Human Cutaneous Fatty Acid-binding Protein Induces Metastasis by Up-Regulating the Expression of Vascular Endothelial Growth Factor Gene in Rat Rama 37 Model Cells

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INTRODUCTION

Using recently developed molecular biology approaches for analysis of differential gene expression (1–3), we have identified a number of genes expressed differentially between benign and malignant human prostates and breast cell lines. One such gene is that coding for human C-FABP.3 Transfection of the C-FABP gene into nonmetastatic Rama 37 cells revealed that expression of the endogenous vascular endothelial growth factor (VEGF) gene was increased by 3.8–5.2-fold in the C-FABP-transfected cells (pSV-CFABP-R37) and in their metastatic sublines (e.g., Met-1) when compared with that in the nonmetastatic control pSV-R37 cells generated by transfection of only plasmid DNA. Higher levels of VEGF immunoreactive protein were also secreted from the malignant C-FABP-expressing cells. Reverse transcription-PCR detected two VEGF transcript isoforms, VEGF164 and VEGF188, in both the nonmetastatic control transfectant pSV-R37 cells and the malignant metastatic Met-1 cells. Chick chorioallantoic membrane assays showed that the conditioned medium of the control pSV-R37 cells possessed only very weak angiogenic activity, whereas conditioned media from the metastatic C-FABP transfectants and their sublines were strongly angiogenic and could be inhibited by antibodies to VEGF. Transfection of VEGF164 cDNA in an expression vector into nonmetastatic Rama 37 cells produced a cell clone (R37-VEGF-2) that expressed high levels of VEGF. Inoculation of R37-VEGF-2 cells into syngeneic Wistar Furth rats produced metastases in a significant number (Fisher's exact test, \( P < 0.01 \)) of animals (18 of 31 animals), whereas the control, vector alone-transfected R37-PSV cells produced no metastases (0 of 30 animals). Immunocytochemical methods demonstrated a strong positive staining for VEGF and an increased microvessel density in the primary tumors produced from PSV-VEGF-2 cells in comparison with tumors produced from control transfectants. Immunocytochemical staining for factor VIII detected a 3.5-fold increase in microvessel density of the primary tumors produced by PSV-VEGF-2 cells when compared with that of the primary tumors developed from the control pSV-R37 cells. Therefore, we suggest that overexpression of the C-FABP gene in the original transfectants induces metastasis through up-regulation of expression of the VEGF gene in this rat Rama 37 model system, and thus VEGF may play a crucial role in this particular metastatic cascade.

ABSTRACT

Human cutaneous fatty acid-binding protein (C-FABP) gene is capable of inducing the metastatic phenotype when overexpressed in nonmetastatic rat Rama 37 cells. However, the mechanism of how it induces metastasis is not clear. Northern and slot blot analyses revealed that expression of the endogenous vascular endothelial growth factor (VEGF) gene was increased by 3.8–5.2-fold in the C-FABP-transfected cells (pSV-CFABP-R37) and in their metastatic sublines (e.g., Met-1) when compared with that in the nonmetastatic control transfectant pSV-R37 cells generated by transfection of only plasmid DNA. Higher levels of VEGF immunoreactive protein were also secreted from the malignant C-FABP-expressing cells. Reverse transcription-PCR detected two VEGF transcript isoforms, VEGF164 and VEGF188, in both the nonmetastatic control transfectant pSV-R37 cells and the malignant metastatic Met-1 cells. Chick chorioallantoic membrane assays showed that the conditioned medium of the control pSV-R37 cells possessed only very weak angiogenic activity, whereas conditioned media from the metastatic C-FABP transfectants and their sublines were strongly angiogenic and could be inhibited by antibodies to VEGF. Transfection of VEGF164 cDNA in an expression vector into nonmetastatic Rama 37 cells produced a cell clone (R37-VEGF-2) that expressed high levels of VEGF. Inoculation of R37-VEGF-2 cells into syngeneic Wistar Furth rats produced metastases in a significant number (Fisher's exact test, \( P < 0.01 \)) of animals (18 of 31 animals), whereas the control, vector alone-transfected R37-PSV cells produced no metastases (0 of 30 animals). Immunocytochemical methods demonstrated a strong positive staining for VEGF and an increased microvessel density in the primary tumors produced from PSV-VEGF-2 cells in comparison with tumors produced from control transfectants. Immunocytochemical staining for factor VIII detected a 3.5-fold increase in microvessel density of the primary tumors produced by PSV-VEGF-2 cells when compared with that of the primary tumors developed from the control pSV-R37 cells. Therefore, we suggest that overexpression of the C-FABP gene in the original transfectants induces metastasis through up-regulation of expression of the VEGF gene in this rat Rama 37 model system, and thus VEGF may play a crucial role in this particular metastatic cascade.

