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The Isocoumarin, NM-3, Enhances Antitumor Effects of Ionizing Radiation

By Helena J. Mauceri, PhD, Rabih M. Salloum, MD, Nora T. Jaskowiak, MD, Mitchell C. Posner, MD, and Ralph R. Weichselbaum, MD

ANGIOGENESIS IS A DISCRETE COMPONENT OF TUMOR PHENOTYPE AND IS REQUIRED FOR tumor progression and metastases formation. Successful vascularization, based on invasion and morphological differentiation of endothelial cells, is controlled by the relative ratios of pro-angiogenic and anti-angiogenic proteins in the tumor microenvironment.[1] Unlike proliferating endothelial cells within tumors, normal endothelium is quiescent, and cell division is rare. Treatments designed to inhibit the angiogenesis process are designed to exploit the differences between proliferating endothelial cells in tumor and those of normal tissues. Also, because endothelium is genetically stable, it is unlikely that endothelial cells will develop resistance to cytotoxic agents.[2] Approaches to inhibit angiogenesis include the use of neutralizing antibodies to angiogenic proteins, integrin molecules, and growth factor receptors, as well as antibiotic derivatives, such as TNP-470 and minocycline.[3-7]

Angiostatin, a plasminogen cleavage product, has been shown to induce tumor regression and prolong tumor dormancy in murine model systems.[8-10] However, when angiostatin is employed as a single treatment modality, tumor regrowth ensues following cessation of treatment, and cure rate is limited. Because radiotherapy improves local tumor control and cure rates,[11-16] we evaluated the interaction between angiostatin and ionizing radiation (IR). We demonstrated that combined treatment with angiostatin and IR enhanced tumor regression in Lewis lung carcinoma (LLC) tumors and three human tumor xenograft model systems.[17] We also studied the interaction between IR and endostatin, a c-terminal proteolytic fragment of collagen XVIII, and reported that combined treatment with IR and endostatin produced significant tumor growth inhibition when compared with either treatment alone.[18]

Another approach to enhance the therapeutic ratio involves blocking a positive regulator of angiogenesis. We conducted experiments in which tumor-bearing mice were treated with an antibody to vascular endothelial growth factor (VEGF). We reported that combined treatment

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with anti-VEGF antibody and IR produced greater than additive antitumor effects when compared to either treatment alone.[19] Collectively, our studies suggest that when combined with IR treatment, angiogenesis inhibition targets both tumor cells and tumor vasculature. Enhancing tumor control without increasing toxicity has important implications for cancer treatment.

The isocoumarin derivative, NM-3 [2-(8-hydroxy-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid], has been reported to inhibit the growth of endothelial cells in vitro and exert modest antitumor effects in vivo.[20,21] Because NM-3 possesses a low toxicity profile, is orally bioavailable, and is easily produced, we conducted studies to evaluate if treatment with NM-3 and IR enhanced the therapeutic ratio of IR. We report that NM-3 increases the antitumor effects of IR without a concomitant increase in acute local or systemic toxicity. The bioavailability and non-toxic profile of NM-3 support the testing of this new isocoumarin in combination with radiotherapy in clinical trials.

NM-3 Is Anti-Angiogenic and Selectively Cytotoxic to Endothelial Cells

We evaluated the effects of NM-3 treatment on the three components of angiogenesis (i.e., endothelial cell survival, migration, and tube formation). Cell survival was analyzed using clonogenic assays in which endothelial cells and tumor cells were exposed to NM-3 alone for four hours or exposed to NM-3 and treated with increasing doses of IR. NM-3 was cytotoxic to endothelial cells but not to tumor cells. When endothelial cells were exposed to NM-3 prior to IR exposure, additive killing was observed. No enhancement of IR-induced cytotoxicity was observed in tumor cell cultures.

Endothelial cell migration was evaluated using an inverted, modified Boyden chamber.[22] Cells were treated with NM-3 for four hours prior to exposure to IR. NM-3 alone did not affect endothelial cell migration when compared to control. However, IR alone inhibited migration by 34% and the combination of NM-3 and IR produced a 49% inhibition in endothelial cell migration.

To evaluate the effects of NM-3 on tube formation, tumor cells were mixed with Matrigel (synthetic basement matrix) and injected subcutaneously into nude mice. Matrigel plugs from mice receiving daily injections of saline showed a robust angiogenic response, as demonstrated by an extensive network of vessels. In contrast, a dramatic reduction in neovascularization was observed in the Matrigel plugs from mice receiving two daily injections of NM-3.

