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DOD Grant "Endothelial Genes"

Background

Breast cancer growth and metastasis are dependent on angiogenesis. Multiple angiogenic peptides have been identified and sequenced. These include basic and acidic fibroblast growth factor (bFGF and aFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor-alpha and beta (TGF-α and TGF-β), hepatocyte growth factor (HGF), tumor necrosis factor-alpha (TNF-α), interleukin-8 (IL-8), granulocyte colony stimulating factor (G-CSF), E-selectin, angiogenin, platelet-derived endothelial cell growth factor (PD-ECGF), placental growth factor and pleiotrophin. Furthermore, several endogenous angiogenic inhibitors have recently been identified. These include thrombospondin, TIMP-1 (tissue inhibitor of metalloproteinase), angiostatin, endostatin, METH-1 and METH-2. Recently, our laboratory has utilized a subtraction hybridization technique to identify two novel genes: endomucin and EG-1 (endothelial-derived gene-1).

Objective/Hypothesis

We ask whether the level of any of these factors could be detected in the serum, and whether their levels have any clinical relevance in monitoring the response of breast cancer patients to anti-angiogenic treatment.

Specific Aims

The objectives for this proposal are as follows: 1) to generate antibody-based assays for endomucin and EG-1 and to further characterize these novel genes; 2) to conduct a clinical trial to assess whether angiogenesis markers correlate with the surgical removal of early stage breast cancer; and 3) to conduct a clinical trial to assess whether angiogenesis markers correlate with the anti-angiogenic therapy of metastatic stage breast cancer.

Study Design

We will characterize the novel genes endomucin and EG-1 in *in vitro* and *in vivo* studies. We will then measure the levels of multiple known angiogenic factors and inhibitors as well as E-selectin, endomucin, and EG-1 in the serum of early stage breast cancer patients prior and after surgical excision of their tumors. Also, we will measure the levels of multiple known angiogenic factors and inhibitors as well as E-selectin, endomucin, and EG-1 in the serum of metastatic breast cancer patients prior and during/after anti-angiogenic treatment.

Relevance

We expect that the results of this study will provide more information on two novel genes that may play a role in angiogenesis. Furthermore, the results from the

clinical trials will point to the identity of those angiogenic marker(s) that is(are) potentially useful in monitoring breast cancer therapy. Such marker(s) could then be further tested in the context of other clinical trials using other types of anti-angiogenic treatments.

Key Research Accomplishments

Introduction

We have made progress in all four tasks outlined in the grant Statement of Work. This progress is detailed in the following body section.

Body

Task 1: to generate antibody-based assays for endomucin and EG-1. Months 1-18

a. Production of polyclonal antibodies: We have generated five different antibodies against EG-1 and five others against endomucin. These were made in rabbits, that were injected with peptide fragments of the above gene products.

b. Western analysis and immunoprecipitation studies of antibodies: We have succesfully carried out Western analysis and immunoprecipitation of in vitro translated products of genes EG-1 and endomucin. This data was included in the previous annual report.

c. ELISA: We have made recombinant EG-1 peptide via E. coli. Work is underway to produce monoclonal antibodies.