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3 The abbreviations used are: C-FABP, cutaneous fatty acid-binding protein; VEGF, vascular endothelial growth factor; RT-PCR, reverse transcription-PCR; CAM, chorioallantoic membrane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rhVEGF, recombinant human VEGF; hVEGF, human VEGF; hpf, high-power field; FABP, fatty acid-binding protein; CM, conditioned medium.
transfected Rama 37 cells with VEGF cDNA to test whether the increased expression of the VEGF gene can promote the malignant dissemination of the resultant transfectants in syngeneic animals.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The nonmetastatic rat Rama 37 model cell line (4) was used as the recipient cell line for the gene transfection experiments. The pSV-R37 cells are controls generated by transfection of the Rama 37 cells with the pSVneo plasmid alone, and the pSV-C-FABP-R37 cells were generated by transfection of the Rama 37 cells with the human C-FABP gene, as described previously (5). The cell lines Met-1 and Met-2 were derived from two metastases produced by pSV-C-FABP-R37 cells in syngeneic rats. The R37-PSV cells are the control transfectants generated in this work by transfection of the Rama 37 cells with the pSVneo plasmid alone. R37-VEGF cells were a pool of cell clones established by transfection of the Rama 37 cells with a VEGF-expressing vector constructed by insertion of VEGF164 into the pSVneo plasmid. R37-VEGF-1, R37-VEGF-2, R37-VEGF-3, R37-VEGF-4, and R37-VEGF-5 are five separate clones of R37-VEGF and express high levels of VEGF. All of the cell lines used in this work were grown as monolayer cultures in DMEM supplemented with 10% (v/v) FCS, penicillin, and streptomycin (100 units/ml), hydrocortisone (5 mg/ml), and insulin (5 mg/ml).

Detection of VEGF mRNA and Protein. For preparation of RNA, cells from each cell line were grown to about 80% confluence in 13.5-cm-diameter Petri dishes (Life Technologies, Inc., Paisley, United Kingdom), washed with PBS (pH 7.4), and scraped into a universal tube. The total RNA was isolated with a RNeasy RNA midi-kit (Qiagen GmbH, Hilden, Germany). The polyadenylated RNA was isolated with an Oligotex mRNA mini-kit purchased from the same company. The integrity of the RNA samples was verified by the presence of an ungraded band after Northern hybridization to a radioactively labeled actin cDNA probe. The VEGF probe used for Northern blot hybridization was a 601-bp cDNA fragment prepared by RT-PCR, as described previously (9, 17). A pair of VEGF-specific primers was used to amplify the VEGF cDNA fragment, and the template was made from 100 ng of mRNA extracted from the malignant rat AT-3 cells of the Dunning model (18), which were previously shown to express high levels of VEGF (9). The primer sequence for the positive (sense) strand was 5′-AACCATGAACTTCTTGCTCTC-3′, and that for the negative (antisense) strand was 5′-GGTGAGGGTTAGTCCCGA-3′. The nucleotide sequence of the amplified rat VEGF probe was confirmed using an automated sequencer (ABI 377; pe, Inc., Foster City, CA). The correct insertion of VEGF cDNA into the pSVneo plasmid DNA. The DNA transfection

ELISA was performed to measure the cell-secreted VEGF protein in the serum-free CM used to culture the cells for 48 h. The mouse monoclonal anti-VEGF antibody, which is specifically reactive to rat VEGF188, VEGF164, VEGF121, and their equivalents in mouse and human (Santa Cruz Biotechnol-

ogy), was diluted to a concentration of 1 μg/ml in 50 mM sodium carbonate (pH 7.6). A 96-well microtiter plate was coated overnight at 4°C with 100 μl of the diluted VEGF antibody and incubated with a blocking buffer [2% (w/v) BSA in PBS] for 2 h at room temperature. The CM or rhVEGF standard (R&D Systems) was added to the wells, and the plate was incubated for another 2 h at room temperature. After washing in washing buffer (0.1% Tween 20 in PBS), goat anti-VEGF polyclonal antibody (R&D Systems) was added (100 μl of 1 μl/ml), and the incubation was continued for 2 h at room temperature. A peroxidase-labeled rabbit antigoat IgG (61-1620; Zymed) was added to the wells and incubated for an additional 2 h at room temperature. The plate was then washed with washing buffer, and the color was developed with 0.04% o-phenylenediamine dihydrochloride in PBS plus 0.4 μl/liter of 30% (v/v) hydrogen peroxide. The results were assessed by quantitating the color intensity relative to a standard curve with rhVEGF using a PLUS kit (Flow Laboratories) at 492 nm. The sensitivity limit was 0.4 ng/ml.

