ANTICANCER RESEARCH *25*: 701-708 (2005)

A Potential Role for Vascular Endothelial Growth Factor-D as an Autocrine Growth Factor for Human Breast Carcinoma Cells

MANABU AKAHANE[1], TAKEMI AKAHANE[1],
AMY SHAH[1], EIJIRO OKAJIMA[2] and UNNUR P. THORGEIRSSON[1]

[1]*Laboratory of Cellular Carcinogenesis and Tumor Promotion (LCCTP), Center for Cancer Research, National Cancer Institute (NCI), National Institute of Health (NIH), Bethesda, MD 20892-4255, U.S.A.;*
[2]*National Cancer Center Hospital, 1-1 Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

Abstract. *Background. Vascular endothelial growth factor-D (VEGF-D) binds and activates vascular endothelial growth factor receptor-2 (VEGFR-2), which signals for angiogenesis, and VEGFR-3, which signals for lymphangiogenesis. Besides endothelial cells, VEGFR-2 has been detected on malignant cells, including human breast carcinoma cells. Materials and Methods. It was examined if ectopic expression of human VEGF-D affected growth of breast carcinoma cell lines in vitro and in vivo. Results. VEGF-D overexpressing clonal MCF-7 and MDA-MB-231 cell lines displayed increased proliferative activities and upregulation of cyclins A, D1 and D3, compared to the vector control. Following subcutaneous inoculation of the MDA-MB-231 cells into nude mice, growth of the VEGF-D overexpressing cells was greatly accelerated. The tumor weight gain was accompanied by increased proliferative activity, cyclin A expression and microvascular density. Conclusions. These findings suggest that VEGF-D functions both as an autocrine growth factor and a stimulator of angiogenesis in breast cancer.*

Members of the vascular endothelial growth factor (VEGF) family are secreted glycoproteins, which serve as important regulators of vascular development, angiogenesis and lymphangiogenesis (1-3). VEGF-A interacts with two high affinity tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (4-6). VEGF-D also interacts with VEGFR-2 and stimulates angiogenesis (7). It was originally identified as a *c-fos*-induced growth factor (8). VEGF-D is structurally related to VEGF-C and both serve as ligands for the lymphatic VEGFR-3 and stimulate lymphangiogenesis (9, 10). Structure-function studies have shown that VEGF-D is produced as a precursor form possessing a receptor-binding domain and N- and C-terminal propeptides (11). Following cellular release, it is reduced through proteolysis to multiple smaller forms, including a mature form, which has high affinity for VEGFR-2 and VEGFR-3 (12). VEGF-D acts as angiogenic and lymphangiogenic factor in experimental tumors and has also been detected in tumor cells of malignant melanoma (13, 14). Following the discovery of VEGFR-2 in malignant cells, VEGF-A was shown to have autocrine growth and survival functions in epithelial malignancies, including breast carcinoma (15-17). VEGF-D is also overexpressed in different types of malignancies and has been implicated as a prognostic factor in ovarian (18), colorectal (19) and breast (20) carcinomas. It was identified in more than 80% cases of invasive breast carcinomas and was associated with lymph node metastases and overall survival (20). Here, we addressed the question whether VEGF-D can act as an autocrine growth factor of the human MCF-7 and MDA-MB-231 cell lines. Because these cell lines did not spontaneously express VEGF-D *in vitro*, they were transfected with full-length human VEGF-D cDNA to examine the effect on *in vitro* and *in vivo* growth.

Correspondence to: Unnur P. Thorgeirsson, Laboratory of Cellular Carcinogenesis and Tumor Promotion, Center for Cancer Research, National Cancer Institute, NIH, Bldg. 37, Room 4032A, Convent Drive, Bethesda, MD. 20892-4255, U.S.A. Tel: (301)-496-1982, Fax: (301)-496-8709, e-mail: thorgeiu@mail.nih.gov

Key Words: VEGF-D, cell proliferation, breast cancer, cyclin, angiogenesis.

Materials and Methods

Materials. The MCF-7 and MDA-MB-231 breast carcinoma cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in ATCC recommended media. G418 was from Sigma (St. Louis, MO, USA). The primary antibodies for Western blot analysis were as follows: VEGF-D and cyclin D1 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); cyclin D3 and cyclin A from Oncogene (San Diego, CA, USA); actin from Chemicon (Temecula, CA, USA). The secondary horseradish peroxidase conjugated antibody was from Dako

(Carpinteria, CA, USA). The Ki67 and cyclin A antibodies, used for immunohistochemistry, were from Novocastra Laboratories (Burlingame, CA, USA) and the von Willebrand factor (vWf) antibody was from Chemicon (Temecula, CA, USA).