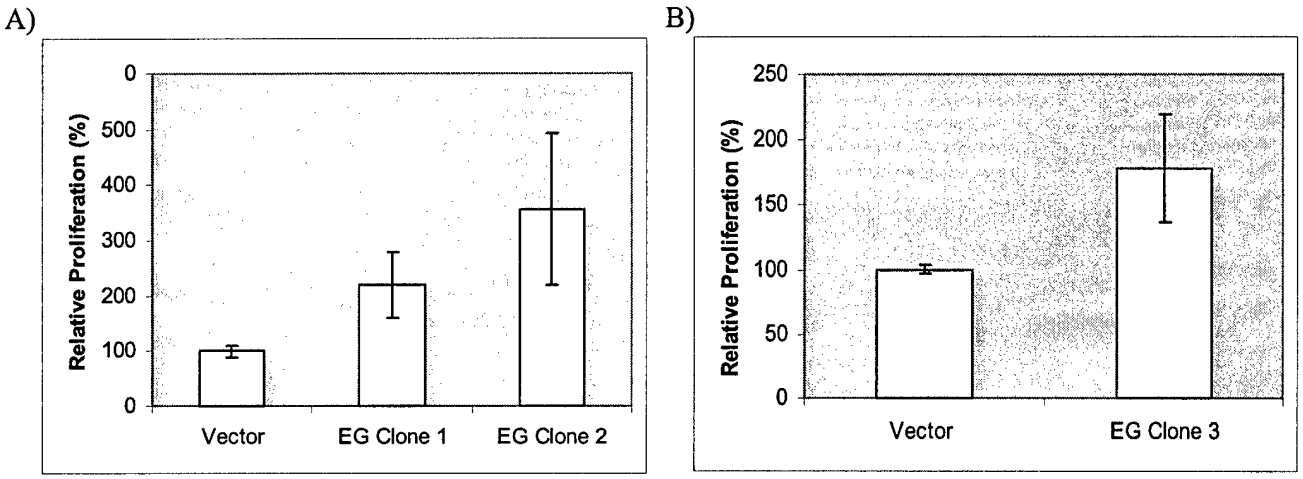
Task 2: to perform studies to characterize the functions of endomucin and EG-1. Months 18-48

a. Antibody studies in assays of angiogenesis and immunohistochemical studies: We have successfully performed immunohistochemistry of human tissue specimens with the polyclonal antibodies against EG-1. This data was included in the previous annual report. In this report, we include our paper in press in Clinical Cancer Research.

We have carried out other assay studies to further understand the functions of the gene EG-1. We transfected a full length cDNA of EG-1 into HEK293 (human embryonic kidney) cells. Succesfully stable clones of transfected cells have increased EG-1 expression by Northern analyses. Subsequent experiments showed that the transfected cells have increased proliferation in comparison to the ones transfected with empty vectors. In Figure 1A, two HEK293 clones (#1 and #2) stably transfected and expressing EG-1 have many-fold increase in proliferation when compared with the empty vector transfected cell line. Subsequent experiments with the malignant breast

cancer cell line MDA-MB-231 (clone #3) also showed that the transfected cells have increased proliferation in comparison to the ones transfected with empty vectors.

Figure 1: Proliferation of (A) HEK-293 cells, and (B) MDA-MB-231 cells. The assay measures the growth ([methyl-^{3}H] thymidine incorporation) of empty vector transfected controls (first bar), and independent EG-1-expressing clones (second and third bars). Values are the means ± standard errors of three experiments, expressed relative to control vector. ** is $p < 0.01$ (Student's t test).



b. Adenoviral vectors and the effect on HUVEC functions: to be accomplished.

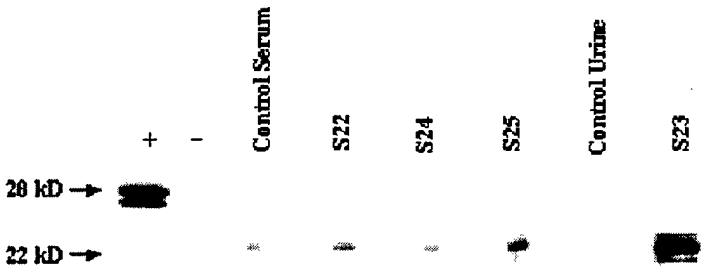
Task 3: to conduct a clinical trial to assess whether angiogenesis markers correlate with the surgical removal of early stage breast cancer. We will measure the levels of multiple known angiogenic factors and inhibitors as well as E-selectin, endomucin, and EDG-1 in the serum of early stage breast cancer patients. Months 1-36

a. Collect serum from surgical patients preop and postop: We have received permission from the UCLA (IRB) Institutional Review Board and ISPRC (Internal Scientific Peer Review Committee) for this protocol, and are starting to enroll patients.

b. Measure the levels of angiogenesis markers and perform statistical comparisons between preop and postop values: to be accomplished.