Detection of Isoforms of the VEGF Gene Transcripts. The potential splice variants of the VEGF mRNA transcripts in the rat cells were analyzed by RT-PCR. Single-stranded cDNA templates were transcribed from the polyadenylated end of the mRNAs (100 ng) extracted from the control pSV-R37 cells and the malignant pSV-C-FABP-R37 cells using a cDNA synthesis kit (Boehringer Mannheim). The products (2 μl from each reaction) of the reverse transcription reactions were amplified by PCR using the same specific primer pair as before, which was designed to allow the amplification of all possible rat VEGF isoforms. The PCR products were subjected to electrophoresis in 0.8% agarose gels, and the separated cDNA bands were visualized by etidium bromide staining. The isoforms of VEGF were initially determined by the molecular sizes of the cDNA bands and then confirmed by nucleotide sequence analysis of the bands recovered from the gel. For the positive control, we used VEGF188 and VEGF164 cDNAs obtained by PCR using the previously constructed vectors pBluescript-V188 and pBluescript-V164 as templates in a way similar to that described previously in hVEGF work (18).

Detection of Angiogenic Activity of VEGF. The angiogenic activity of the rat cell lines was tested in the chick CAM assay, as described previously (21). The rhVEGF121 protein was used as a positive control and was diluted to 100 ng/ml with PBS. Serum-free medium without exposure to cells was used as a negative control. The CM was obtained from serum-free medium exposed to cells for 48 h. Both control and CM were concentrated 10-fold by centrifugal ultrafiltration (3 kDa cutoff). A 40-μl aliquot was mixed with 50 μl of 1% sterile methylcellulose (M0512; 4000 centipoises; Sigma Chemical Co.) and 1 μl of PBS or 1 μl of anti-hVEGF neutralizing antibody (MAB293; R&D Systems) at a 50-fold molar excess to rhVEGF protein. This mixture (10 μl) was then applied to a 2-mm-diameter Teflon column and dried under sterile conditions to give a clear disc. The samples were then applied to the CAM on day 10, when vessel growth had ostensibly finished. The angiogenic reaction was determined on day 14, and the response was scored as follows: (a) negative, 0; (b) moderate, 0.5, 1, and 2; and (c) strong and full spokewheel (9). For photography, the membrane was fixed in situ with ice-cold 4% (v/v) paraformaldehyde-PBS, excised, placed on a fresh glass slide, and photographed under a Leitz binocular dissection microscope using indirect fiberoptic illumination. The accumulated responses for the test samples in each group were calculated, and the statistical analysis was performed using the Mann-Whitney U test.

VEGF Gene Transfection and the in Vivo Assay for Metastasis. The VEGF164 cDNA amplified by RT-PCR with the specific primers was first inserted into a PCR-Blunt vector using a Zero Blunt PCR Cloning Kit (In-vitrogen). After the VEGF nucleotide sequence was confirmed by automatic sequencing analysis (ABI 377; Applied Biosystems), the VEGF cDNA was excised from the PCR-Blunt vector by HindIII and XhoI and inserted into the pSVneo plasmid, which had been linearized by digestion with HindIII and XbaI. The correct insertion of VEGF cDNA into the pSVneo vector was confirmed by automated nucleotide sequence analysis through the junctions. The DNA transfection experiment was performed similar to those described previously (22, 23). Exponentially growing benign Rama 37 cells were harvested, seeded at a density of 0.5–0.75 × 106 cells/10 ml DMEM in each 10-cm-diameter Petri dish, and cotransfected with 1.8 μg of pSV-VEGF construct DNA and 0.2 μg of pSVneo plasmid DNA. The DNA transfection
was performed using an Effectene Transfection Reagent kit (Qiagen Ltd.) following the manufacturer’s instructions. The resultant cells were passaged at a 1:10 dilution in a selective medium containing 1 mg/ml Geneticin (G418), which was replaced every 3–4 days thereafter. Five stably transfected clones were isolated from a single experiment by ring cloning. The rest of the colonies of cells were pooled and grown in culture to yield R37-VEGF transfectants. In a separate experiment, Rama 37 cells were transfected only with the pSVneo plasmid DNA to form control transfectants.

The R37-VEGF-2 (highest VEGF expresser) and control R37-PSV cells were injected at 2 × 10^6 cells/0.2 ml PBS into experimental and control groups (each group consisted of 35 animals) of 4–6-week-old female Wistar Furth rats (Wistar OLA strain) at a single s.c. site in the right inguinal mammary fat pad. At the time of inspection, animals that developed ulcerations in their primary tumors were killed and excluded from further study. At autopsy, each primary tumor’s length (L), width (W), and thickness (H) were measured. The tumor volumes were calculated according to the following formula: \( V = \frac{1}{2} \times L \times W \times H \times 0.5236 \). All surviving animals were autopsied 5 weeks after injection, and the lungs, liver, spleen, kidney, heart, and axillary lymph nodes were examined for gross metastases. Samples of primary tumors and tissues taken at autopsy were fixed in Methacarn (methanol:inhibisol:acetic acid, 6:3:1) or neutral buffered formol saline, processed conventionally, embedded in paraffin wax, sectioned, and stained with H&E. Sections of each tissue were examined for micrometastases by two independent observers.

