

New Doctorial Cancer Research

**Essential Role of Angiogenic Factors
and Bone Marrow-Derived
Endothelial/Hematopoietic Cells in the
Growth of Solid Tumors**



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The current thesis examines the role of vascular endothelial growth factor receptor-1 (VEGFR-1) autocrine and paracrine loops in different subsets of cells, namely, breast tumor, endothelial and bone marrow (BM)-derived cells, and their role on tumor growth regulation. These studies highlight the functional relevance of the biological pathways mediated by this receptor and the importance of therapies against VEGFRs as antitumor and antiangiogenesis targets. These findings open new perspectives for the study and development of novel and more effective strategies to fight tumor growth and invasion, and lay the foundation for further development of clinical trials.

It is well established that angiogenesis is essential for tumor development. Although the process is controlled by several molecules, the vascular endothelial growth factor (VEGF), by signaling through its receptors, VEGFR-1 and VEGFR-2, seems to be the main mediator. Whereas VEGFR-2 biological functions on tumor endothelium are clearly defined, VEGFR-1 roles are mostly unknown. Moreover, besides being initially described as endothelial specific, VEGFR-1 has also been reported in other types of cells of nonendothelial origin, such as subsets of breast tumor cells and hematopoietic stem and progenitor cells.

These observations led us to hypothesize the following: (1) If breast tumor cells express VEGFR-1 and produce VEGF, VEGF/VEGFR-1 autocrine loops could occur and promote breast tumor growth in an endothelial cell-independent fashion. (2) Tumor-produced VEGF could also activate, in a paracrine fashion, VEGFR-1 on tumor-associated endothelium promoting angiogenesis, a feature still unclarified. (3) Tumor cells, by releasing VEGF, may be able to induce in a paracrine way the mobilization of populations of VEGFR-1, expressing BM-derived hematopoietic cells that, in addition to VEGFR-2+ endothelial progenitor cells (EPCs) recruitment to tumor sites, might influence malignant cell growth and neovascularization.

The in vitro and in vivo functional expression of VEGFR-1 in human breast cancer cells, and the relevance of the specific impairment of this receptor as a therapeutic target, using monoclonal antibodies (mAbs) as strategy, was first evaluated. VEGFR-1 was functionally expressed on subsets of primary human invasive ductal breast carcinoma tissues and breast cancer cell lines, directly promoting their in vitro proliferation by the

downstream activation of the p44/42 MAPK signaling pathway. Stimulatory proliferative effects were abolished by a high-affinity neutralizing monoclonal antibody (mAb) against human VEGFR-1. In vivo studies demonstrated that specific inhibition of human VEGFR-1 signaling on breast tumor cells by mAb treatment significantly delayed the growth of human breast carcinoma xenografts in immunocompromised mice. This outcome was due to considerable direct mAb-induced tumor cell apoptosis.

In addition, the role of angiogenesis mediated by VEGFR-1 on breast tumor associated endothelium was also assessed in vivo by specific non-cross-reactive antimurine VEGFR-1 mAb immunotherapy. These studies showed that hampering the VEGFR-1 signaling network on tumor-associated endothelium induced a moderate, although not very efficient, antiangiogenic and antivascular effect, when compared with breast xenografts treated with a known antivascular VEGFR-2 mAb. However, a complete and efficient tumor remission was achieved only by selective target of both VEGFR-1 on human breast cancer cells and by blockade of host VEGFR-2-mediated angiogenesis. Close histological examination indicated that the underlying mechanism leading to total breast tumor regression involved the induction of both tumor (human VEGFR-1 dependent pathway) and endothelial cell (murine VEGFR-2 dependent pathway) apoptosis.

