Expression Pattern of the Novel Gene EG-1 in Cancer

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ABSTRACT

Purpose: **We recently discovered a novel gene responsive to tumor-conditioned media: endothelial-derived gene 1 (EG-1). Its transcript has been shown to be present in epithelial cells, as well as in endothelial cells. In this study, we examined the levels of EG-1 protein expression in breast, colon, prostate, and lung cancers, which constitute the four most common solid malignancies in the United States.**

Experimental Design: **Polyclonal antibodies were generated that recognize the EG-1 peptide. These antibodies were used in immunoblot analysis, as well as immunohistochemistry of multiple human clinical specimens of cancer.**

Results: **In immunoblots of whole cell lysates, EG-1 antibodies revealed the presence of a 22-kDa peptide. Immunohistochemistry of breast, colon, and prostate specimens showed higher levels of EG-1 peptides in cancer tissues, in comparison with their benign counterparts. However, EG-1 expression was minimal in both benign and malignant lung tissues.**

Conclusions: **Here, we demonstrated that the expression of EG-1 is elevated in cancerous in comparison to benign epithelial cells, as seen in immunohistochemistry of human pathological specimens. These observations collectively support the hypothesis that the novel gene EG-1 is associated with the malignant phenotype of the common epithelial-derived cancers of the breast, colon, and prostate.**

INTRODUCTION

Cancer is a major cause of morbidity and the second leading cause of death in the American population. Overall, cancer incidence and mortality began to stabilize in the mid to late 1990s but have not improved significantly in recent years (1). Several major oncogenes and tumor suppressor genes have been identified to contribute to the neoplastic transformation of epithelial cells. These include p53, c-myc, ras, retinoblastoma, BRCA-1 and BRCA-2 (breast cancer susceptibility genes), Her-2, cyclin D1, and phosphatase and tensin homologue (2). Other alterations in the cell such as DNA methylation contribute to the overall genetic instability, whereas abnormal maintenance of telomerases results in replicative immortality (3).

Another important biological phenomenon in the tumorigenic and metastatic phenotype involves the process of angiogenesis. Three decades of experimental evidence has demonstrated that the growth and metastasis of solid tumors is dependent on their ability to initiate and sustain new capillary growth, *i.e.*, angiogenesis (4). Angiogenesis is a complex multistep process, which includes endothelial cell proliferation, migration, and differentiation into tube-like structures. These steps involve multiple growth factors, proteases, and adhesion molecules among endothelial cells, as well as those with other supporting cells (5). In the healthy human adult, the endothelium is generally quiescent, and turnover of endothelial cells is extremely slow. An exception to this is the angiogenesis that occurs during wound healing and endometrial proliferation. Abnormal angiogenesis occurs in rheumatoid arthritis, diabetic retinopathy, and in cancer growth and metastasis.

Multiple clinical observations in human cancer have added support to the hypothesis that tumors are angiogenesis dependent. The number of vessels in a tumor specimen correlates with the disease stage and can add prognostic value independent of other routinely used markers (6). Furthermore, the levels of various angiogenic factors in bodily fluids have been demonstrated to correlate with prognosis in cancer patients (7–9). Many agents have been developed to inhibit tumor angiogenesis, and there have been reports of some encouraging results (10, 11).

Several researchers, including our laboratory, have investigated the difference between molecules of the proliferating tumor endothelium from those in the normal quiescent endothelium (12, 13). To closely mimic a tumor environment, we have attempted to identify endothelial gene products expressed in response to a mixture of growth factors found in tumor conditioned media. Toward this goal, we used a subtraction hybridization method called suppression subtractive hybridization (14). In human umbilical vein endothelial cell (HUVEC) populations exposed to conditioned media from human cancer cells (15) for 4 h, we have isolated ~300 up-regulated and another 300 down-regulated clones (16, 17). One of these differentially expressed genes is endothelial-derived gene 1 (EG-1; Ref. 18). In the present study, we show that EG-1 expression is elevated in several cancer cell types. These results suggest that EG-1 may be a novel marker of the malignant phenotype of common epithelial-derived cancers, including breast, colon, and prostate.