**Immunocytochemical Staining.** For detection of VEGF, the rat tissue sections were first incubated with the primary antibodies to VEGF (Santa Cruz Biotechnology) diluted 1:500 and then incubate with the EnVision System, a horseradish-peroxidase labeled polymer (Dako). To detect factor VIII, the sections were incubated with rabbit anti-factor VIII serum (Dako) at 1:1000 and then incubated with donkey antirabbit IgG conjugated to biotin (Amersham). VEGF and factor VIII proteins were visualized with an antibody complex/horseradish peroxidase staining procedure (Dako). Control sections incubated with preimmune serum and with antisera preincubated with preparations of antigens yielded no specific staining. The sections were counterstained with Gill’s hematoxylin.

**RESULTS**

**Analysis of VEGF Gene Expression in C-FABP Transfectants.** Northern hybridizations were performed to detect C-FABP (Fig. 1A) and VEGF (Fig. 1B) mRNA in control and malignant rat cell lines. Although C-FABP mRNA was not detectable in nonmetastatic control transfectant pSV-R37 cells, which were generated from plasmid DNA alone, its expression was greatly increased in the C-FABP transfectant pSV-C-FABP-R37 cells and in the metastatic cell lines Met-1 and Met-2, which were respectively established from two metastases developed from pSV-C-FABP-R37 cells. Incubation with [α-32P]dCTP-labeled VEGF cDNA probe detected a major hybridizing band of about 3.9 kb in all four cell lines examined, consistent with the expected size of VEGF mRNA (25, 26). The relative intensities of the hybridizing VEGF bands in the metastatic C-FABP-expressing cell line pSV-C-FABP-R37 was 4 times higher than that in the control transfectant pSV-R37 cells, which do not express C-FABP.

In the metastatic sublines Met-1 and Met-2, which express high levels of C-FABP, the intensities of the VEGF hybridizing bands were 4.8 and 5.6 times higher, respectively, than that in the nonmetastatic control cell line pSV-R37 (Fig. 1C). More accurate quantitative measurements by slot blot analysis (Fig. 1C) showed that the levels of hybridizing VEGF mRNA in the transfectant cell pool pSV-C-FABP-R37 and its sublines Met-1 and Met-2 were 3.8 ± 0.6, 5.2 ± 0.4, and

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**Fig. 1. Detection of C-FABP or VEGF mRNA and VEGF transcript isoforms in rat cells.** Northern blot analyses of C-FABP (A) or VEGF (B) mRNA in the control transfectant cell line pSV-R37 (Lane 1), the malignant transfectant pooled cell line pSV-C-FABP-R37 (Lane 2) and their metastasis-derived sublines Met-1 (Lane 3) and Met-2 (Lane 4) are shown. Total RNA samples (20 μg each) were electrophoresed through a formaldehyde-agarose (0.8%, w/v) gel, transferred to a nylon membrane, and hybridized with a [32P]dCTP-labeled C-FABP or VEGF cDNA probe. The radioactivity bound to the washed membrane was detected by exposure to Kodak XAR-5 film with an intensifying screen for 48 h. A radioactively labeled GAPDH probe was hybridized to RNAs on the membrane to standardize the hybridization. C, quantitative analysis of the levels of VEGF mRNA in different rat cell lines. The intensities of the hybridizing bands in the Northern blot were scanned with a digital image system to determine the relative VEGF mRNA levels in each cell line. More accurate measurement of the mRNA levels was performed by slot blot analysis (D). Increasing amounts of mRNA (0.1–4.8 μg) from each cell line were loaded onto duplicated membranes. One membrane was used for hybridization with the VEGF cDNA probe, and the other was used for hybridization with GAPDH cDNA. The levels of mRNA in different cell lines were compared by relative hybridization. The mean relative levels and SDs are shown for three separate experiments. 1, pSV-R37; 2, pSV-C-FABP-R37; 3, Met-1; 4, Met-2. The level of the VEGF mRNA expressed in control R37-PSV cells is set at 1. D, detection of the isoforms of VEGF in control and metastatic transfectant cells by RT-PCR. The first strand of the cDNA was synthesized from 100 ng of extracted RNA from the nonmetastatic control cell line pSV-R37 (Lane 2) and the malignant metastatic cell line Met-1 (Lane 3), respectively. A same PCR reaction without any template DNA was loaded as a negative control (Lane 1). VEGF114 and VEGF164 cDNAs were used as positive controls (Lane 4). Equal volumes of the semiquantitative RT-PCR products were subjected to electrophoresis in a 0.8% agarose gel and stained with ethidium bromide.
5.3 ± 0.6 times higher, respectively, than that in the control transfectant pSV-R37. ELISA analysis (four separate measurements) showed that 2.6 ± 0.4 ng of VEGF protein were secreted per milliliter of CM in 48 h from the control transfectant pSV-R37 cells. In the CM from the representative metastatic Met-1 cells, the amount of the secreted VEGF protein increased to 10.9 ± 1.0 ng/ml medium in 48 h; 4.2-fold higher than that detected in the nonmetastatic control pSV-R37 cells. RT-PCR analysis detected two forms of VEGF transcripts in the nonmetastatic control transfectant pSV-R37 cells and in a representative malignant cell line, Met-1 (Fig. 1D). Nucleotide sequence analyses of these two transcript forms revealed that they coded for two rat VEGF isoforms, VEGF188 and VEGF164. Scanning the intensities of the semiquantitative RT-PCR bands showed that the levels of VEGF188 and VEGF164, respectively, in the pSV-C-FABP-R37 cells were nearly 4-fold higher than those detected in the control transfectant pSV-R37 cells.