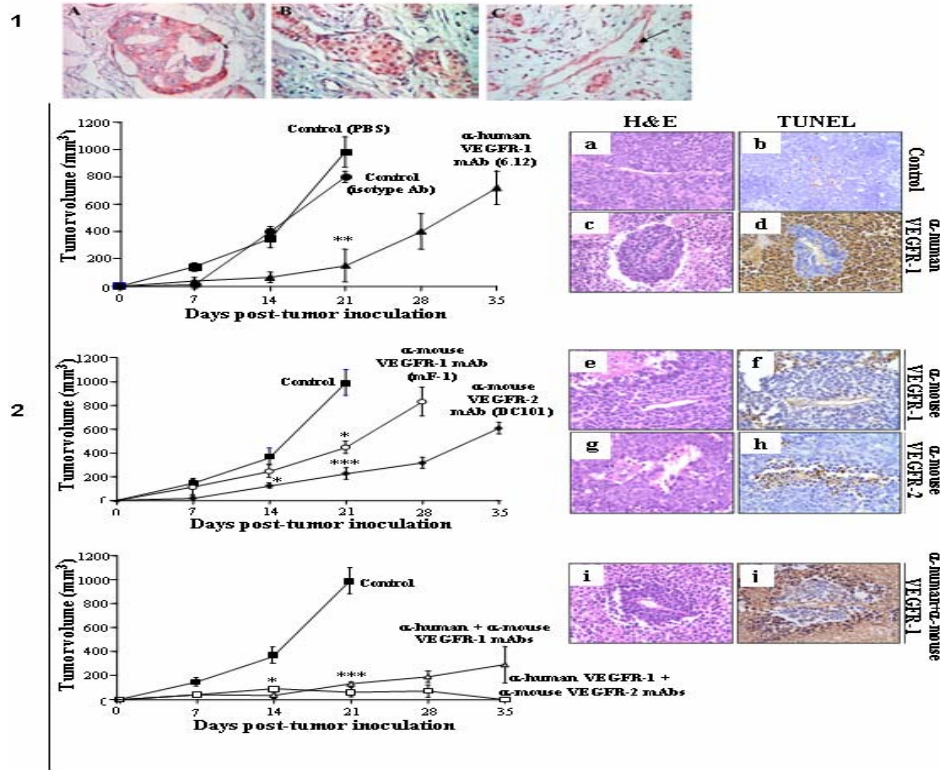


FIGURE 1. VEGFR-1 functional expression in breast tumor cells, endothelial cells, and bone marrow-derived cells: Relevance for tumor development and neoangiogenesis. (1) Expression of VEGFR-1 in breast tumor cells. VEGFR-1 is expressed on epithelial components of 54% of human breast carcinoma samples analyzed, as well as in intratumoral-associated endothelium. (2) Therapeutic strategies and histologic outcome of

Furthermore, it was demonstrated, using several mice transgenic strains and bone marrow (BM) transplantation studies, that a population of BM-derived myelomonocytic VEGFR-1-expressing cells are mobilized to sites of tumor neovascularization, playing a crucial role in tumor angiogenesis, development, and growth. This cell population is corecruited from the BM along with VEGFR-2+ EPCs (endothelial progenitor cells), contributing to rapid tumor neovascularization, especially in early phases of tumor devel-