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MATERIALS AND METHODS

Cell Culture. Human embryonic kidney cells, HEK-293 and HEK-293T, and the human breast cancer cell, MDA-MB-231, were purchased from American Tissue Type Culture Collection (Manassas, VA), and maintained in DMEM (Life Technologies, Inc., Grand Island, NY) with 10% heat-inactivated FCS, 100,000 units/liter penicillin, and 100 mg/liter streptomycin, at 37°C in 5% CO_2. HUVECs were obtained from Cascade Biologics (Portland, OR). The cells were plated on tissue culture flasks coated with 1.5% gelatin (Difco, Detroit, MI) in PBS. They were maintained in endothelial growth media completed with low serum growth supplement (Cascade Biologics), penicillin, and streptomycin.

Transfection. We used the pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA) and pShuttle-IRES-hrGFP-1 (Stratagene, La Jolla, CA) vectors to carry the full-length EG-1 gene. Empty vectors were used as negative controls. Liposomal reagents were used to transfect the pcDNA3.1D/V5-His-TOPO vectors into cells (19). pShuttle-IRES-hrGFP-1 vector with a 3xFLAG tag was transfected into HEK-293 or HEK-293T cells using the MBS Mammalian Transfection kit according to the manufacturer's protocol (Stratagene).

Generation of Antibodies. Polyclonal antibodies that recognize five different epitopes on human EG-1 were generated by Washington Biotechnology (Baltimore, MD). Briefly, different antigenic peptide fragments of human EG-1 were synthesized and used to immunize the rabbits. Preimmune and immune sera were harvested. Polyclonal antibodies were also affinity purified. For Western analysis, the secondary antibody used was horseradish peroxidase-conjugated goat antirabbit IgG from Jackson ImmunoResearch (West Grove, PA). The anti-FLAG M2 antibodies were obtained from Sigma (St. Louis, MO).

Western Analysis. Cell pellets were lysed in preheated 0.025 M Tris (pH 7.4), 0.001 M EDTA, and 0.3% SDS and then boiled for 5 min. The cell lysate was centrifuged at 12,000 $\times$ *g* for 10 min, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA).

For Western analysis, ~40 μg of protein were separated by a 10% Tris-HCl Ready Gel (Bio-Rad) and transferred to a nitrocellulose membrane by electrophoretic blotting. The membrane was blocked overnight (4°C) with 5% nonfat dry milk in TBST (Tris-buffered saline, 0.1% Tween 20) and then incubated with a 1:500 dilution of EG-1 antiserum for 2 h. The blots were then washed three times over 30 min in TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody goat antirabbit IgG (1:10,000) and then washed in PBS-Tween as before. The membranes were then developed using the Supersignal West Pico Chemiluminescent Western blotting detection system according to the manufacturer's instructions (Pierce, Arlington Heights, IL).

Human Tissue. Human archival tissue samples were obtained from the University of California at Los Angeles Department of Pathology. As for all studies involving human tissue, this study was conducted in compliance with the rules and regulations of the University of California at Los Angeles Institutional Review Board.

Immunohistochemistry. Immunohistochemical procedures were performed similarly to previously described methods (13, 20). Briefly, paraffin-embedded specimens were cut into 5-μm sections, then baked at 65°C for 30 min. H&E preparations of each specimen were performed to confirm the presence of nonnecrotic tumor. The paraffin was removed by incubation in xylene, followed by graded alcohols.