Analysis of the Angiogenic Activity of the C-FABP Transfectants. To test whether the VEGF produced and secreted by the rat cell lines is angiogenic, the CAM assay was performed on the CM from transfectant cells and compared with that produced by rhVEGF121 protein as a positive control. The results were obtained from visual assessment (Fig. 2A) and from semiquantitative methods (Fig. 2B), as described in “Materials and Methods.” The rhVEGF induced a strong angiogenic response that was inhibited by a neutralizing antibody to VEGF. The CM from the nonmetastatic pSV-R37 cells induced a very weak angiogenic response, which was not completely suppressed by anti-VEGF. However, the CM derived from all three metastatic cell lines (pSV-C-FABP-R37, Met-1, and Met-2) induced moderate angiogenic responses, all of which were completely inhibited by the anti-VEGF.

DNA Transfection and Metastasis. VEGF164 cDNA was inserted into the mammalian expression vector pSVneo, and the construct was cotransfected with small amounts of pSVneo plasmid DNA into benign rat Rama 37 cells. In a control, the pSVneo vector alone without the insert was also transfected into the Rama 37 cells. When both sets of transfectant cells were cultured in a selection medium containing Geneticin, small cell colonies became visible after about 7 days. The colonies from the control transfection experiment were combined to form a separate pool of transfectant cells; these were termed R37-PSV cells. For the Rama 37 cells transfected with the VEGF expression construct, the ring cloning method was used to isolate five separate colonies from single cells, and the rest of the colonies were combined to form a transfectant pool. The five cell lines established from the five separate colonies were termed R37-VEGF-1, R37-VEGF-2, R37-VEGF-3, R37-VEGF-4, and R37-VEGF-5, and the cell line from the pooled transfectants was termed R37-VEGF. No significant differences (Student’s t test, P > 0.05) were observed in the growth rates between the transfectant cell pool and the control cell pool or among the cloned cell lines, the pooled transfectants, and the parental Rama 37 cells (data not shown). Northern blot hybridization and analysis of intensities of hybridizing bands showed that the level of VEGF mRNA was much higher in the VEGF-transfected cells than in both the control transfectants and the parental Rama 37 cells (Fig. 3A). The VEGF mRNA level in the transfectant pool and the five colonies was 3.1, 2.7, 5.4, 2.8, 3.9, and 2.6 times higher, respectively, than that in the control R37-PSV cells. The corresponding level in the parental Rama 37 cells was 0.99. Thus R37-VEGF-2, or clone 2, was identified as the highest VEGF expresser, and transfection with the expression plasmid alone failed to increase VEGF mRNA.

The VEGF protein secreted by the Rama 37 cells or by the transfectant cells was determined by ELISA analysis of the CM removed from the cell cultures (Fig. 3B). The amount of protein produced per milliliter of medium in 48 h by the parental Rama 37 cells (2.95 ng) was almost the same as that produced by the control transfectants R37-PSV (3.0 ± 0.2 ng). The amount of VEGF produced per milliliter of medium in the same period of time by the R37-VEGF cells (8.1 ± 0.5 ng) and the R37-VEGF-2 cells (13.7 ± 0.4 ng) was 2.7 and 4.5 times greater, respectively, than that secreted by the control R37-PSV cells.

The control transfectants and the R37-VEGF-2 cells were tested for their metastatic capability in syngeneic rats. Two groups of 35 Wistar Furth (OLA strain) female 4–6-week-old rats were injected with either R37-VEGF-2 or control R37-PSV cells. Four animals from the experimental group and five animals from the control group developed ulcerated primary tumors and had to be killed and dissected prematurely and thus were excluded from further study. All of the remaining 61 animals (31 animals in the experimental group and 30 animals in the control group) developed primary tumors (100%; Table 1). The mean latent period before the appearance of primary tumors in the experimental group is significantly shorter (Student’s t test, P < 0.05) than that observed in the control group, with means of 10 and 14 days, respectively. At autopsy, the average size of primary tumors in the experimental group (8.9 ± 1.7 cm3) was significantly larger (Student’s t test, P < 0.05) than that in the control group (5.2 ± 2.6 cm3). The tumors consisted predominantly of spindle cells with some glandular elements. Among the 31 animals inoculated with R37-VEGF-2 cells, a significant number of rats (18 of 31 animals,
C-FABP INDUCES METASTASIS BY UP-REGULATING VEGF