The Combination of NM-3 and IR Inhibits Primary Tumor Growth

The effects of NM-3 and IR were assessed in one murine tumor and two human tumor xenograft models. In the LLC models, no significant tumor regression was observed following treatment with NM-3 alone. Tumors in the IR alone group regressed initially, but then regrew. However, combined treatment with NM-3 and IR significantly reduced mean tumor volume when compared to IR alone. Equivalent treatment effects were observed in xenografts of human espohageal adenocarcinoma (Seg-1) cells. Treatment with NM-3 alone did not inhibit tumor growth, whereas combined treatment with NM-3 and IR produced a significant reduction in mean tumor volume. The combined treatment group demonstrated a growth delay of 14 days compared to the control group and three days compared to the IR alone group. Human squamous cell carcinoma (SQ-20B) control tumors and tumors treated with NM-3 alone grew steadily for 20 days, at which time animals in these two groups were sacrificed due to tumor burden. Tumors in the IR treatment group initially doubled in volume, regressed, and then regrew to twice original volume (day 0 volume). Animals treated with NM-3 and IR regressed to 77% of original volume but, unlike IR treated tumors, never regrew to original volume. These studies demonstrate

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that when NM-3 is combined with IR, significant growth inhibition of primary tumors can be achieved.

Primary Tumor Growth Delay Translates to Enhanced Local Cures

We employed the LLC tumor model system to examine the effects of treatment with NM-3 and IR on local cure rate, defined as the absence of measurable tumor at the site of implantation. Mice bearing LLC tumors were treated with a higher dose of NM-3 (50 mg/kg/day) and a higher dose of IR (66 Gy). On day 21 after initiation of treatment, five of 12 mice in the IR alone group were cured locally, whereas nine of 11 animals in the NM-3/IR group were tumor-free.

Combination Treatment with NM-3 and IR Reduces Microvascular Density

To assess the effect of NM-3 and IR on tumor vessels, LLC tumors were excised on days 5 or 11 from mice treated with NM-3, IR, or the combination of NM-3 and IR. Employing rat anti-mouse-CD31 (PECAM-1) monoclonal antibody, immunohistochemistry was performed and microvascular density was determined using light microscopy. At day 5, tumors in the combined treatment group (NM-3 and IR) had fewer vessels (14.2 ± 1.2) than either the NM-3 alone group (19.2 ± 3.6) or the IR group (18.7 ± 2.7). No further reduction in the total number of vessels was observed in any treatment group at day 11. However, fewer distinct small vessels were present in the IR and combined treatment groups compared to the control and NM-3 alone groups.

NM-3 Alone or in Combination with IR Is Not Toxic to Experimental Animals

We also evaluated the effects of treatment with NM-3 alone and in combination with IR on regional and systemic toxicity. Mice bearing Seg-1 tumor xenografts and mice bearing LLC tumors were employed in these studies. With regard to systemic toxicity, no weight loss or mortality resulted from NM-3 treatment in either experiment. Local toxicity was evaluated in treated hind limbs. When the hind limbs of mice bearing Seg-1 xenografts were scored for superficial injury and scab formation, no differences between treatments were observed. Mice bearing LLC tumors were treated for 11 days with higher doses of both NM-3 and IR. In these animals, hind limbs were scored for superficial injury, scab formation, ulceration, hair loss, and limb shortening. Both the IR alone and the NM-3 and IR groups demonstrated similar local effects of treatment. A greater increase in foot swelling was noted in the combined treatment group at days 17 and 20, but by day 21 no difference between IR alone and NM-3 and IR was evident.

Conclusion

The present studies demonstrate that NM-3, an orally bioavailable isocoumarin, increases the antitumor effects of IR. Combined treatment with NM-3 and IR increased tumor regression and local cure rates when compared to IR alone, without a concomitant increase in toxicity. This enhancement of the therapeutic ratio of IR is attributable to the selective effects of NM-3 on the tumor vasculature, as indicated by the effects of NM-3 on endothelial cell survival, migration, and tube formation. Clinical radiotherapy focuses on targeting the tumor, employing techniques to deliver higher doses of IR to the tumor volume while sparing normal tissues. However dose escalation IR is required to achieve tumor control. The use of chemical modifiers and conventional cytotoxic agents combined with radiotherapy has yielded poor clinical results, mainly due to acute tissue toxicity.

Our studies employing treatment with NM-3 describe a new paradigm in which a non-toxic agent potentiates the effects of IR. We demonstrate selective antitumor effects by employing combined treatment with NM-3 and IR, without local or systemic toxicity. Importantly, the combination of NM-3 and radiotherapy improves both tumor growth delay and radiocurability. The present findings are supported by reports of angiogenesis inhibitors potentiating the effects of IR and strengthen the concept of targeting both tumor cells and the tumor microvasculature to improve the therapeutic ratio. *(Dr. Mauceri is Research Associate and Dr. Weichselbaum is Professor and Chairperson, Department of Radiation and Cellular Oncology; Drs. Salloum and Jaskowiak are Surgical Oncology Fellows and Dr. Posner is Associate Professor, Department of Surgery, University of Chicago.)* ❖

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Prostate Cancer-Associated Overexpression of *PSGR*

By Linda L. Xu, MD, and Shiv Srivastava, PhD

PROSTATE CANCER (CAP) IS THE MOST COMMON MALIGnancy and the second leading cause of cancer mortality in American men.[1] Outward symptoms are not always apparent; when untreated, CaP may spread, or metastasize, to other parts of the body or vital organs. When this happens, the patient has fewer treatment options than he would have if the disease had been discovered earlier.