Immunostaining was performed with the DAKO Envision peroxidase rabbit ready-to-use system. The slides were sequentially incubated at room temperature as follows: (*a*) in DAKO antigen block reagent to block nonspecific antibody binding; (*b*) with the specific primary antibody for 1 h; (*c*) with the DAKO secondary antibody to rabbit for 30 min; and (*d*) developed with DAKO 3,3′-diaminobenzidine solution. The tissues were then stained with Gill's hematoxylin, dehydrated through graded alcohols, and mounted. For EG-1 studies, we used antigen retrieval with 0.01 M sodium citrate (pH 6.0) in a 95°C water bath for 20 min. The EG-1 antiserum was used at 1:400 dilution and EG-1 affinity-purified polyclonal antibodies at 1:2000. The negative control was preimmune rabbit serum at 1:400 dilution.

The histological slides were reviewed and scored by three pathologists (J. Rao, S. Apple, and D. Seligson). Both the staining intensity and percentage of staining were taken into consideration. The intensity of staining was graded from − to +++. Because the percentage of tumor cells staining correlated strongly with the staining intensity, the staining intensity was used as an indicator for EG-1 expression. Photography was carried out with a Leica DMLS microscope (McBain Instruments, Chatsworth, CA) and a Nikon CoolPix 995 digital camera (Tokyo, Japan).

Confocal Microscopy. Immunofluorescence labeling was performed in a Lab-Tek chamber slide (Nalge Nunc, Naperville, IL). Cells were fixed in 4% formalin, permeabilized in acetone, and washed in 1× PBS. Cells were placed in 75% ethanol for 5 min, 3% H_2O_2 for 20 min, and washed in 1× PBS. Cells were blocked in 5% goat serum in PBS for 30 min and incubated with EG-1 antiserum at a 1:400 dilution. Secondary antibodies, biotinylated antirabbit IgG (DAKO), were used at 1:200 dilution and Streptavidin-conjugated Texas Red (DAKO) as the final reporter. Confocal microscopy was performed with an Olympus AX 70 Confocal Microscope (Melville, NY) and the same Nikon digital camera.

RESULTS

The EG-1 Antibodies Recognized a 22-kDa Peptide. We generated five sets of rabbit antiserum against different antigenic synthetic peptide fragments of EG-1. Two of these five sets detected EG-1 bands on Western analysis and EG-1 signals in immunohistology studies. Western analysis of cell lysates demonstrated the presence of a 22-kDa peptide in MDA-MB-231 cells and two bands (28 and 30 kDa) in the HEK-293 cells transfected with the full-length EG-1 cDNA carrying the 3xFLAG signal (Fig. 1). The blots were also probed with anti-FLAG antibodies for confirmation. In previous analysis, the negative control using preimmune rabbit serum did not detect any EG-1 bands. The signal was slightly larger in the lysates from transfected cells because of the additional weight of the three FLAG proteins (6–8 kDa). In other studies, *in vitro* transcription and translation was carried out with the full-length

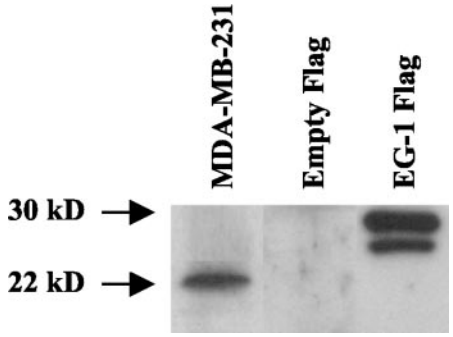

Fig. 1 Western analysis of endothelial-derived gene 1 (EG-1) expression in cell lysates. Forty μg of protein was loaded/lane. *Lane 1* represents cell lysates from the human breast cancer cells MDA-MB-231. *Lanes 2* and *3* contain cells lysates from HEK-293 cells transfected with empty vector and with EG-1 and 3xFLAG tag vector, respectively.

EG-1 cDNA without FLAG and yielded a single protein product at 20 kDa (data not shown).