Establishment of VEGF-D overexpressing clonal lines. Full length VEGF-D cDNA was isolated from a human lung cDNA library. After sequence verification, it was cloned into the pcDNA3.1 mammalian vector (Invitrogen, Carlsbad, CA, USA). The human MCF-7 and MDA-MB-231 cell lines were transfected with either the full-length human VEGF-D cDNA or the pcDNA3.1 vector alone, using SuperFect Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The transfected cells were cultured in G418 selection medium (500 μg/ml). G418 resistant colonies were lifted from the culture plates and grown individually to make the stable VEGF-D overexpressing clones. VEGF-D and VEGF-A ELISA (R&D Systems, Minneapolis, MN, USA) were used to quantitate VEGF-D and VEGF-A in the culture supernatants.

Cell proliferation assay. In vitro growth was measured by using a BrdU cell proliferation assay kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. The VEGF-D overexpressing and vector control MCF-7 and MDA-MB-231 clonal lines were seeded in a 96-weel plate at a density of 3 x 10^3 cells/well (5 wells for each clone) in 100 μl of culture medium containing 10% FBS. Cell proliferation was measured on days 1 and 3 with the addition of BrdU 4 hours before harvesting the cells. Proliferation was also measured in the presence of VEGF-D antibodies against epitopes at the C-terminus and N-terminus, or a neutralizing VEGFR-2 antibody (500-5000 ng/ml) (Santa Cruz, Santa Cruz, CA, USA). It was examined if recombinant human VEGF-D (rhVEGF-D) (R&D Systems, Minneapolis, MN, USA) stimulated growth of the MCF-7 and MDA-MB-231 cells. The cells were seeded in a 96-well plate at 3 x 10^3 cells/well) and incubated overnight in 100 μl culture medium containing 10% FBS. This was followed by starvation in the basal medium without FBS for 24 h to allow synchronization of cells in G_1/G_0. Consequently, the cells were cultured in medium containing 1% FBS with or without rhVEGF-D (20, 50, or 100 ng/ml) for 72 h (culture medium was replaced every 24 hours), followed by the BrdU cell proliferation assay.

Western blot. Expression of VEGF-D and the cell cycle associated cyclins A, D1 and D3 was evaluated by Western blot analysis. Cell lysates were prepared using M-PER Mammalian protein extraction reagent (Pierce, Rockford, IL, USA) containing protease inhibitor cocktail tablets, 1 mM sodium orthovanadate and 25 mM NaF. Protein concentration was measured in duplicate samples using Pierce's Micro BCA reagent and bovine serum albumin as a standard. Samples (30 μg protein) of the cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Invitrogen). The membrane was incubated with VEGF-D, cyclin A, cyclin D1, cyclin D3, or actin antibodies diluted in PBS-T with 5% non-fat dry milk at 4˚C overnight. The membrane was then washed in PBS-T and incubated with the appropriate secondary antibodies at room temperature for 1 h. The immune complexes were visualized with enhanced chemiluminescence by using the ECL kit (Amersham Biosciences, Piscataway, NJ, USA). The signal intensities of protein expression were measured by densitometric analysis and normalized against actin expression. The densitometric unit of the vector-transfected control was arbitrarily set as 1.

Tumorigenicity in nude mice. The VEGF-D overexpressing and vector control MCF-7 and MDA-MB-231 cells were trypsinized, rinsed twice in PBS, resuspended at a concentration of one million cells in 100 μl of PBS, and injected subcutaneously into the flanks of 7 week-old female nude mice. The MCF-7 group consisted of 7 mice injected with the MCF-7 vector control and 8 mice injected with the VEGF-D overexpressing clone 7. The MDA-MB-231 group consisted of 15 mice injected with the vector control and 12 mice injected with the VEGF-D overexpressing clone 10. The mice were examined weekly for palpable tumors. All the mice were euthanized 9 weeks after tumor cell inoculation and macroscopic tumors were harvested and weighed.

Immunohistochemistry. Proliferative activity, cyclin A expression and microvascular density were assessed in serial histological sections from all of the VEGF-D overexpressing and vector control MDA-MB-231 tumors. The formalin fixed, paraffin embedded tumor sections were deparaffinized, microwaved in citrate buffer for antigen retrieval, and treated with 3% H_2O_2 dissolved in methanol to inactivate endogenous peroxidase. The sections were blocked with Power Block (Universal blocking reagent, BioGenex, San Ramon, CA, USA) for 20 min and treated with MOM immunodetection kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instruction. They were then incubated with Ki67 (1:200 dilution), cyclin A (1:100 dilution) and von Willebrand factor vWf (1:100 dilution) antibodies for 30 min at room temperature. Staining was visualized by using the avidin-biotin-complex (ABC) system and NovaRED substrate kit (Vector Laboratories). The sections were then counterstained with methyl green (Vector Laboratories) for 1 min. Ki67 and cyclin A positive cells were counted in 5 random microscopic fields per section at x200 magnification. Vascular density was determined by counting VWF positive microvessels in 5 random microscopic fields per section at x400 magnification.

Statistical analysis. Data were analyzed for statistical significance by the Student's *t*-test or one factor ANOVA. Results were expressed as mean ± SD. p-value <0.05 reflected a significant difference between groups.