Diagnostic Tools

Serum prostate-specific antigen (PSA) and other diagnostic tools have been very successful in the early detection of CaP.[1] However, the wide spectrum of biologic behavior exhibited by prostatic neoplasms poses a difficult problem in predicting the clinical course for the individual patient.[2] Traditional prognostic markers such as grade, clinical stage, and pretreatment PSA have limited prognostic value for individual men.[3] Molecular studies have shown a significant heterogeneity between multiple cancer foci present in a cancerous prostate gland. These studies also have documented that metastatic lesions can arise from cancer foci other than dominant tumors. Approximately 50-60% of patients treated with radical prostatectomy for localized CaP have microscopic disease that is not organ-confined, and a significant portion of these patients relapse.[4] Therefore, identification and characterization of genetic alterations defining CaP onset and progression are crucial to understanding the biology and clinical course of disease.

PSA has been used clinically as a biomarker for CaP diagnosis and post-treatment follow-up due to its specific expression in prostate epithelial cells. Over the past 10 years, the PSA test has revolutionized the early detection of CaP; organ-confined disease can be cured effectively by surgical intervention or radiation treatment. Since the introduction of the PSA test, there has been a sharp decline in the incidence of metastatic CaP. However, the PSA blood test is not always entirely accurate, and it is not CaP-specific. PSA testing may identify men with CaP, but often PSA is elevated in men with benign prostate hyperplasia, prostatitis (inflammation of the prostate), and other non-malignant, non-life threatening prostate disorders. Current figures on this common diagnostic test show that about 25% of men with CaP will have normal PSA levels and more than one-half of men with higher PSA levels may be cancer-free.

Discovery of Prostate-Specific Genes

The discovery of additional prostate-specific genes has resulted in enthusiasm for evaluating their potential utility in the diagnosis and disease progression of CaP. *HK2*, a member of kallikrein gene family, currently is being evaluated for its role in CaP.[5] Prostate-specific membrane antigen (PSMA) is a membrane-bound glycoprotein that is expressed in prostate and few other tissues. Expression of PSMA is increased in CaP, particularly in hormone-refractory disease. PSMA has been exploited as a marker for tumor detection and treatment by immunoscintiscanning with the ^{111}indium-labeled, anti-PSMA monoclonal antibody 7E11.C5. Increased concentrations of 7E11.C5-reactive antigen are present in the serum of CaP patients compared with healthy individuals. Also, hematogenous circulating CaP cells are detectable with reverse transcriptase-polymerase chain reaction analysis.[6]

Current strategies for defining CaP-specific genetic alterations include positional cloning of candidate genes from chromosome loci frequently altered in CaP and comparison of the global gene expression profiles in cancer cells and corresponding normal cells by differential display (DD), serial analysis of gene expression (SAGE), and cDNA microarrays.[7-9] The database of cDNA sequence libraries from defined tissues or cells also have provided impetus for identifying unique expression patterns in specific target tissues. These gene discovery approaches recently have led to the identification of several new prostate-specific/abundant genes, such as *NKX3.1*, prostase, prostate stem cell antigen (PSCA), *TMPRSS2*, *STEAP*, *PDEF*, *PART-1*, *HOXB13*, *DD3*, *PCGEM1*, *PMEPA1*, and *PSGR*, all of which exhibit diverse characteristics.[10-20] Of special interest is the identification of prostate-specific expression markers, which not only are prostate-specific but also show elevated expression in CaP. Furthermore, several laboratories, including ours, are addressing the potential utility of new prostate-specific molecules as biomarkers of CaP onset and progression and as cancer vaccine targets.

Our laboratory has employed DD, SAGE, DNA microarrays, and electronic subtraction of cDNA sequence libraries to study CaP-associated gene expression alterations.[19-21] These techniques led us to identify new prostate-specific genes that are overexpressed in CaP, such as *PCGEM1* and *PSGR*, novel androgen-regulated prostate-specific or abundant genes, such as *PMEPA1*, and other promising candidates.[19-22] Here we briefly review our first observations on isolation and characterization of *PSGR*, a seven-transmembrane G-protein coupled receptor.[21]