Immunohistochemistry Revealed Increased Expression of EG-1 in Human Cancer. To examine the involvement of EG-1 in the malignant progression of human epithelial-derived cancers, the expression of EG-1 in multiple clinical samples was analyzed by immunohistochemistry. The histological slides were reviewed independently by three pathologists. The staining intensity of the slides was scored from − to +++ (Table 1). The archival pathological specimens were obtained from surgical resection of invasive breast, colon, prostate, and lung cancer cases. Corresponding benign areas from the same patient specimens were available for analysis in almost all cases. Fig. 2*A–H* shows representative sections of breast, colon, and prostate tissues, which demonstrate higher expression of the EG-1 protein in the cancer cells, in comparison to the benign epithelial cells from the same surgical specimens. The first specimen was obtained from a 1.5-cm invasive ductal breast carcinoma case, poorly differentiated with high nuclear grade, extensive comedo ductal carcinoma *in situ* with estrogen receptor positive, progesterone receptor positive, Her2 positive, and negative axillary lymph nodes. The second specimen was derived from a colon adenocarcinoma case, 7 cm in length, moderately differentiated, with lymphovascular invasion, extending to the serosa, 4/12 positive lymph nodes, and liver metastasis. The third specimen was obtained from a 3.5-cm prostate adenocarcinoma case, Gleason grade 4 + 3 = 7, extending into but not through the capsule, with perineural invasion, and negative nodes. Table 1 summarizes the characteristics of these cancer cases and their observed staining intensities for the EG-1 peptide. Cancer stage was assigned by the standard Tumor-Node-Metastasis classification of malignant tumors. We observed minimal expression of EG-1 in seven lung cancer cases (four squamous and three adenocarcinoma), both in the malignant and corresponding normal epithelial cells.

We also observed minimal EG-1 staining in inflammation or wound healing situations. Fig. 2*I–J* shows no staining in specimens from inflamed breast tissue and granulated healing breast tissue.

Observations from several immunohistochemical specimens showed that the EG-1 protein appeared to be localized mostly in the cytoplasm of the cells and partially in the nucleus. Confocal microscopy performed on HUVECs in culture also confirmed this observation (Fig. 2, *K* and *L*).

Table 1 Immunohistochemistry of EG-1 in human cancerous tissues and their benign counterparts (in the same specimens): breast; colon; and prostate

Specimen no.	Histology	Stage	EG in cancer	EG in benign
Breast				
1	Invasive ductal	1	+++	−
2	Invasive	3	+++	−
3	Inv ductal/lobular	2	+++	−
4	Invasive ductal	3	+++	+
5	Invasive ductal	2	+++	+
6	Invasive ductal	2	+++	+
7	Invasive ductal	1	+++	+
8	Invasive ductal	2	+++	+
9	Invasive lobular	2	+++	+
10	Invasive ductal	2	+++	+
11	Invasive ductal	3	+++	+
12	Invasive ductal	2	+++	++
13	Invasive ductal	1	+++	+++
14	Invasive ductal	2	++	−
15	Invasive ductal	2	++	−
16	Invasive ductal	2	++	−
17	Invasive ductal	2	++	+
18	Invasive ductal	2	++	+
19	Invasive ductal	1	++	+
20	Invasive lobular	1	++	+
21	Inflammatory	3	++	+
22	Invasive ductal	1	++	++
23	Invasive tubular	1	++	++
24	Invasive ductal	1	++	+++
25	Invasive ductal	2	++	+++
26	Invasive ductal	2	++	N/A[a]
27	Squamous	3	++	N/A
28	Invasive ductal	2	+	−
29	Invasive ductal	1	+	+
30	Invasive ductal	2	+	++
31	Invasive ductal	3	+	+++
32	Invasive ductal	1	±	−
Colon				
1	Adenocarcinoma	4	+++	−
2	Adenocarcinoma	3	+++	−
3	Adenocarcinoma	3	+++	−
4	Adenocarcinoma	4	+++	+
5	Adenocarcinoma	1	+++	+
6	Adenocarcinoma	4	+++	+
7	Adenocarcinoma	3	+++	+
8	Adenocarcinoma	3	+++	+
9	Adenocarcinoma	2	++	+
Prostate				
1	Adenocarcinoma	2	++	+
2	Adenocarcinoma	2	+	−
3	Adenocarcinoma	2	+	−
4	Adenocarcinoma	3	+	−
5	Adenocarcinoma	2	+	−
6	Adenocarcinoma	3	+	N/A
7	Adenocarcinoma	2	−	−
8	Adenocarcinoma	2	−	−
9	Adenocarcinoma	2	−	−
10	Adenocarcinoma	2	−	−
11	Adenocarcinoma	2	−	−

[a] N/A, not available.