Results

Ectopic expression of VEGF-D is associated with increased proliferative activity of MCF-7 and MDA-MB-231 cells *in vitro*. The untransfected MCF-7 and MDA-MB-231 cells expressed VEGF-D and VEGFR-2 by RT-PCR but VEGFR-3 was expressed only by the MCF-7 cells (Figure 1A). The VEGF-D protein was not detected by Western blot analysis of cell lysates of the untransfected or VEGF-D transfected cells, although a minute amount of VEGF-D ($\sim$1 pg/ml) was detected by ELISA in 3-day culture supernatants of the MDA-MB-231 cells (not shown). Due to the lack of VEGF-D production *in vitro*, full-length VEGF-D cDNA was stably transfected into the MCF-7 and MDA-MB-231 cells. Of the three transfected MCF-7 clonal lines, which expressed

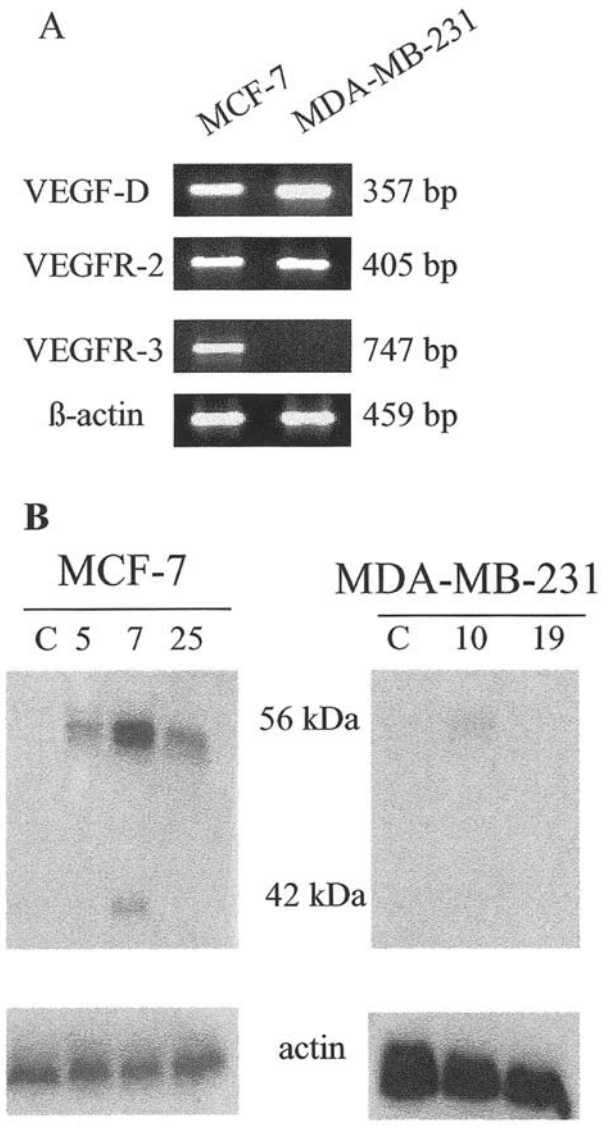

Figure 1. *Establishment of VEGF-D overexpressing clonal breast carcinoma cell lines. (A) Constitutive expression of VEGF-D and its receptors in MCF-7 and MDA-MB-231 cells, determined by RT-PCR. (B) Western blot analysis of VEGF-D expression by the vector controls (C), VEGF-D transfected MCF-7 clones 5, 7, 25, and VEGF-D transfected MDA-MB-231 clones 10 and 19. The data represent three separate experiments with similar results.*

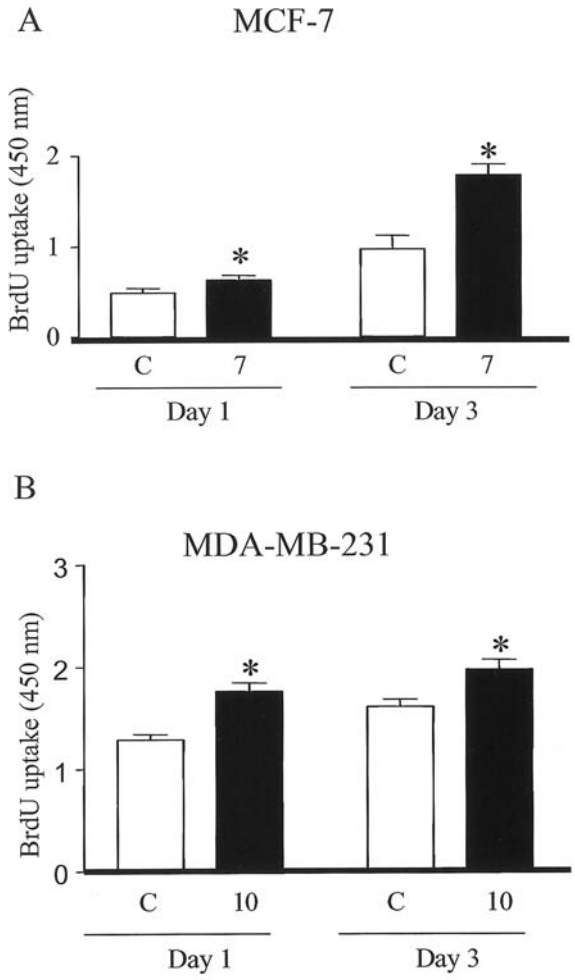

Figure 2. *VEGF-D overexpression stimulates proliferation of breast cancer cells in vitro. Proliferative activity of the VEGF-D overexpressing (A) MCF-7 clone 7 and (B) MDA-MB-231 clone 10 and their respective vector controls was quantitated by BrdU uptake at days 1 and 3. The data represent three separate experiments. * indicates significant difference ($p<0.05$) between the VEGF-D overexpressing clones and the vector controls.*

