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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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 transfected 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 transfected 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 transfecants 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-VEGF164) that expressed high levels of VEGF. Inoculation of R37-VEGF2 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 transfecants. 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 transfecants 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.

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 prostate and breast cell lines. One such gene is that coding for human C-FABP. 1 Transfection of the C-FABP gene into nonmetastatic rat mammary Rama 37 model cells (4) and inoculation of the transfecants into syngeneic Wistar Furth rats demonstrated that overexpression of the C-FABP gene can induce the nonmetastatic Rama 37 cells to metastasize to the lungs and lymph nodes. Therefore, the C-FABP gene is suggested to be a metastasis-inducing gene (5). Because the C-FABP gene is also overexpressed in most human prostate carcinomas and some squamous carcinomas of the bladder and skin (5–7), it is possible that the increased expression of this gene may be involved in the malignant dissemination of some human cancers.

Although the mechanism underlying the metastasis-promoting activity of the C-FABP gene is not clear, our past work in Rama 37 cells suggested that it may be related to an effect on the VEGF gene. Previously, we have generated several metastatic variants of Rama 37 cells by transfection of the genomic DNA extracted from carcinoma cells of rat prostate (8). Northern and slot blot analyses demonstrated that expression of VEGF was greatly increased in the metastatic variants when compared with that in the parental nonmetastatic Rama 37 cells. When tested in the chick CAM assay, the CM from the metastatic variants also showed increased angiogenic activity, and this could be suppressed with antibodies to VEGF (9). Because increased VEGF expression and angiogenic activity are associated with metastatic ability in Rama 37 cells transfected with genomic DNA from carcinoma cells of the rat prostate, it is possible that VEGF may play an important role in metastasis in this rat cell model.

Metastasis is the major reason in preventing effective treatment of cancer. Although its molecular cascade may involve complicated and multiple genetic changes, angiogenesis is an essential common mechanism for the development and formation of metastases in solid tumors. A number of both stimulating and inhibiting factors are involved in regulating angiogenesis, but VEGF is one of the most potent factors for stimulating angiogenesis (10). However, although VEGF can stimulate angiogenesis, which is an essential step for cancer metastasis, its direct role in causing metastasis has not been consistent in different experimental cell models. On one hand, overexpression of VEGF has been shown to enhance malignant dissemination in some tumor cell models (11–15), but on the other hand, VEGF has been demonstrated to be necessary but not sufficient to produce metastasis (16). Thus the precise effect of the increased expression of VEGF on the metastatic potential of the Rama 37 cells is not clear.

In the work described in this report, we have studied the possible mechanism involved in the metastasis-promoting activity of the C-FABP gene by testing the hypothesis that the C-FABP gene may have induced metastasis by up-regulating the expression of the VEGF gene in this Rama 37 cell model. First, we have investigated whether or not the elevated expression of C-FABP is associated with an increase in the level of VEGF expression and angiogenic activity. Then we have...
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 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. The 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 (100 units/ml), streptomycin (100 μg/ml), hydrocortisone (5 ng/ml), and insulin (5 ng/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 undegraded band after Northern hybridization to a radioactively labeled actin cDNA probe. The bound radioactivity was detected by autoradiography, and autoradiographic images were scanned with an IS-1000 digital image system (Alpha Innotech, San Leandro, CA). 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.

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 pSVC-FABP-R37 cells using a cDNA synthesis kit (Boehringer Mannheim). The products (2 μl each of reaction) were separated by agarose gel electrophoresis, and 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 8% agarose gels, and the separated cDNA bands were visualized by ethidium 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.

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 methylene cellulose (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).

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 VEGF, was isolated from mouse hybridoma (San Carlos, CA) and purified by affinity chromatography on a VEGF-Sepharose column (Pharmacia). The antibody was diluted to 10 ng/ml with PBS. The VEGF concentration was measured by ELISA according to the manufacturer’s instructions (eBioscience).