DISCUSSION

The growth and expansion of primary tumors and the development of secondary metastases require a large supply of blood. Thus, increased numbers of blood vessels and hence increased angiogenic activity are essential for the growth and metastasis of cancer. VEGF is one of the most potent factors that can stimulate angiogenesis and thus facilitate the malignant dissemination of cancer cells (27). The increased expression of the VEGF gene has been detected in various cancers, including that of the prostate (28–30). The malignant progression of some rat model cells has also been associated with increased VEGF expression (9, 31). In previous work on the generation of metastatic variants from Rama 37 cells through transfection of genomic DNA extracted from rat prostate carcinoma cells (8), we found that the levels of VEGF expression in the three metastatic variants were increased by 2.5-, 3.5-, and 4.5-fold (9). This finding suggests that the increase in VEGF expression might also be associated with malignant progression in this Rama 37 model.

Cancer metastasis is a complicated process, which involves a number of changes in the expression patterns of different genes. These changes include the diminished activity of metastasis-suppressing genes or the increased activity of metastasis-inducing genes. One such metastasis-inducing gene identified recently is that for C-FABP, which, when overexpressed, is able to cause Rama 37 cells to metastasize (5). To investigate the possible molecular mechanisms involved in the metastasis-inducing activity of the C-FABP gene, we have, in this work, performed several sets of experiments on Rama 37 model cells to find out whether or not up-regulation of the potent angiogenic factor VEGF is one of the major biological effects by which the C-FABP gene has caused the malignant dissemination of the transfectant cells.

Analyses of the VEGF expression by slot blot measurements in the C-FABP transfectant cells detected increased levels of VEGF mRNA in cells expressing high levels of C-FABP (Fig. 1C). The levels of VEGF mRNA in the transfectant cell pool pSV-C-FABP-R37 and its sublines Met-1 and Met-2 were respectively 3.8 ± 0.6, 5.2 ± 0.4, and 5.3 ± 0.6 times that in the control transfectant pSV-R37 generated by plasmid DNA alone. These differences were confirmed by Northern blotting (Fig. 1, A and B). On the protein level, the amount of VEGF produced per milliliter of CM from the metastatic Met-1 cells in 48 h is 4.2 times higher than that produced from the nonmetastatic control transfectant pSV-R37 cells under the same condition. It has been demonstrated previously that the control transfectant pSV-R37 did not express detectable levels of C-FABP mRNA, whereas high levels of

58.8%) developed metastases (Fisher’s exact test, P < 0.01). These metastases were similar in histological appearance to the corresponding primary tumors. The sizes of metastases ranged from 0.5 to 2 mm in diameter, and all developed within the lung tissue (Fig. 4D). The mammary epithelial origin of the metastases was confirmed by their positive immunocytochemical staining with antibodies to human cal- lus keratin and to rat milk fat globule (data not shown). Antibodies to vimentin also stained these metastases and their primary tumors (data not shown), confirming their origin from individual cells of the primary tumors. No metastases were identified at autopsy and on subsequent histological examinations in the remaining 30 animals inoculated with control R37-PSV cells.

Detection of VEGF Protein in Primary Tumors and Metastases.

The presence of VEGF in the primary tumors and metastases was sought using immunocytochemical staining with antibodies against VEGF (Fig. 4). Moderate staining was observed in the primary tumors produced by the control transfectant R37-PSV cells (Fig. 4A). However, strong staining was observed in the primary tumors produced by the R37-VEGF-2 cells (Fig. 4B) and in all their pulmonary metastases (Fig. 4C). Both the moderate staining in the R37-PSV tumors and the strong staining in the primary tumors developed from R37-VEGF-2 cells and the metastases were almost completely inhibited by prior incubation of the antibody with VEGF (Fig. 4D). Both the primary tumors and the metastases showed a mainly heterogeneous, speckled extracellular staining. There was also some stain located in the cytoplasm.

Determination of Microvessel Density in the Primary Tumors.

Microvessel density of the primary tumors was determined after immunocytochemical staining for factor VIII. The average number of vessels (± SD) in the primary tumors developed from the R37-PSV cells (Fig. 5A) was 7.6 ± 1.6 vessels/hpf. The average number of vessels in the primary tumors produced by R37-VEGF-2 cells (Fig. 5B) was increased by 3.5-fold to 26.6 ± 5.7 vessels/hpf (Fig. 5C).