VEGF-D by Western blot, clone 7 displayed two forms of VEGF-D at molecular weights of ~56 and 42 kDa (Figure 1B) and was selected for further studies. Of the stably transfected MDA-MB-231 cells, VEGF-D was detected in only one clonal line (clone10), which displayed a weak band of the ~56 kDa form by Western blot (Figure 1B) and released 8-9 times less VEGF-D into the supernatants (5.41 pg/ml/10^5cells) than the MCF-7 clone 7 (46.6 pg/ml/10^5cells) after three days in culture.

The MDA-MB-231 cells displayed approximately 3 times higher constitutive proliferative activity by the BrdU assay than the MCF-7 cells after one day in culture (Figure 2). The proliferative activity was significantly higher in the VEGF-D overexpressing MCF-7 and MDA-MB-231 clones than their vector controls ($p<0.05$), both after one and three days in culture (Figure 2). At three days, the increase in proliferative activity was 81% for the MCF-7 clone 7 and 19% for the MDA-MB-231 clone 10. It was also examined if proliferation of untransfected MCF-7 and MDA-MB-231 cells was stimulated by rhVEGF-D. Similar to the results on the VEGF-D overexpressing transfectants, a significant increase in proliferative activity ($p<0.05$) was observed following three day treatment of the MCF-7 cells with a

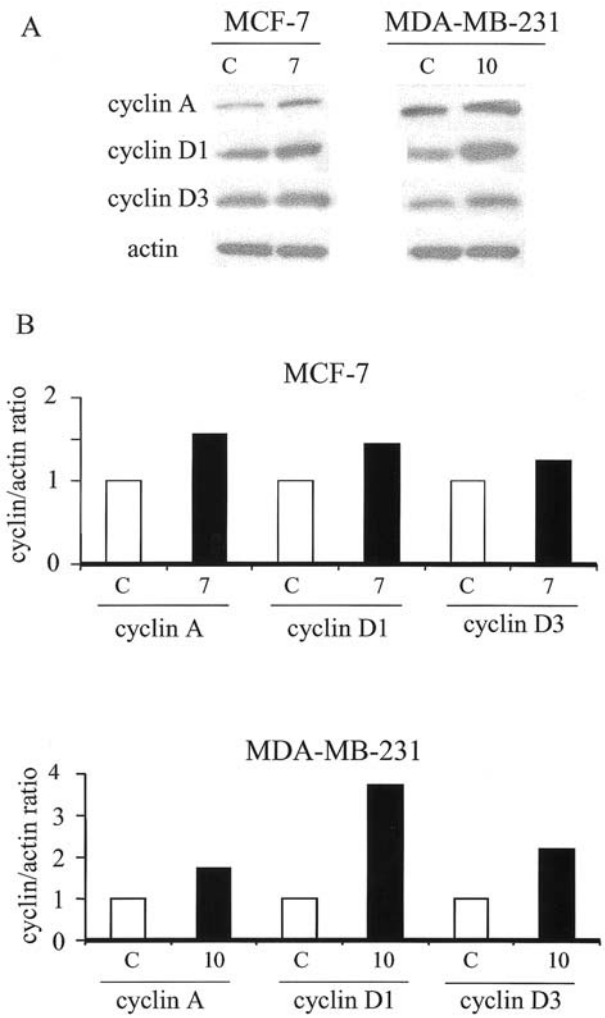

Figure 3. *VEGF-D overexpression stimulates cyclins A and D. (A) Western blot shows cyclin A, D1 and D3 expression by the vector controls, VEGF-D transfected MCF-7 clone 7 and MDA-MB-231 clone 10. Actin served as a loading control. (B) The columns represent the cyclin/actin ratios of cyclin A, D1 and D3 expression, which were arbitrarily set as 1.0 for the controls. The data show one of three experiments with similar results.*

minimum of 20 ng/ml rhVEGF-D and the MDA-MB-231 cells with a minimum of 100 ng/ml rhVEGF-D (data not shown). Antibodies, which recognize epitopes at either the C-terminus or N-terminus of VEGF-D, did not block the increased proliferation observed for the VEGF-D overexpressing clones or following exposure to rhVEGF-D (not shown). Unfortunately, we did not have access to a neutralizing antibody to the mature form of VEGF-D, which binds VEGFR-2 with much higher affinity than the unprocessed form (11). The possibility was considered that VEGF-A was stimulated in the VEGF-D overexpressing clones and be responsible for the growth stimulation in an autocrine fashion. This turned out not to be the case based on VEGF-A ELISA. Also, a neutralizing VEGF-A antibody did not suppress the proliferative activity of the VEGF-D overexpressing clones in the BrdU assay (not shown). Furthermore, increasing concentrations of a neutralizing VEGFR-2 antibody (500-5000 ng/ml) failed to alleviate the VEGF-D-associated growth stimulation (not shown).

