siRNAs or a mock transfection. Cell cycle analysis showed a G2-M phase arrest in OVCAR3 cells 24 hours after siRNA-1 administration (Fig. 2B). The cell cycle arrest was reflected by a significant decrease in the numbers of viable cells at 48 hours after apoE siRNA treatment. The number of viable OVCAR3 cells was 139 ± 33 × 10^4 /mL (average ± SD) which was lower than 298 ± 12 × 10^4 /mL in the mock treated cells and 247 ± 21 × 10^4 /mL in the control siRNA treated cells (P < 0.01, t test). Nocodazole trapping was then used to distinguish G2-phase versus M-phase arrest by counting the mitotic index in OVCAR3 cells 24 hours after apoE siRNA targeting. There was no substantial increase in the mitotic index among nocodazole treated cells at 3, 6, and 12 hours after adding nocodazole (P > 0.1). The cell cycle arrest was associated with an increase in apoptotic OVCAR3 cells. As compared with the control siRNAs, an increased number of apoptotic cells were observed in OVCAR3 cells treated with apoE siRNAs (P < 0.01) as detected by annexin V and DAPI staining (Fig. 3). The annexin V–positive cells occurred as early as 24 hours after treatment when rare cells showed morphologic features of apoptosis.
by DAPI staining. In contrast, the phenotypic changes seen in the apoE siRNA–treated OVCAR3 cells were absent in apoE siRNA–treated ovarian cancer cells such as SKOV3, OV-90, and CAOV3, which did not express detectable apoE. A representative cell cycle analysis of SKOV3 cells after apoE siRNA treatment was shown in Fig. 2C.

To validate the specificity of the siRNA approach, we tested if the phenotypes observed in apoE siRNA–treated OVCAR3 cells could be rescued by expression of an engineered mutant version of apoE containing silent mutations in the target sequence that were refractory to siRNA silencing. As compared with native wild-type apoE (Fig. 2A), expression of full-length mutant apoE with silent mutations was not suppressed by apoE siRNA based on Western blot analysis (Fig. 4). Cell cycle analysis showed a similar cell cycle distribution with minimal apoptotic cell (sub-G1 fraction) in apoE mutant-transfected OVCAR3 cells 48 hours after treatment of apoE and control siRNA, indicating that cell cycle arrest was not detected in the cells expressing the mutant form of apoE with silent mutations in the siRNA targeted sequence (Fig. 4).

ApoE Tag Distribution in Other Human Cancers. In order to extend the apoE expression profile to other common types of carcinomas, we did a tag counting analysis based on the SAGE libraries that were available in the public database (http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd=tagsearch). In addition to ovarian cancer in which the apoE expression is up-regulated, almost all the libraries, including pancreas, prostate, breast, stomach, and colon carcinomas, showed an increase in the number of apoE tags compared with their normal counterparts, indicating that apoE was overexpressed in these cancer types (Fig. 5).

Clinical Significance of apoE Expression. In this study, we carefully designed our experiments and controls to avoid the specificity problems. We used two apoE siRNAs and several control siRNAs in experiments to show similar phenotypes observed in apoE siRNA–treated cells such as SKOV3, OV-90, and CAOV3, which did not express detectable apoE protein. A representative cell cycle analysis demonstrates that cell cycle appears not arrested 48 hours after apoE siRNA-1 treatment in OVCAR3 cells that express the mutant apoE.

**Discussion**

The results in this report showed that most ovarian serous carcinomas express apoE, thus confirming the previous SAGE result (7). Overexpression of apoE may not be confined to ovarian serous carcinomas because analysis of the SAGE database (http://www.hlm.nih.gov/SAGE/) showed that apoE expression was also up-regulated in other tumor types, including breast carcinomas, pancreatic carcinomas, stomach carcinomas, colon carcinomas, and prostate carcinomas (Fig. 5). The above findings suggest that apoE represents a tumor-associated marker in a wide variety of human cancers. The significantly higher frequency of apoE expression in high-grade compared with low-grade ovarian serous carcinomas is of great interest, and the finding suggests that both low-grade and high-grade tumors are distinct in gene expression, further supporting a dualistic pathway for the development of ovarian serous carcinoma (29). In that model, low-grade and high-grade ovarian serous carcinomas develop independently and are characterized by different molecular genetic changes and gene expression profiles (30–32).

Although siRNA is an effective and convenient approach to assess functional roles of genes by silencing their expression, it is essential to ensure that the phenotype changes after siRNA treatment are the results of specific gene knockdown rather than the “off-target” effects associated with the siRNA approach (33). In this study, we carefully designed our experiments and controls to avoid the specificity problems. We used two apoE siRNAs and several control siRNAs in experiments to show similar phenotypes in growth inhibition and apoptosis induction by the
targeting siRNAs. The phenotype changes can only be shown in ovarian cancer cells with apoE expression but not in those without detectable apoE expression. More importantly, we have shown that expression of a mutant version of apoE that cannot be recognized by the siRNA reverts the phenotypes. Overexpression of the engineered mutant apoE with silent mutations abolished the apoE related pathway(s), causing cell cycle arrest and apoptosis.

In summary, this study shows a new role of apoE in human cancer. Cancer cells may depend on apoE for cell survival in apoE-expressing tumors. ApoE knockdown in OVCAR3 cells represents an experimental model for the future studies aimed at elucidating the basic mechanisms by which apoE expression contributes to cell proliferation and survival in cancer. Finally, apoE expression has clinical relevance by serving as a prognostic marker in ovarian cancer patients.

Figure 5. Analysis of apoE expression pattern in different carcinomas based on an analysis of SAGE database. All the SAGE libraries from carcinomas and normal counterparts from ovary, pancreas, colon, breast, stomach, and prostate in the SAGE database are selected. The expression level is shown as the number of apoE tag per one million of total tag counts (y-axis). Circle, SAGE library; T, tumor; N, normal counterpart.

Figure 6. Kaplan-Meier survival analysis shows that previously untreated patients with carcinoma cells in effusion showing nuclear immunoreactivity of apoE (n = 24, solid line) had significantly better survival than those with effusions showing negative expression (n = 121, dashed line; 43 versus 28 for median overall survival; P = 0.004).
Acknowledgments

References


Apolipoprotein E Is Required for Cell Proliferation and Survival in Ovarian Cancer

Yu-Chi Chen, Gudrun Pohl, Tian-Li Wang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/1/331

Cited articles
This article cites 39 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/1/331.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/1/331.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/65/1/331.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.