Fig. 3. Analyses of VEGF expression in VEGF cDNA transfectants. A, identification of the clone with the highest VEGF expression by Northern hybridization. Samples (20 µg each) of total RNA extracted from different cell lines were subjected to agarose (0.8%) gel electrophoresis. The separated RNAs in the gels were transferred onto nylon membranes and cross-linked by a brief exposure (3 min) to an UV light. The [32P]dCTP-labeled VEGF cDNA probe was used to hybridize to the RNA on the membranes. The washed membranes were exposed to Kodak XAR-5 film with an intensifying screen for 48 h. To standardize the VEGF hybridization, the membrane was hybridized with a radioactively labeled GAPDH cDNA probe, and images were visualized by exposure to the Kodak films for 3 h. B, detection of the amount of cell-secreted VEGF protein. ELISAs used to measure the cell-secreted VEGF protein in the CM (serum free) were exposed to the cultured cells for 48 h. The results were quantitated by measuring the color intensity and relating it to a standard curve established by using rhVEGF protein with a TiterTek Multiskan PLUS (Flow Laboratories) at a wavelength of 492 nm. The results shown in the figure are the means ± SD of six measurements from two separate experiments.

Cancers, including that of the prostate (28–30). The malignant progression of some rat model cells has also been associated with increased VEGF expression (9, 31). In previous work on the generation of metastatic variants from Rama 37 cells through transfection of genomic DNA extracted from rat prostate carcinoma cells (8), we found that the levels of VEGF expression in the three metastatic variants were increased by 2.5-, 3.5-, and 4.5-fold (9). This finding suggests that the increase in VEGF expression might also be associated with malignant progression in this Rama 37 model.

Cancer metastasis is a complicated process, which involves a number of changes in the expression patterns of different genes. These changes include the diminished activity of metastasis-suppressing genes or the increased activity of metastasis-inducing genes. One such metastasis-inducing gene identified recently is that for C-FABP, which, when overexpressed, is able to cause Rama 37 cells to metastasize (5). To investigate the possible molecular mechanisms involved in the metastasis-inducing activity of the C-FABP gene, we have, in this work, performed several sets of experiments on Rama 37 model cells to find out whether or not up-regulation of the potent angiogenic factor VEGF is one of the major biological effects by which the C-FABP gene has caused the malignant dissemination of the transfectant cells.

Analyses of the VEGF expression by slot blot measurements in the C-FABP transfectant cells detected increased levels of VEGF mRNA in cells expressing high levels of C-FABP (Fig. 1C). The levels of VEGF mRNA in the transfectant cell pool pSV-C-FABP-R37 and its sublines Met-1 and Met-2 were respectively 3.8 ± 0.6, 5.2 ± 0.4, and 5.3 ± 0.6 times that in the control transfectant pSV-R37 generated by plasmid DNA alone. These differences were confirmed by Northern blotting (Fig. 1, A and B). On the protein level, the amount of VEGF produced per milliliter of CM from the metastatic Met-1 cells in 48 h is 4.2 times higher than that produced from the nonmetastatic control transfectant pSV-R37 cells under the same condition. It has been demonstrated previously that the control transfectant pSV-R37 did not express detectable levels of C-FABP mRNA, whereas high levels of
A moderate positive staining was detected in the primary tumors developed from R37-PSV cells, which were generated by transfection of Rama 37 cells with plasmid DNA alone. B, strong positive staining was seen in the tissue sections of primary tumors developed from R37-PSV cells, which were generated by transfection of Rama 37 cells with the VEGF expression construct. C, VEGF was detected in the pulmonary metastasis (Met) developed from R37-VEGF-2 cells but was barely detected in the adjacent lung tissue (Lu). D, representative result from the antibody suppression experiments. The positive staining in the primary tumors developed from R37-VEGF-2 cells was suppressed by the addition of neutralizing anti-VEGF antibody. Magnification, ×200.

Fig. 5. Vessel density analysis using immunocytochemical staining for factor VIII. The tissue sections were counterstained with Gill’s hematoxylin. A, primary tumor produced by control R37-PSV cells established by transfection of benign Rama 37 cells with plasmid DNA alone. B, primary tumor produced by transfectant R37-VEGF-2 cells, which were established by transfection of Rama 37 cells with a VEGF-expressing vector and selected from a single cell clone expressing a high level of VEGF. C, quantitative analysis of vessel density in different primary tumor tissues. The finding of three or more contiguous factor VIII-positive cells was defined as a microvessel. Microvessels were counted in 40 high-powered (×40) fields. For each section, 10 randomly chosen fields were counted. The means ± SD were calculated from eight tumors developed from each cell line used. The average number of vessels in the R37-PSV tumors was set at 1.