VEGF-D upregulates cyclin expression. Cell proliferation is regulated by cell cycle proteins, including cyclins. We evaluated the expression of cyclins A, D1 and D3 by Western blot and found that they were stimulated in the VEGF-D overexpressing MCF-7 and MDA-MB-231 clonal lines, compared with their vector controls (Figure 3A). The cyclins A, D1 and D3 levels were increased 1.56-fold, 1.44-fold and 1.25-fold in the MCF-7 clone 7 and 1.73-fold, 3.73-fold and 2.20-fold in the MDA-MB-231 clone 10 (Figure 3B). However, expression of the cell cycle regulators, CDK2 and CDK4, was not altered in the VEGF-D overexpressing clones (data not shown).

VEGF-D overexpression results in increased in vivo tumor growth. The VEGF-D overexpressing MCF-7 clone 7 and MDA-MB-231 clone 10, as well as their vector controls, were inoculated subcutaneously into nude mice. At the end of the 9-week experimental period, neither the MCF-7 clone 7 nor the vector control MCF-7 cells had produced palpable tumors. However, there was increased tumor incidence and greatly accelerated *in vivo* growth of the VEGF-D overexpressing MDA-MB-231 clone 10, compared to that of the vector control. The first palpable VEGF-D overexpressing tumor was detected at 2 weeks. At nine weeks, tumor incidence was 41.7% for the mice inoculated with the VEGF-D overexpressing MDA-MB-231 cells and 26.7% for the mice inoculated with the control cells. There was a striking increase in weight of the tumors derived from the VEGF-D overexpressing cells with an average weight of 693 mg, compared to 11.8 mg in the vector control group ($p<0.05$) (Figure 4A).

Increased proliferative activity, cyclin A expression and microvascular density in the VEGF-D overexpressing MDA-MB-231 tumors. H&E stained histological sections showed that the VEGF-D overexpressing tumors were more cellular than the vector control, but no other discernible microscopic differences were observed (not shown). The increased cellularity in the VEGF-D overexpressing tumors was associated with higher number of Ki67 positive cells (271 cells/200x field) than the vector control tumors (122 cells/200x field) (Figure 4B). Similarly, the number of cyclin A immunopositive cells was higher in the VEGF-D overexpressing tumors (155 cells/200x field) than the vector control tumors (58 cells/200x field) (Figure 4C). Microvascular density was evaluated by immunostaining of