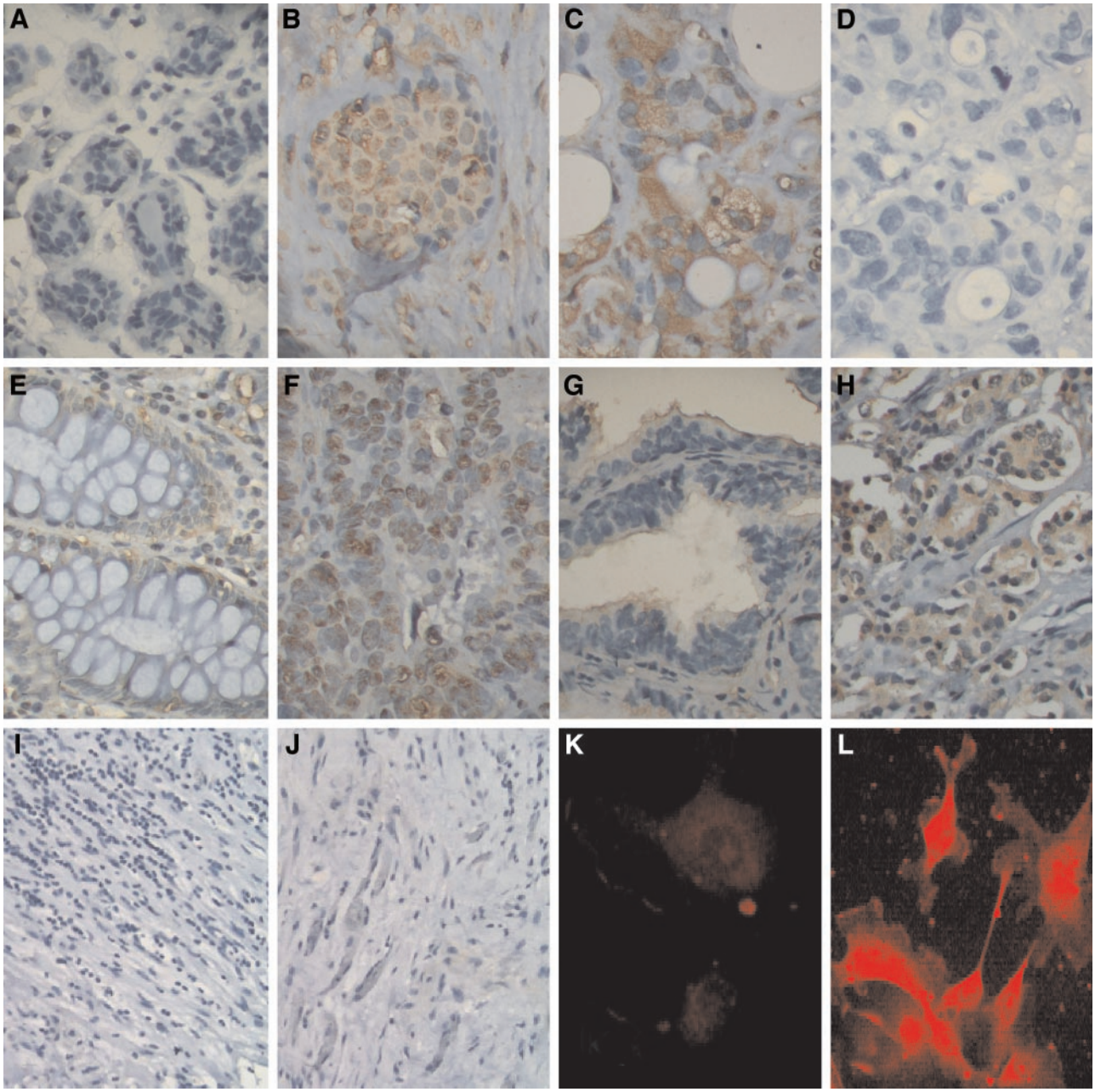

Fig. 2 *A–J*, immunohistochemistry of human specimens, with positive staining in *brown: A*, benign breast, endothelial-derived gene 1 (EG-1) antibody; *B*, breast ductal carcinoma *in situ*, EG-1 antibody; *C*, breast invasive cancer, EG-1 antibody; *D*, breast invasive cancer, control preimmune serum; *E*, benign colon, EG-1 antibody; *F*, colon adenocarcinoma, EG-1 antibody; *G*, benign prostate, EG-1 antibody; *H*, prostate adenocarcinoma, EG-1 antibody; *I*, inflamed breast, EG-1 antibody; *J*, granulated healing breast, EG-1 antibody. *K* and *L*, confocal immunofluorescence of human umbilical vein endothelial cells, with positive staining in *red: K*, control preimmune serum and (*L*) EG-1 antiserum.

DISCUSSION

We show here that the expression of EG-1 is elevated in cancerous in comparison to benign epithelial cells, as seen in immunohistochemistry of several human pathological specimens. These observations collectively support the hypothesis that the novel gene EG-1 is associated with the malignant phenotype of the common epithelial-derived cancers of the breast, colon, and prostate. In this small sample size, colon cancer seems to consistently have elevated EG-1 signals, whereas the increased staining pattern is more variable in breast and prostate cancer types. Lung cancer does not appear to express much EG-1, as detected by our first generation of polyclonal antibodies. It is possible that this staining pattern may change with future new and improved antibodies against EG-1, as well as a larger sample size.

The first and only publication to date on EG-1 came from

our laboratory (18). Suppression subtractive hybridization revealed an RNA sequence (GenBank accession no. AW735731), the expression of which is increased in HUVECs treated with tumor conditioned media derived from human cancer cells. Subsequent cloning of the full-length cDNA from a HUVEC library (AF358829), and a Basic Local