Table 1 Incidence of primary tumors and metastases produced by transfectants in vivo

<table>
<thead>
<tr>
<th>Cell lines established by transfection</th>
<th>No. of animals inoculateda</th>
<th>Incidence of primary tumors</th>
<th>Median latent period (days)</th>
<th>Average volume of primary tumors at autopsy (cm³)b</th>
<th>Incidence of metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>R37-PSV</td>
<td>30</td>
<td>30</td>
<td>100%</td>
<td>14 (10–16)</td>
<td>0</td>
</tr>
<tr>
<td>R37-VEGF-2</td>
<td>31</td>
<td>31</td>
<td>100%</td>
<td>10 (7–13)</td>
<td>18</td>
</tr>
</tbody>
</table>

a Thirty-five animals were initially injected with R37-PSV and R37-VEGF cells in each of two groups, but nine rats (five from the control group and four from the experimental group) whose primary tumors were ulcerated in the early stages of the experiment were excluded from further testing.

b At autopsy, each tumor’s length (L), width (W), and thickness (H) were measured. The tumor volume was calculated according to the formula of Janik et al. (24). L × W × H × 0.5236.

Both C-FABP mRNA and protein were detected in the C-FABP transfectants pSV-C-FABP-R37, Met-1, and Met-2 (5). Therefore, the increased expression of VEGF is associated with the increase in C-FABP. Thus, the up-regulation of the VEGF expression may be caused by the increased expression of the C-FABP gene in the metastatic C-FABP transfectants and their sublines.

The CAM assay showed that the CM removed from the benign pSV-R37 cells induced only a very weak angiogenic response, which was not completely suppressed by the anti-VEGF antibody. However, the CM derived from the C-FABP transfectant pSV-C-FABP-R37 cells and their metastasis-derived sublines Met-1 and Met-2 induced moderate angiogenic responses that were completely inhibited by anti-VEGF neutralizing antibody (Fig. 2A). These results indicate that the weak angiogenic response in the CM of the control cells might be caused in part by factors other than VEGF because anti-VEGF could not suppress it. In contrast, the result that the moderately strong angiogenic activities, which were completely neutralizable by anti-VEGF in pSV-C-FABP-R37, Met-1, and Met-2 cells, indicates that this angiogenic activity is predominantly due to VEGF.

According to the RT-PCR analysis, there were two VEGF isoforms, VEGF₁₈₈ and VEGF₁₆₄, which were expressed in the representative metastatic Met-1 cells derived from the C-FABP transfectants and in control pSV-R37 cells (Fig. 1D). Although different isoforms of VEGF have been shown to be involved in the development and growth of cancer, VEGF₁₆₄ may be more active than other isoforms in promoting tumorigenic neovascularization (32, 33). To find out whether increased expression of VEGF would be responsible for the malignant dissemination of the C-FABP transfectants, we transfected the Rama 37 cells with the cDNA of the VEGF₁₆₄ isoform and
selected the highest VEGF-expressing clone (R37-VEGF-2) from five separate colonies growing in selective medium. The level of VEGF mRNA in R37-VEGF-2 cells was increased by 5.4-fold when compared with that in the control transfectants (Fig. 3A). Similar levels of increase (about 4.5-fold) were detected in the amount of immunoreactive VEGF protein secreted in the CM (Fig. 3B). When the control transfectants and the R37-VEGF-2 cells were tested for their metastatic capability in syngeneic rats, all 61 animals tested (31 animals in the experimental group and 30 animals in the control group) developed primary tumors (100%; Table 1). The mean latent period before the appearance of primary tumors in the experimental group was significantly shorter (Student's t test, \( P < 0.05 \)) than that observed in the control group, with means of 10 and 14 days, respectively. At autopsy, the average size of primary tumors in the experimental group of animals \( (8.9 \pm 2.6 \, \text{cm}^3) \) was significantly larger (Student's t test, \( P < 0.05 \)) than that in the control group \( (5.2 \pm 1.7 \, \text{cm}^3) \). These results indicate that the increased expression of VEGF has greatly promoted the growth of the primary tumors. Among the 31 animals inoculated with R37-VEGF-2 cells, a significant number of rats (18 of 31) developed metastases from the VEGF cDNA transfectants (Fig. 4). Whereas the control R37-PSV cells. These results suggest that the increased expression of VEGF gene has not only facilitated the growth and development of primary tumors but has also initiated and promoted the metastasis of the transfectants in the Rama 37 model cells.

Immunocytochemical staining with antibodies against VEGF confirmed that VEGF was still produced in the primary tumors and metastases from the VEGF cDNA transfectants (Fig. 4). Whereas moderate positive staining was observed in the primary tumors produced by the control transfectant R37-PSV cells (Fig. 4A), strong positive staining was observed in both the primary tumors (Fig. 4B) produced by the R37-VEGF-2 cells and all their pulmonary metastases (Fig. 4C). These results further confirm that it is the overexpression of VEGF that is responsible for metastasis. Immunocytochemical staining with factor VIII showed that the number of vessels/hpf in the metastatic capability in Rama 37 model cells.

RESULTS

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Human Cutaneous Fatty Acid-binding Protein Induces Metastasis by Up-Regulating the Expression of Vascular Endothelial Growth Factor Gene in Rat Rama 37 Model Cells

Chun Jing, Carol Beesley, Christopher S. Foster, et al.


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