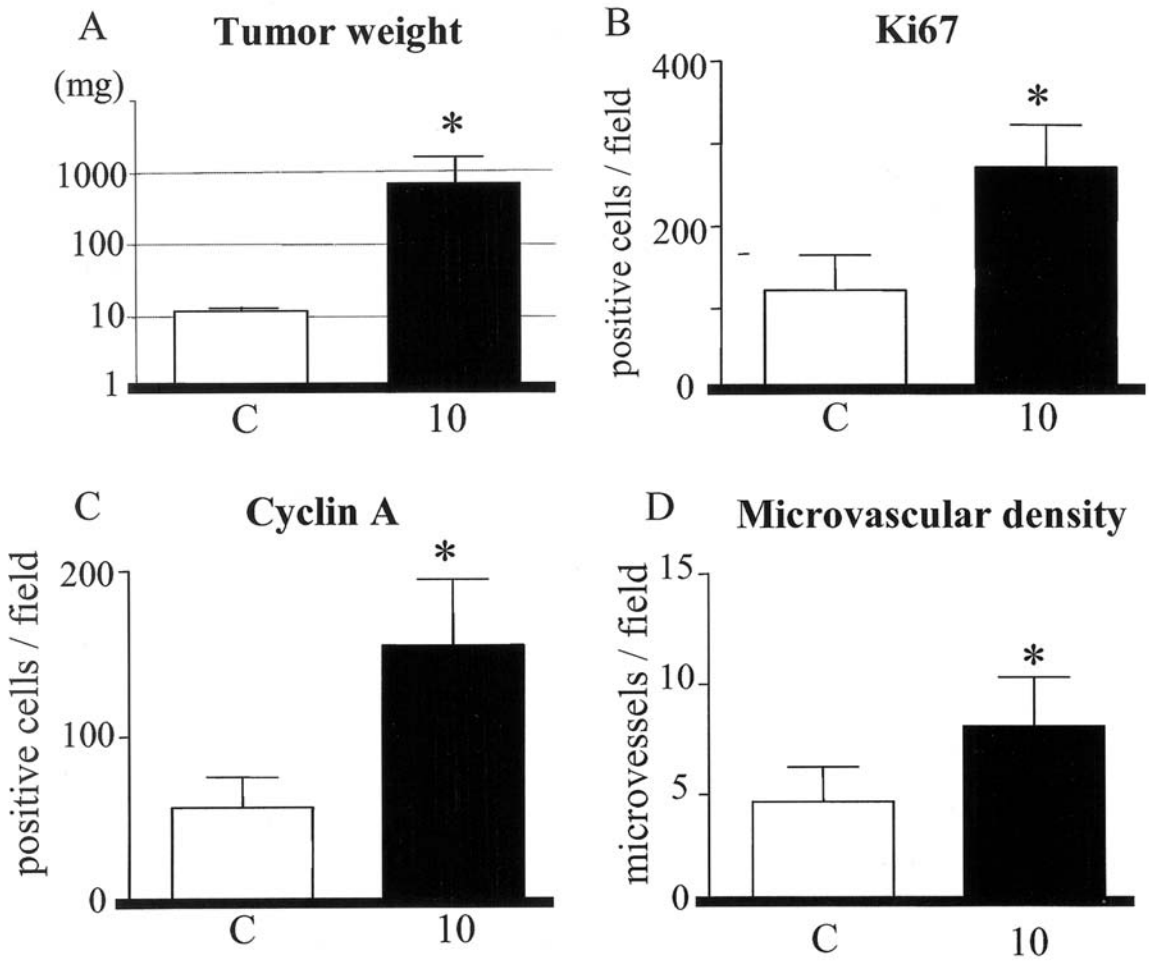

Figure 4. *Accelerated growth of VEGF-D overexpressing MDA-MB-231 cells in nude mice. (A) Tumor weights at 9 weeks after inoculation of the VEGF-D overexpressing MDA-MB-231 clone 10 and the corresponding vector control. * indicates a significant difference ($p<0.05$) between the VEGF-D overexpressing tumors ($n= 5$) and the vector control tumors ($n= 4$). Results of immunostained serial sections of these tumors show the number of (B) Ki67 and (C) cycline positive tumor cells/ field at x200 magnification. Tumor sections were also stained for vWf for quantitation of microvascular density within the tumors. (D) shown are vWf positive microvessels/field at x400 magnification.*

the endothelial marker, vWf. The VEGF-D overexpressing tumors contained 8.1 microvessels/400x field and the vector control tumors 4.7 microvessels/400x field (Figure 4D). Statistical analysis showed that the number of Ki67 and cyclin A positive cells, as well as the microvascular density, was significantly higher in the VEGF-D overexpressing tumors than the vector control tumors ($p<0.05$).

Discussion

Until recently, studies of VEGF family members have focused primarily on their paracrine functions as angiogenic and/or lymphangiogenic stimulators. Following the discovery of VEGF receptors on malignant cells, it was shown that VEGF-A can as an autocrine growth factor in different types of epithelial cancers (15-17). The main finding of the present study suggests that VEGF-D may also act as an autocrine growth factor in breast carcinoma cells. Overexpression of VEGF-D caused *in vitro* growth stimulation of the MCF-7 and MDA-MB-231 cell lines. In addition, there was greatly accelerated *in vivo* growth of the VEGF-D overexpressing MDA-MB-231 cells, which was associated with increased neovascularization, proliferative avtivity and cyclin A expression. These findings suggest that VEGF-D acts both as an autocrine growth factor and a stimulator of angiogenesis in human breast carcinoma cell lines.

The MCF-7 and MDA-MB-231 cell lines differ in their malignant behavior and this was reflected in increased proliferative activity of the MDA-MB-231 cells. Ectopic expression of VEGF-D resulted in significant growth

stimulation in both cell lines. Positive correlation was found between VEGF-D production and proliferative activities of the VEGF-D overexpressing clones after three days in culture. Proliferation was assessed by BrdU labeling, which identifies cells in S phase of the cell cycle (21). The G_1/S checkpoint is regulated by cell cycle proteins, including cyclins A, D1 and D3 (22-24). Increased expression of these cyclins by the VEGF-D overexpressing clones provided evidence that they were involved. Similarly, VEGF-A growth stimulation of neuronal precursor cells was found to entail stimulation of cyclins A and D (25).