Alignment Search Tool for Nucleotide search in the GenBank database shows that EG-1 is on chromosome no. 4. It spans four exons and three introns. The human EG-1 sequence has significant homology to a murine cDNA (94%) and a Drosophila cDNA (31%). From the nucleotide sequence, the predicted peptide has 178 amino acids and weighs 19.5 kDa. This is consistent with our Western analysis results which reveal a protein at slightly higher weight than that predicted above, suggesting some degree of posttranslational modifications.

A Basic Local Alignment Search Tool for Nucleotide search for sequence homology performed in the GenBank database reveals that EG-1 has no significant homology to any gene with a known function. A Profile Scan search reveals a long proline-rich region, one *N*-glycosylation site, two *O*-glycosylation sites, four casein kinase II phosphorylation sites, and two *N*-myristoylation sites. A search looking for motif match shows some alignment with the following: disheveled specific domain; Wilms' tumor protein signature; phosphoinositide 3-kinase family; ras-binding domain; C2 domain; p85-binding domain; breast cancer type I susceptibility protein signature and BRCA2 repeat; C-C chemokine receptor type 9 signature; cadherin-2; xeroderma pigmentosum group B protein signature; and SKI/SNO proto-oncogene. Although the function of EG-1 is to be determined, its sequence alignment with multiple oncogenes and cancer-related genes is consistent with our hypothesis that it may be involved in tumorigenesis.

In summary, based on its expression profile in human tissues, EG-1 appears to be particularly relevant to those cancer types of ductal epithelial origin such as breast, colon, and prostate. These results will form the basis for additional studies of this interesting gene and the possible translation of the discovery of this molecule into potential use in cancer diagnosis and/or treatment.

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