PSGR

PSGR was identified as a prostate tissue-specific cDNA during a search of the expressed sequence tag database at Human Genome Sciences. Analysis of the 1,474 bp *PSGR* cDNA sequence revealed an open reading frame (ORF) of 963 bp nucleotides encoding a 320 amino acid protein with a predicted molecular mass of 35.4 kDa. The *PSGR* ORF revealed intriguing homology (~ 50% identity and ~ 70% similarity) to the G-protein coupled odorant receptor (OR) family. A protein motif search using ProfileScan indicated the existence of seven transmembrane domains between amino acid residues 22 and 293 that are characteristic of G-protein coupled receptors. ORs belong to a superfamily of G-protein coupled receptors (GPCRs) that are transmembrane proteins mediating cellular responses to diverse extracellular stimuli, including light, neurotransmitters, hormones, and odorants.[23] GPCRs are the largest gene family known to exist in a given animal genome. Through selective ligand binding, the GPCRs discriminate between multiple signals. GPCRs amplify and transduce the information inherent in ligand binding to the cell interior by interacting with the heterotrimeric G-proteins. The ligand-bound GPCR activates the G-protein complex, which in turn modulates a number of effector proteins such as adenylyl cyclase, phospholipase C-beta, G-protein-gated calcium and potassium channels, and membrane proximal components of the MAP kinase pathway. ORs are localized in nasal epithelium and are highly selective in expression. However, OR-like genes have been detected in testis, and their functions are not well understood. It has been suggested that OR-like genes may serve a chemosensory role in sperm chemotaxis during fertilization. Prostate-specific expression of

PSGR, suggests an as yet undiscovered function in the prostate. Also of note is the overexpression of *PSGR* in about two-thirds of CaP specimens analyzed.

The distribution of *PSGR* mRNA in 50 different normal human adult and fetal tissues examined by Northern blot and slot blot analyses showed that *PSGR* expression was detected only in prostate tissue. In situ RNA hybridization analysis of *PSGR* expression in prostate tissues revealed that *PSGR* expression was localized predominantly to epithelial cells of the gland. Comparison of *PSGR* expression in normal and tumor tissues by laser capture microdissection-derived normal/tumor cell RNAs, using semiquantitative or real time PCR assays and RNA in situ hybridization, revealed overexpression of *PSGR* in 62% (32/52) of the tumor specimens.

Conclusion

We have demonstrated that prostatic epithelial cells restrict expression of *PSGR* and tumor cells exhibit significantly increased expression of this putative seven-transmembrane G-protein coupled receptor. However, it is not yet clear how *PSGR* overexpression may play a role in the process of tumorigenesis. G-protein coupled receptor may play important roles in cell signaling and cell proliferation. Therefore, future experiments will focus on biologic function of *PSGR* in tumorigenesis of CaP and its potential utility as a CaP biomarker. Membrane localization of *PSGR* makes *PSGR* protein an attractive target for immunotherapy approaches and antibody-based imaging of metastatic CaP. It also will be very important to determine the signals transduced by *PSGR* in the prostate gland and future studies will address these issues. Using the well-studied prostate-specific gene (PSA or PSMA) paradigm, further studies of *PSGR*, focusing on basic research and preclinical models, hold promise in evaluating the utility of this novel prostate-specific membrane protein in CaP diagnosis, prognosis, and therapy. *(Dr. Xu is Staff Scientist and Dr. Srivastava is Professor and Scientific Director, Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Rockville, MD.)* ❖

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The Role of *RET* in Pheochromocytoma

By Christian A. Koch, MD, Karel Pacak, MD, PhD, Steve C. Huang, PhD, Alexander O. Vortmeyer, MD, and Zhengping Zhuang, MD, PhD

PROTO-ONCOGENES AND ONCOGENES SUCH AS *MET* AND *RET* play important roles in the development of tumors. Often, however, the exact mechanisms of tumorigenesis are unknown. There are many in vitro studies and animal models on the study of tumor formation in oncogene-driven disorders, but only a few, recent in vivo studies on hereditary tumor syndromes. Zhuang and associates reported on hereditary papillary renal carcinomas associated with germline mutations of *MET* and showed that a subset of these tumors develop by duplication of mutant *MET* in trisomy 7.[1] Based on these findings, in multiple endocrine neoplasia type 2 (MEN 2)-related pheochromocytomas Huang and Koch tested whether *RET*, another proto-oncogene with structural and functional homology to *MET*, would lead to tumorigenesis through a similar mechanism.[2] This work will elucidate tumor formation in MEN 2-related tumors and may lead to a better understanding of tumorigenesis of other oncogene-related hereditary tumor syndromes.

Background

Pheochromocytoma is a neuroendocrine tumor that often leads to paroxysmal hypertension, headaches, stroke, cardiac arrhythmias, and so-called spells.[3] Most of these tumors occur in the sporadic form, but about 10% are hereditary. Among hereditary syndromes, pheochromocytoma most commonly occurs in MEN 2, followed by von Hippel-Lindau disease and neurofibromatosis type 1. The gene responsible for MEN 2 is the *RET* proto-oncogene.