VEGF-D is a ligand for VEGFR-2 and VEGFR-3 but not VEGFR-1(7). Proteolytic processing produces multiple forms of VEGF-D, including a fully processed form, which binds VEGFR-2 with high affinity (12). Studies have shown that VEGF-D is proteolytically processed and bioactive in human malignancies (13). VEGF-D was reported to phosphorylate endothelial VEGFR-2 more slowly and less effectively than VEGF-A at early time points but had more sustained effect and was as effective at later time points (26). Both MCF-7 and MDA-MB-231 cell lines were shown to express VEGF-A but only MCF-7 cells expressed the VEGFR-2 protein (27, 28). The MCF-7 and MDA-MB-231 cell lines used in the present study produced VEGF-A by ELISA, which was not altered by the ectopic expression of VEGF-D, making it unlikely that VEGF-A was responsible for the increased proliferative activity of the VEGF-D overexpressing clones *in vitro*. Like previously reported (27, 28), we did not detect VEGFR-2 by Western blot analysis of the control or the VEGF-D overexpressing MDA-MB-231 cell lysates. Moreover, VEGFR-2 protein was not detected by Western blot analysis of our MCF-7 cells. Thus, it is not likely that VEGFR-2 serves as the signaling receptor for the VEGF-D associated growth stimulation. Because VEGFR-3 was only expressed by the MCF-7 cells, it is not a likely candidate either. The VEGF165 specific receptor, neuropilin-1, was implicated in inhibition of apoptosis mediated by VEGF-A in MDA-MB-231 cells (16). However, neuropilin-1 does not possess a binding domain for VEGF-D (1). It remains to be resolved if yet unknown VEGF-D autocrine pathway exists that stimulates proliferation of breast carcinoma cells.

Recently, VEGF-D was found to be the most potent angiogenic VEGF family member following adenoviral delivery into skeletal muscle (29). Although increased angiogenesis undoubtedly contributed to the accelerated growth of the VEGF-D overexpressing MDA-MB-231 carcinoma cells in the nude mice, the 2-fold increase in microvascular density seemed insufficient to account for the almost 100-fold increase in the mean terminal weight of the VEGF-D overexpressing tumors. Tumor cell lines generally do not constitutively express VEGF-D in culture but it is induced *in vivo* through tumor cell-host cell interactions (30). Presumably, the induction of VEGF-D *in vivo* ought to affect control and VEGF-D transfected cells to the same extent and assessment of VEGF-D expression by the MDA-MB-231 carcinoma cells *in vivo* was therefore not undertaken. VEGF-A is known to stimulate tumor cell VEGFR-2 expression *in vivo* (31, 32). It remains to be resolved if VEGF-A contributes to the VEGF-D-mediated increase in tumor weight *in vivo* through stimulation of MDA-MB-231 VEGFR-2 expression. It is also worth bearing in mind that VEGF-D, like VEGF-A, causes vasodilatation through nitric oxide-mediated mechanism (33). Increased tissue perfusion due to vasodilatation may help explain the early emergence of the VEGF-D overexpressing tumors at 2 weeks.

Lymphatics are the primary route of dissemination of human breast carcinomas. VEGF-D stimulates lymphatic formation and metastatic spread *via* lymphatics in experimental models (34, 35). It has been implicated as a prognostic factor in ovarian, colorectal and breast carcinomas (18-20) and positive correlation was found between VEGF-D expression and lymph node metastases of breast carcinomas (20). Given the functions of VEGF-D as both vascular and lymphatic endothelial mitogen, as well as a potential autocrine growth factor for breast carcinoma cells, it is conceivable that it may play an important role in the growth and metastatic dissemination of human breast cancer.

References

1 Neufeld G, Cohen T, Gengrinovitch S and Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. FASEB J *13*: 9-22, 1999.

2 Ferrara N: Molecular and biological properties of vascular endothelial growth factor. J Mol Med *77*: 527-543, 1999.

3 Ferrara N and Alitalo K: Clinical applications of angiogenic growth factors and their inhibitors. Nature Medicine *5*: 1359-1364, 1999.

4 Cross MJ, Dixelius J, Matsumoto T and Claesson-Welsh L: VEGF-receptor signal transduction. Trends Biochem Sci *28*: 488-494, 2003.

5 Petrova TV, Makinen T and Alitalo K: Signaling *via* vascular endothelial growth factor receptors. Exp Cell Res *253*: 117-130, 1999.

6 Shibuya M: Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. Cell Struct Funct *26*: 25-35, 2001.

7 Achen MG, Jeltsch M, Kurk E, Makinen T, Vitali A, Wilks AF, Alitalo K and Stacker SA: Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci USA *95*:548-553, 1998.

8 Orlandini M, Marconcini L, Feruzzi R and Oliviero S: Identification of a *c-fos*-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family. Proc Natl Acad Sci *93*: 11675-11680, 1996.