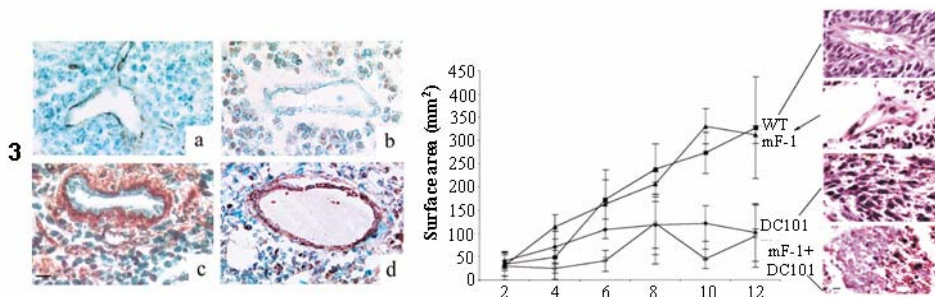


FIGURE 1. CONT'D. treatments using monoclonal antibodies (mAbs) targeting: VEGFR-1 on human breast tumor cells and VEGFR-1 and VEGFR-2 on tumor-associated endothelium. Immunodeficient mice were injected with human breast cancer cells and treated with: PBS (vehicle treated controls, ■); antihuman VEGFR-1 (6.12, ▲) mAb; a control antibody given at the same concentration (●); antimouse VEGFR-1 (mF-1, ○) mAb; antimouse VEGFR-2 (DC101, ◆) mAb; a combination of antihuman VEGFR-1 (6.12) plus antimouse VEGFR-1 (mF-1) mAbs (Δ) and 6.12; plus antimouse VEGFR-2 (DC101) mAbs (□). Data represents two individual experiments. Mean ± SD (t-test; *p < 0.05; **p < 0.01; ***p < 0.001). *In vivo* impairment of human VEGFR-1 on breast carcinoma cells resulted in significant tumor growth inhibition when compared to the control groups, mainly due to an overwhelming mAb-induced tumor cell apoptosis throughout the tissue. The few areas of viable tumor cells, localized surrounding viable and intact blood vessels, forming a perivascular cuffing of neoplastic cells (c, d). Xenotransplanted mice treated with antimurine VEGFR-1 mAb showed significant, although moderate antitumor properties, mainly to loss of tumor cell viability, all through the tissue; however, tumor vascular integrity did not seem to be largely affected (e, f). Blocking angiogenesis by host VEGFR-2 impairment on endothelial cells exhibited a statistically significant, although not complete, antitumor efficacy by mAb-induced apoptosis, severely affecting the tumor vasculature (g, h). Furthermore, treatment of tumor-bearing mice with combined mAb regimens of antihuman and antimurine VEGFR-1 was more effective than monotherapies alone, promoting profound apoptosis and alteration in tumor architecture, very similar to the result obtained with antihuman VEGFR-1 mAb alone (i, j). Remarkably, in mice submitted to treatment regimens with mAbs against human-VEGFR-1 and murine-VEGFR-2, targeting both tumor growth and host angiogenesis-dependent pathways, tumors achieved complete regression, suggesting that the efficacy of these combination therapies has involved, respectively, tumor and endothelial cell apoptosis. (3) Contribution of VEGFR-1⁺ bone marrow-derived hematopoietic cells in early tumor neovascularization. Transplantation of BM cells from β-gal⁺ mice into lethally irradiated wild type recipient animals that were challenged with B6RV2 tumors showed at day 2 posttumor inoculation: (a, b) Vessels surrounded by LacZ⁺VEGFR-1⁺ BM-derived cells. (c) Most mononuclear cells found in association with the vessels were of myeloid origin expressing LacZ⁺MOMA⁺, (d) whereas the cells lining the lumen were of endothelial origin expressing LacZ⁺VEcadherin⁺. Blocking the recruitment of BM-derived cells with the use of neutralizing antibody treatments against mouse VEGFR-1 resulted in generation of vessels without significant perivascular mononuclear infiltrates. Inhibition of murine VEGFR-2 resulted in disruption of all vessels. Treatment of mice with both neutralizing antibodies resulted in widespread tumor necrosis and complete absence of viable tumor cells and blood vessels, indicating once more the efficiency of targeting both VEGFR-1 and VEGFR-2 to completely block tumor angiogenesis and growth.

opment. Whereas VEGFR-2+ EPCs incorporate and differentiate in mature endothelium at tumor vascular sites, the VEGFR-1+ myelomonocytic population was present in close association with the tumor neovessels. Blocking the recruitment of these cells in mouse models using an anti-VEGFR-1 mAb showed significant antiangiogenic and antitumor effects, indicating the crucial role of these BM-derived cells in tumor development and neoangiogenesis.

Overall, this thesis has challenged the notion that the function of VEGF/VEGFRs in cancer is limited to angiogenesis, highlighting the important biological function of this system in breast malignant cells and bone marrow-derived cells, and their role for tumor development. Furthermore, this work has demonstrated the importance of anti-VEGFRs therapies, where multiple targets of different cells and biological pathways involved in tumor growth may warrant more effective anticancer and antiangiogenic treatments.

Comment by David Lyden

The current thesis investigates the important role of vascular endothelial growth factor receptor-1 (VEGFR-1) in different cell types that comprise a growing tumor. This is a pioneering research work, where the clinical significance of the functional expression of VEGFR-1 on human breast carcinomas was unveiled and the relevance of the specific impairment of this receptor as a potential anticancer therapeutic target was evaluated. It was demonstrated that VEGFR-1 is functionally expressed on subsets of breast cancer cells, directly promoting in vitro and in vivo tumor cell proliferation. Moreover, hampering of the VEGFR-1 signaling network on tumor cells by a specific neutralizing monoclonal antibody induced significant tumor regression in xenotransplanted human breast tumors. However, complete tumor remission was achieved only by impairment of both tumor VEGFR-1 and by tumor-associated angiogenesis blockade, emphasizing the fact that combined therapies conduce to more effective outcomes. Furthermore, demonstrated in detail was the essential involvement of bone marrow progenitor cells that are mobilized to sites of tumor neovascularization playing a crucial role in tumor development and growth. Blocking the recruitment of these cells using monoclonal antibodies as therapeutic strategy showed significant antiangiogenic and antitumor effects. This work is an important motivator on the use of monoclonal antibodies against VEGFRs, highlighting the importance of targeting multiple pathways as therapy to treat and prevent tumor growth and metastasis. Overall, this scientific work has opened new perspectives for the future development of new anticancer and antiangiogenesis strategies to treat cancer patients, and has been published or submitted for publication in high-impact journals, such as *Nature Medicine*, and presented in prestigious international scientific meetings.