RET has been mapped to chromosome 10q11.2 and subsequently has been identified.[4-6] Its name stems from transfection studies and stands for "rearranged during transfection." *RET* consists of 21 exons, with six exons called "hot spots." These hot spots are areas where germline mutations of *RET* frequently are detected in patients with MEN 2. Germline mutations of *RET* are responsible for the familial tumor syndrome, MEN 2, which is subdivided into three groups: familial medullary thyroid carcinoma; MEN 2A, including medullary thyroid carcinoma, pheochromocytoma, and parathyroid hyperplasia/adenoma; and MEN 2B, consisting of medullary thyroid carcinoma, pheochromocytoma, and mucosal and other neuromas, as well as certain body features such as a marfanoid body habitus. *RET* encodes a receptor tyrosine kinase, the ligands of which are glial cell line-derived neurotrophic factor (GDNF) and neurturin.[4] GDNF is a member of the transforming growth factor (TGF)-β family. *RET* activation by GDNF appears to occur via a membrane-bound protein, GFRα, which seems to function as the ligand-binding domain of the ligand-receptor complex.

Tumorigenesis in MEN 2-Related Pheochromocytoma

Although it has been known that patients with *RET* germline mutations develop hyperplasia of the parafollicular C-cells in the thyroid gland and of the adrenal medulla, the mechanisms of tumor formation in patients with MEN 2-related medullary thyroid carcinoma and pheochromocytoma are widely unknown. Whereas tumor suppressor genes such as *VHL* are believed to initiate tumorigenesis according to Knudson's two-hit model,[7] oncogenes such as *RET* may lead to tumor formation by other mechanisms. In a recent study, two possible mechanisms of tumorigenesis in MEN 2-related pheochromocytoma have been suggested.[2]

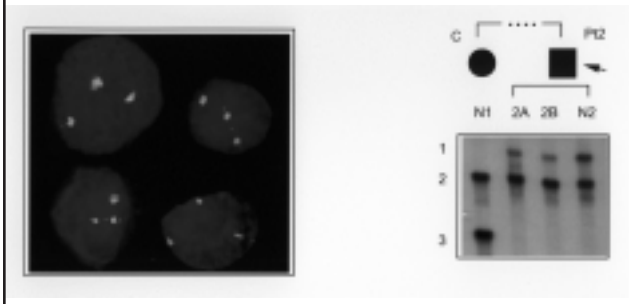
Hereditary tumor syndromes are ideal for studying the mechanisms of tumor formation. Huang and Koch investigated nine pheochromocytomas from five unrelated patients with MEN 2.[2] By performing microdissection of frozen tumor tissue, fluorescent in situ

hybridization, quantitative PCR, linkage analyses, phosphorimage densitometry, and mutation analyses by single-strand conformation polymorphism and restriction enzyme digestion, they found that these pheochromocytomas showed duplication of the mutant *RET* allele or loss of the wild-type allele *(see Figure)*. These results indicate a dominant effect of mutant *RET* and suggest this effect as a mechanism of tumor formation in MEN 2-related pheochromocytoma. This dominant effect of *RET* could represent the so-called "second hit" in tumor formation analogous to the two-hit model in tumor suppressor gene-associated tumors.[7] In ongoing studies, researchers found that patients with MEN 2 might have more than one *RET* mutation.[8-10] In this scope, however, Koch and Huang also discovered that somatic *RET* mutations in pheochromocytomas from patients with MEN 2 likely represent a phenomenon of tumor progression (unpublished data). The biological/functional significance of more than one *RET* mutation in patients with MEN 2 must be elucidated.

Analysis of RET mutations in sporadic pheochromocytomas has revealed somatic mutations in up to 15% of tumors, with the codon 918 somatic *RET* mutation (MEN 2B mutation) as the one most commonly found (in 3-10% of cases).[11-15] On the other hand, exon 10 and 11 somatic *RET* mutations (as seen in MEN 2A) occur in less than 1% of tumors. Based on this low number of somatic *RET* mutations in sporadic pheochromocytomas and our findings in MEN 2-related pheochromocytoma, we propose that other mechanisms of tumor formation, in addition to a somatic *RET* mutation, must play a role in the development of sporadic pheochromocytomas.

Figure

Duplication of mutant *RET* in trisomy 10 in MEN 2-related pheochromocytoma



On the left side, a pheochromocytoma tumor has been analyzed by FISH. Three signals (lighter in color) in the tumor cells are shown, indicating trisomy 10. On the right side, linkage analysis shows that allele number 2 is the inherited mutant *RET* allele. This mutant *RET* allele number 2 demonstrates strong intensity in lanes 2A and 2B (both pheochromocytoma) compared to lanes N1 (blood DNA from the patient's cousin "C") and lane N2 (blood DNA from the patient "Pt2"), indicating allelic imbalance between mutant and wild-type *RET*, which occurred in a ratio of 2:1 by phosphorimage densitometry.[7]