9 Jussila L and Alitalo K: Vascular growth factors and lymphangiogenesis. Phys Rev *82*: 673-700, 2002.

10 Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel TB, Nice BEC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG and Alitalo K: Isolated lymphatic endothelial cells transduce growth, survival and migratory signals *via* the VEGF-C/D receptor VEGFR-3. EMBO J *20*: 4762-4773, 2001.
11 Stacker SA, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson RJ, Moritz R, Karpanen T, Alitalo K and Achen MG: Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. J Biol Chem *274*: 32127-32136, 1999.
12 Baldwin M, Roufail S, Halford MM, Alitalo K, Stacker SA and Achen MG: Multiple forms of mouse vascular endothelial growth factor-D are generated by RNA splicing and proteolysis. J Biol Chem *276*: 44307-44314, 2001.
13 Achen MG, Williams RA, Baldwin ME, Lai P, Alitalo K and Stacker SA: The angiogenic and lymphangiogenic vascular endothelial growth factor-D exhibits a paracrine mode of action in cancer. Growth Factors *20*: 99-107, 2002.
14 Achen MG, Williams RA, Minekus MP, Thornton GE, Stenvers K, Rogers RAW, Lederman F, Roufail S and Stacker SA: Localization of vascular endothelial growth factor-D in malignant melanoma suggests a role in tumour angiogenesis. J Pathol *193*: 147-154, 2001.
15 Mazood R, Cai J, Zheng T, Smith DL, Hinton DR and Gill PS: Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. Blood *98*: 1904-1913, 2001.
16 Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G and Mercurio AM: Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells, Cancer Res *61*: 5736-5740, 2002.
17 Jackson MW, Roberts JS, Heckford SE, Ricciardelli C, Stahl J, Horsfall DJ and Tilley D: A potential autocrine role for vascular endothelial growth factor in prostate cancer. Cancer Res *62*: 854-859, 2002.
18 Yokoyama Y, Charnock-Jones DS, Licence D, Yanaihara A, Hastings JM, Holland CM, Emoto M, Umemoto M, Sakamoto T, Sato S, Mizunuma H and Smith SK: Vascular endothelial growth factor-D is an independent prognostic factor in epithelial ovarian carcinoma. Br J Cancer *88*: 237-244, 2003.
19 White JD, Hewett PW, Kosuge D, McCulloch T, Enholm BC, Carmichael J and Murray JC: Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. Cancer Res *62*: 1669-1675, 2002.
20 Nakamura Y, Yasuoka H, Tsujimoto M, Yang Q, Imabun S, Nakahara M, Nakao K, Nakamura M, Mori I and Kakudo K: Prognostic significance of vascular endothelial growth factor D in breast carcinoma with long-term follow-up. Clin Cancer Res *9*: 716-721, 2003.
21 Iatropoulos MJ and Williams GM: Proliferation markers. Exp Toxicol Pathol *48*: 175-181, 1996.
22 Johnson DG and Walker CL: Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol 39:295-312, 1999.
23 Tapon N, Moberg KH and Hariharan IK: The coupling of cell growth to the cell cycle. Curr Opin Cell Biol *13*: 731-737, 2001.
24 Vermeulen K, Van Bockstaele DR and Berneman ZN: The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif *36*: 131-149, 2003.
25 Zhu Y, Jin K, Ou X and Greenberg DA: Vascular endothelial growth factor promotes proliferation of cortical neuron precursors by regulating E2F expression. FASEB J *17*: 186-193, 2003.
26 Jia H, Bagherzadeh A, Bicknell R, Duchen MR, Liu D and Zachary I: Vascular endothelial growth factor (VEGF)-D and VEGF-A differentially regulate KDR-mediated signaling and biological function in vascular endothelial cells. J Biol Chem *279*: 36148-36157, 2004.
27 Tian X, Song S, Wu J, Meng L, Dong Z and Shou C: Vascular endothelial growth factor: acting as an autocrine growth factor for human gastric adenocarcinoma cell MGC803. Biochem Biophys Res Commun *286*: 505-512, 2001.
28 Soker S, Fidder H, Neufeld G and Klagsbrun M: Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 *via* its exon 7-encoded domain. J Biol Chem *271*: 5761-5767, 1996.
29 Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI, Kholova I, Kauppinen RA, Achen MG, Stacker SA, Alitalo K and Yla-Herttuala S: VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle *via* adenoviruses. Circ Res *92*: 1098-1106, 2003.
30 Krishnan J, Kirkin V, Steffen A, Hegen M, Weih D, Tomarev S, Wilting J and Sleeman JP: Differential *in vivo* and *in vitro* expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats. Cancer Res *63*: 713-722, 2003.
31 Woessner RD, Loudy DE, Wallace CD, Montgomery LR, Cross-Doersen DE, Bush TL, Lewis MT, Prakash N, Bitonti AJ and Wright PS: Decreased vascular endothelial growth factor expression associated with tumor regression by (E)-2'-deoxy-2'(fluoromethylene)cytidine (MDL 101, 731). Oncol Res *9*: 543-552, 1997.
32 Matsumoto H, Iino Y, Koibuchi Y, Andoh T, Horii Y, Takei H, Horiguchi J, Maemura M, Yokoe T and Morishita Y: Antitumor effect of 22-oxacalcitrol on estrogen receptor-negative MDA-MB-231 tumors in athymic mice. Oncol Rep *6*: 349-352, 1999.
33 Rissanen TT, Rutanen J and Yla-Herttuala S: Gene transfer for therapeutic vascular growth in myocardial and peripheral ischemia. Adv Genet *52*: 117-164, 2004.
34 Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H and Achen MG: VEGF-D promotes the metastatic spread of tumor cells *via* the lymphatics. Nat Med *7*: 186-191, 2001.
35 Alitalo K and Carmeliet P: Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell *1*: 219-227, 2002.

Received February 2, 2005
Accepted February 25, 2005