We'd like to present some preliminary data obtained this past year for this Task 3. Western analysis of serum and urine shows that the 22 kD protein bands that are recognized by the EG-1 antibodies are more intense in specimens from breast cancer patients, in comparison to serum and urine from one normal control (Figure 2).

Figure 2: Western analysis of EG-1 expression, probed with anti-EG-1 antibodies. Lane 1 represents cell lysates from HEK-293T transfectants with EG-1 3x FLAG tag, and lane 2 cell lysates from transfectants with empty vector (approximately fifty μg of protein is loaded per lane). Lanes 3-6 are serum samples from a control subject and three breast cancer patients (S22, S24, S25). Lanes 7-8 are urine samples from a control subject and one breast cancer patient (S23). The urine samples are concentrated, and approximately equivalent amounts of proteins are loaded onto the lanes of the gel.



Task 4: to conduct a clinical trial to assess whether angiogenesis markers correlate with the anti-angiogenic therapy of metastatic stage breast cancer. We will measure the levels of multiple known angiogenic factors and inhibitors as well as E-selectin, endomucin, and EDG-1 in the serum of metastatic breast cancer patients. Months 1-36

a. Collect serum from metastatic cancer patients prior and during/after anti-angiogenic therapy: We have received permission from the UCLA (IRB) Institutional Review Board and ISPRC (Internal Scientific Peer Review Committee) for this protocol, and are starting to enroll patients.

b. Measure the levels of angiogenesis markers and perform statistical comparisons between pre-treatment and post-treatment values: to be accomplished.

Conclusion

Data thus far support possible important roles for our novel genes, EG-1 and endomucin. These genes do code for peptide products. Immunohistochemical studies of EG-1 show that its expression is elevated in human cancer specimens. EG-1 may be relevant to the malignant process of tumorigenesis. The clinical protocols have been approved by the appropriate agencies and subject accrual has begun.

Reportable Outcomes

We include here a paper in press in Clinical Cancer Research. We are also submitting two manuscripts to peer-reviewed journals, both of which acknowledge the DOD grant support.

Appendix

"Expression Pattern of the Novel Gene **EG-1** in Cancer"

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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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Note: L. Zhang and R. Maul contributed equally to this article. Research was performed at the University of California at Los Angeles Medical Center, Los Angeles, CA.

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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 × *g* for 10 min, and the supernatant was saved. Protein concentration was measured by the Bradford assay (Bio-Rad, Hercules, CA). AQ: C

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 F1

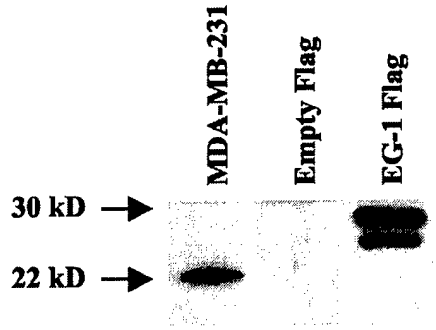

Fig. 1 Western analysis of endothelial-derived gene 1 (EG-1) expression in cell lysates. Forty μg of protein was loaded/lane. *Lane 1* represents cell lysates from the human breast cancer cells MDA-MB-231. *Lanes 2* and *3* contain cells lysates from HEK-293 cells transfected with empty vector and with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