Conclusion

Pheochromocytomas are neuroendocrine tumors that occur most often in the adrenal glands. Duplication of mutant *RET* in trisomy 10 or loss of wild-type *RET* in MEN 2-related pheochromocytoma can lead to a dominant effect of mutant *RET*. This effect likely represents the "second hit" of tumor formation, the first hit being hyperplasia of the adrenal medullary cells caused by the effect of one mutant *RET* allele. Further studies are needed to elucidate mechanisms of tumor formation in pheochromocytoma. *(Drs. Koch and Pacak are Clinical Investigators, Pediatric Reproductive and Endocrinology Branch, National Institute of Child Health and Human Development, NIH; Dr. Vortmeyer is Staff Attending, Neuropathology, Dr. Huang is Research Fellow, and Dr. Zhuang is Head, Molecular Pathogenesis Unit, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, MD.)* ❖

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VE-Cadherin: A Novel, Potential Target for Anti-Angiogenic Therapy

By Fang Liao, PhD, Elisabetta Dejana, PhD, Peter Bohlen, PhD, and Daniel J. Hicklin, PhD

OVER THE PAST DECADE, THE CONCEPT OF ANGIOGENESIS has come of age since it was first described by Dr. Judah Folkman in the early 1970s.[1,2] The complex nature of the cellular and molecular events associated with angiogenesis provides an abundant source of molecular targets for therapeutic design.

It is now widely accepted that unnecessary angiogenesis contributes to the pathogenesis of a variety of diseases, including solid tumors. Blocking angiogenesis has been shown to be a promising way of treating cancer. A significant number of angiogenic inhibitors have been reported that show promise in animals or clinical trials.[3] Some of these inhibitors act via known mechanisms, e.g., endothelial cell growth/proliferation (inhibitors of vascular endothelial growth factor [VEGF] and its receptors), adhesion and migration/invasion (integrins and matrix metalloproteinase inhibitors), as well as the intracellular signaling pathways mediating cell growth and survival (kinase inhibitors).[3] However, many others act via undefined mechanisms, e.g., angiostatin and endostatin, and may or may not be specific to endothelium. Herein, we will describe a novel target/mechanism for blocking angiogenesis, i.e., blocking assembly of capillary tube structures by preventing vascular endothelial (VE)-cadherin-mediated adherens junction formation among endothelial cells.

Background

Endothelial cell-specific adhesion molecules play a crucial role in cell-cell and cell-extracellular matrix interaction and thus, have important vascular functions related to angiogenesis, such as mediation of cell migration and invasion. Certain members of the integrin family (e.g., $\alpha v\beta 3$ and $\alpha v\beta 5$) gained the attention of tumor biologists due to their restricted tissue distribution and differential regulation in tumor endothelium.[4,5] Another type of adhesive molecule, the cadherins, also are involved in facilitating tumor growth, metastasis, and angiogenesis. Cadherins, a rapidly growing family of molecules that mediate calcium-dependent homophilic adhesion between cells of the same type, are transmembrane glycoproteins that typically contain five ectodomains and a short, highly conserved cytoplasmic portion anchoring the cadherin to the cytoskeleton. The ectodomains mediate homotypic cell-cell interaction and clustering.[6] In addition to the classical roles that the cadherins play in "sorting" and maintaining the integrity of tissues,[7] a number of cadherins are implicated in tumor biology. For example, loss or mutation of E-cadherin has been associated with increased invasiveness and metastasis in certain human tumors;[8] switching of gene expression from E- to N-cadherin in melonocytes is a turning point in the development of malignant melanomas;[9] and differential levels of H/T cadherin are observed in tumors exhibiting various amounts of vascularization.[10] More recently, our studies showed that VE-cadherin (VE-cad) is involved in tumor angiogenesis.[11]

VE-cad is an endothelial-specific molecule and mediates adherens junction formation.[12] Accumulating evidence implicates VE-cad in various aspects of vascular biology related to angiogenesis, most notably, endothelial cell assembly into tubular structures.[13] *(See Figure 1.)* VE-cad null mouse embryos exhibit severely impaired assembly of vascular structures, which results in embryonic lethality at day E9.5,[14] implicating VE-cad as an important mediator in developmental angiogenesis. Its restricted distribution and unique biological function

distinguish VE-cad as a potential target for inhibition of endothelial cell-specific events, such as angiogenesis.

Monoclonal Antibody to VE-Cad Blocks Angiogenesis, Tumor Growth, and Metastasis

We tested one monoclonal antibody (BV13) directed against the extracellular region of murine VE-cad as a novel anti-angiogenic strategy designed to inhibit the assembly of capillary structures and, thus, tumor angiogenesis.[15] This antibody has been studied extensively in mouse models of angiogenesis, mouse tumors, and human tumor xenografts, and has demonstrating potent anti-angiogenic and antitumor activity.[11] Moreover, BV13 treatment inhibited the growth of metastases following removal of the primary tumor. Typically, treatment of human xenograft tumors with BV13 resulted in nearly complete inhibition of tumor growth and prevented tumor metastases in lungs. Histological examination of BV13-treated tumors showed evidence of decreased microvessel density, tumor cell apoptosis, decreased tumor cell proliferation, and extensive tumor necrosis. All these effects were observed as early as two weeks after treatment and gradually increased as antibody treatment continued. The decrease in tumor cell proliferation and necrosis in BV13-treated tumors likely reflects the lack of neovasculature needed to supply the rapidly growing tumor mass. Interestingly, antibody BV13-induced apoptosis was observed only in the tumors (mostly tumor cells and certain tumor endothelium), and not in normal endothelium.[16] This selective effect of the anti-VE-cad antibody on tumor cell apoptosis may be due to its ability to inhibit tumor angiogenesis. Taken together, these findings indicate that VE-cad plays a crucial role in post-natal angiogenesis, and thus, validates VE-cad as a target for anti-angiogenic therapy.