Detection of VEGF Protein in Serum-Free CM. The nonmetastatic rat Rama 37 cells were cultured in serum-free CM used to culture the cells for 48 h. The mouse monoclonal

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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 were 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...
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,
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).

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
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

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### 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 inoculated</th>
<th>Incidence of primary tumors</th>
<th>Median latent period (days)</th>
<th>Average volume of primary tumors at autopsy (cm$^3$)</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 (59%)</td>
</tr>
</tbody>
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$^a$ 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).

$L \times W \times H \times 0.5236.$

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Fig. 4. Immunocytochemical staining with antibodies to VEGF of lesions produced in rats by transfected cells. The yellow color represents specific immunocytochemical staining for VEGF, and the blue color is the hematoxylin counterstain. A, moderate positive staining was detected in 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-VEGF-2 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, $\times$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 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 ($\times$40) fields. For each section, 10 randomly chosen fields were counted. The means $\pm$ 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.
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 ± 2.6 cm³) was significantly larger (Student’s t test, P < 0.05) than that in the control group (5.2 ± 1.7 cm³). 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 animals, 59%) developed metastases (Fisher’s exact test, P < 0.01). However, no metastases were identified at autopsy and on subsequent histological examinations in the remaining 30 animals inoculated with 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 R37-VEGF-2 cells was increased by 3.5-fold in vessel density and hence promotes the metastasis through its angiogenic activity.

Human C-FABP was originally identified as being overexpressed in the psoriatic skin, and it shares similarity with other fatty acid-binding family proteins (34). The FABPs present in skin are thought to play a role in the storage and transport of fatty acids (35, 36), thus the dramatic increase of C-FABP may indicate an altered transport or metabolism of fatty acids in this disease state (34). C-FABP and its fatty acid-binding activity have been detected in endothelial cells of the microvascularity of such organs as placenta, heart, skeletal muscle, small intestine, lung, and renal medulla, as well as in Clara cells and goblet cells of the colon (37). Prior to the finding that C-FABP was overexpressed in prostate and breast cancer cells, it had also been found to be overexpressed in bladder and squamous carcinoma cells (5–7). In addition to C-FABP, adipocyte FABP, another member of the FABP family, was also shown to be increased initially in transitional cell carcinomas (38, 39), indicating its possible role in initiating malignant transformation of bladder cells. In our previous work, transfection of the Rama 37 cells with the C-FABP gene and inoculation of the pool of transfectants into syngeneic rats produced a significant number (23%) of animals with metastases. The second round of inoculation of the animals with the cell lines (Met-1 and Met-2) established from the metastases originated from C-FABP transfectant cells produced 50% of animals with metastases (5). This result indicated that the most metastatic clones, which express high levels of both C-FABP and VEGF, had been selected from the pool of transfectants through the first round of inoculation in the animals. In this work, transfection of VEGF cDNA into Rama 37 cells and the subsequent injection of the transfectants into the syngeneic rats produced 58% of animals with metastases. The overexpression of the C-FABP gene in the C-FABP transfectant-derived cell lines (Met-1 and Met-2) can result in a 5.2–5.3-fold increase in the VEGF mRNA levels (Fig. 1C). The level of VEGF in R37-VEGF-2, a clone selected from the VEGF transfectant pool, is 4.5-fold higher than that in the control transfectant (Fig. 3A). C-FABP transfectant-derived cell lines (Met-1 and Met-2) and the VEGF transfectant R37-VEGF-2 expressed a similar level of VEGF and produced a similar percentage of animals with metastases. Therefore, the increased expression of VEGF in these transfectants has facilitated angiogenesis of the primary tumors and promoted their malignant dissemination. Thus, C-FABP has probably induced metastasis by up-regulating the expression of its possible downstream mediator, VEGF, in these rat Rama 37 model cells.

ACKNOWLEDGMENTS

We thank Christine Javis and Andrew Dodson for technical assistance.

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*Cancer Res* 2001;61:4357-4364.

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