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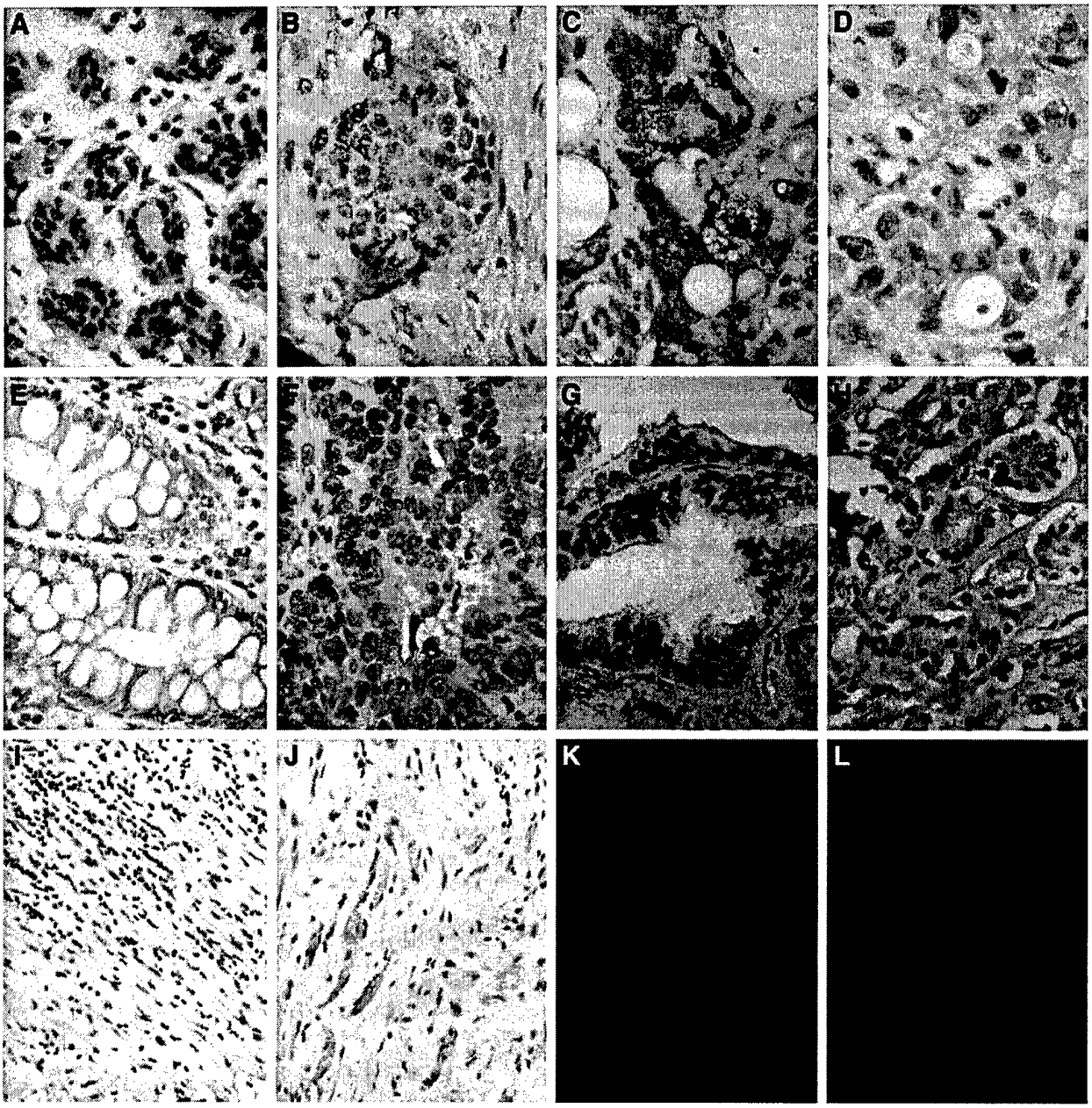

Fig. 2 *A–J*, immunohistochemistry of human specimens, with positive staining in *brown: A*, benign breast, endothelial-derived gene 1 (EG-1) antibody; *B*, breast ductal carcinoma *in situ*, EG-1 antibody; *C*, breast invasive cancer, EG-1 antibody; *D*, breast invasive cancer, control preimmune serum; *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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