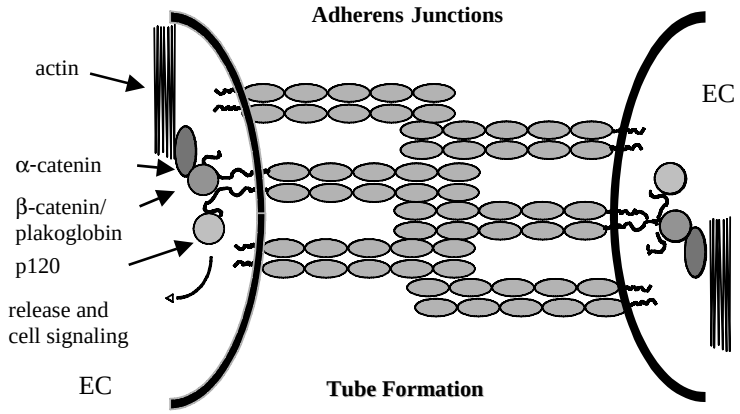
Antibody BV13 Increases Vascular Permeability

It should be noted that BV13 has potent in vivo antitumor activity at 10-fold lower doses (50 mcg/dose) than other anti-angiogenic antibodies, such as those that block the functions of VEGF, VEGF receptor (VEGFR), or $\alpha v\beta 3$.[3] However, higher doses of BV13 (> 75 mcg, i.p.) resulted in increased vascular permeability and edema in the lung and was followed by the death of some animals within 24-48 hours. Moreover, the BV13-induced permeability effect is long-lasting and irreversible as compared to other permeability agents such as thrombin and histamine. Histological examination of BV13-treated lungs showed a noticeable pathology: bleb formations of endothelial and alveolar epithelial cells, aggregates of degranulated platelets, and the formation of platelet microthrombi, as well as leukocyte activation and infiltration resulting from endothelial cell retraction.[15] These events were likely the consequence of vascular leakage caused by BV13 and further compound the vascular damage. Interestingly, BV13-induced vascular leakage could be alleviated by treating mice with an antibody (DC101)[3,16] against VEGFR-2/Flk-1 due to the anti-permeability effect of this antibody (Liao and associates, unpublished data). Furthermore, we demonstrated that antibody DC101 abolishes the VEGF-induced tyrosine phosphorylation on both VEGFR-2 and VE-cad. These findings link the two pathways mediated by VE-cad and VEGF/VEGFR-2, and provide direct evidence for the notion that increased vascular permeability is the primary cause of the pathologic effect of antibody BV13. It remains to be determined whether a combination of these two antibodies will be beneficial therapeutically.

The permeability effect of BV13 on normal tissues is not entirely unexpected, since VE-cad is expressed equally in tumor and normal vasculature and BV13 does not preferentially distribute to tumor blood vessels, but also binds to vessels in several tissues (Liao et al, unpublished data), including lung, kidney, and heart).[17] Therefore, anti-VE-cad antibodies may not only prevent the

Figure 1

Organization of endothelial adherens junctions



The ectodomains of VE-cadherin mediate the homophilic adhesion between endothelial cells. The cytoplasmic domain interacts directly or indirectly with the cytosolic proteins p120, α- and β-catenins, and plakoglobulin, and thus, associates with the cytoskeleton via α-catenin. This intracellular complex mediates a series of signaling events in the endothelial cells. The homophilic interaction among the extracellular domains of VE-cadherin is involved in the assembly of new capillary structures.

formation of adherens junctions in nascent vasculature (junction formation), but also may interfere with established adherens junctions and, thus, cause increased permeability of the affected vasculature (junction disruption). We hypothesize that antibody blockade of VE-cad molecules on tumor vasculature is more likely to result in a therapeutic effect due to the tumor vasculature's poor structural integrity (fenestration) and active angiogenesis. Indeed, we did not observe significant increased permeability in the lung or other tissues at the therapeutically efficacious dose of 50 mcg, nor did we observe other overt signs of toxicity during the course of treatment in a number of animals studies.

Is It Possible to Identify Antibodies That Selectively Inhibit Tumor Angiogenesis Without Affecting Existing Vasculature?

Our results indicate that antibody BV13 would not be an appropriate agent for therapeutic use due to its narrow therapeutic window. Thus, we aimed to identify a more desirable VE-cad inhibitor that preferentially affects ongoing angiogenesis. As for other cadherins, VE-cad clustering involves multiple adhesive contacts between different ectodomains. [18,19] Therefore, it may be possible to target regions of the VE-cad accessible only in growing vessels where junctions are not fully organized ("angiogenic epitopes"), but not exposed in existing vessels with mature junctions *(see Figure 2)*. Indeed, in preliminary work, we have identified unique VE-cad antibodies that appear to inhibit adherens junction formation but do not disrupt existing junctions.[19] Detailed analyses of these antibodies in animal models, and of their corresponding epitopes, may lead to identification of reagents with a more selective anti-angiogenic potential.

Conclusion

Antibodies represent a unique class of therapeutics due to their high specificity toward defined antigens. The recent success of commercial, antibody-based cancer therapeutics has greatly revitalized enthusiasm in this field. With the advance and maturation of antibody engineering technologies, fully humanized antibodies with desired specificity can be readily obtained for cancer therapeutics. The use of a therapeutic antibody against VE-cad will be particularly appealing because of the unique localization of these target molecules on endothelium. It seems likely that an antibody to VE-cad with desired activity may be generally applied to all angiogenic settings, regardless of the angiogenic stimuli or tumor type. *(Dr. Liao is Senior Scientist, Dr. Hicklin is Associate Vice President, and Dr. Bohlen is Senior Vice President, ImClone Systems, Inc., New York, NY; Dr. Dejana is a Consultant and Professor of Vascular Biology, Institute of Pharmacological Research, Milan, Italy.)* ❖

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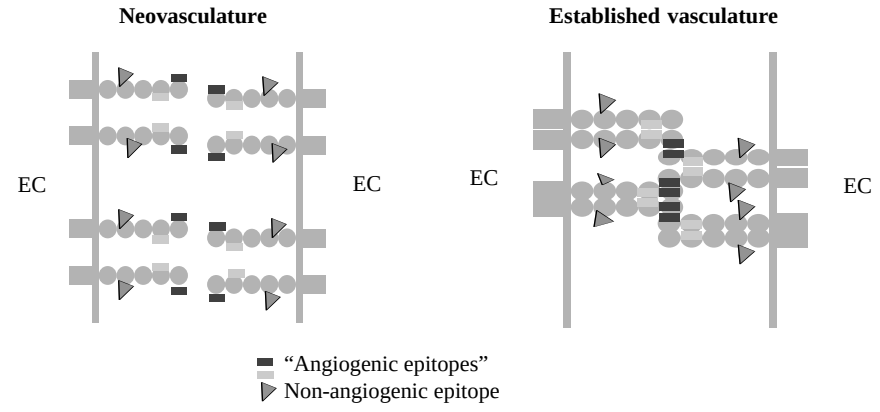
Figure 2

Hypothesis for the angiogenic epitopes



The VE-cad-mediated adhesion is a complex, dynamic process, which likely involves multiple adhesive contacts along the extracellular region. It is possible to target regions that are involved in certain stages during the formation of adherens junctions (the angiogenic epitopes in neovasculature), but are no longer accessible/affected once the junctions are formed.

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Funding News

Non-Mammalian Organisms as Models for Anticancer Drug Discovery

The National Cancer Institute is seeking to fund projects that identify key genes and gene products that are altered in human cancer and could potentially be exploited as intervention points or targets for the discovery of new cancer prevention or treatment drugs. Funding is available through the R21 and R01 mechanisms. The R21 grants will be made in amounts of up to $100,000 in direct costs for four budget modules per year for two years. Researchers at foreign and domestic for profit and non-profit organizations are eligible. The deadline for letter of intent is June 4, 2001, with an application deadline of July 12, 2001, for the current cycle, and letter of intent deadline of Feb. 5, 2002, with application deadline of March 13, 2002, for the next cycle. More information is available at: http://www.nci.nih.gov/initiatives/non-mam.html. ❖

CME Questions

14. Angiostatin:
a. promotes tumor growth in a murine model.
b. shortens the length of tumor dormancy in a murine model.
c. is a plasminogen cleavage product.
d. is a fibronectin cleavage product.

15. Odorant receptors:
a. belong to a superfamily of G-protein coupled receptors.
b. are transmembrane proteins.
c. mediate cellular responses to diverse extracellular stimuli, including light, neurotransmitters, hormones, and odorants.
d. All of the above

16. Germline mutations of the gene *MET* are associated with:
a. angiomyolipomas.
b. hereditary papillary renal carcinomas.
c. sarcomatoid renal cell carcinomas.
d. oncocytomas.

17. Cadherins:
a. are phospholipids.
b. typically contain 10 ectodomains.
c. have a short, highly conserved cytoplasmic portion anchoring the cadherin to the cytoskeleton.
d. are nuclear proteins.

In Future Issues: The Functional Significance of the Polo-Like Kinase Family