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Improved Therapeutic Effectiveness of Photodynamic Therapy Combined with Antiangiogenic Therapy in a Mouse Tumor Model

By Angela Ferrario, PhD and Charles J. Gomer, PhD

PHOTODYNAMIC THERAPY (PDT) IS AN EFFECTIVE CLINICAL CANCER TREATMENT THAT UTILIZES tumor-localizing photosensitizers activated by tissue-penetrating laser light to mediate cytotoxic events that lead to the eradication of solid malignancies.[1-3] The porphyrin photosensitizer, Photofrin porfimer sodium (PH), recently received Food and Drug Administration approval for PDT treatment of esophageal and endobronchial carcinomas.[1] PDT applications continue to be encouraging for bladder, head and neck, brain, intrathoracic, and skin malignancies, as well as for non-oncological disorders such as age-related macular degeneration.[1,4] Nevertheless, recurrences are observed after PDT, and methods to improve the therapeutic efficacy of this procedure are needed.

Introduction

PDT elicits direct tumor cell damage via the photochemical generation of cytotoxic singlet oxygen as well as microvascular injury within exposed tumors. Vascular effects induced by PH-mediated PDT include perfusion changes, vessel constriction, macromolecular vessel leakage, leukocyte adhesion, and thrombus formation.[1,5] Reduction in vascular perfusion occurring during and following PDT leads to a significant blood flow disruption, which can lead to tumor tissue hypoxia.[6,7]

Tissue hypoxia can serve as a catalyst for an inducible response associated with gene activation.[8] An initial step in hypoxia-mediated gene activation is the formation of the hypoxia-inducible transcription factor-1 (HIF-1) transcription factor complex.[8,9] HIF-1 is a heterodimeric complex of two helix-loop-helix proteins: HIF-1β, which is expressed constitutively; and HIF-1α, which is degraded rapidly by the ubiquitin-proteasome system under normoxic condition.[8-11] Hypoxia induces the stabilization of the HIF-1α subunit, allowing the

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formation of the transcriptionally active protein complex.[10,12] Several HIF-1-responsive genes have been identified, including vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter-1.[10]

VEGF is an endothelial cell-specific mitogen involved in the induction and maintenance of the neovasculature in solid tumors.[10,12] VEGF expression in areas of tumor necrosis originally led to the suggestion that hypoxia is a major regulator of tumor angiogenesis.[12,13] VEGF expression in hypoxic tumor tissue increases as a result of both transcriptional activation and increased stabilization.[10,13] Exposure of rat endothelial cells to hydrogen peroxide-mediated oxidative stress and exposure of various mouse and human tumor cells to ionizing radiation also have been shown to up-regulate VEGF expression.[14,15]

In the current study using a murine BA mammary carcinoma, we examined whether PDT-mediated cytotoxicity could serve as an activator of molecular events leading to increased VEGF expression within treated tumor tissue.[16] Interestingly, a growing number of reports have indicated that antiangiogenic agents can enhance the tumoricidal effectiveness of chemotherapy and radiation treatments.[14,17,18] Therefore, we also examined whether antiangiogenic compounds that counter the actions of VEGF would improve PDT responsiveness.[16]

Results

Since hypoxia induces expression and stabilization of the HIF-1α subunit and activates the HIF-1 transcription complex, we initially investigated whether PDT-induced microvascular damage and resulting tumor tissue hypoxia also would stabilize HIF-1α and initiate HIF-1-mediated transcription. BA mammary carcinoma tumors growing in C3H mice were collected immediately after PDT treatment and evaluated for HIF-1α expression by Western immunoblot analysis. HIF-1α was not detectable in control tumors; however, both PDT (5 mg/kg PH; 200 J/cm^2) and 45 minutes of tumor clamping (used as a positive control for hypoxia) resulted in induced expression of HIF-1α. The response was rapid, being observed within the first five minutes following treatments.

At 24 hours following treatment, both PDT and tumor clamping induced significant increases in VEGF expression within exposed lesions compared to control levels as documented by Western and ELISA analysis.

In vitro PDT of BA mammary carcinoma cells growing in culture dishes was not as effective as in vivo tumor treatments in inducing VEGF expression. The in vitro PDT conditions used in our study would be expected to involve singlet oxygen-mediated oxidative stress but not induced hypoxia. A 210 J/m^2 PDT dose resulted only in a small increase in VEGF levels when measured 24 hours after treatment. These results suggest that the increased VEGF expression observed in tumors after in vivo PDT may be associated with treatment-induced hypoxia and to a lesser extent with treatment-induced oxidative stress.

Next we examined whether antiangiogenic treatments, using IM862 or endothelial monocyte-activating polypeptide-II (EMAP-II), could enhance the tumoricidal action of PDT. IM862, obtained from Cytran Inc. (Kirkland,WA), is a synthetic dipeptide of L-glutamyl-L-tryptophan that initially was isolated from the thymus.[18] Preclinical studies have shown that the dipeptide inhibits angiogenesis in chorioallantoic membrane assays and VEGF production in monocytic lineage cells. IM862 also inhibits tumor growth in xenograft models not by direct cytotoxic effect on tumor cells, but by inhibiting VEGF production and activating natural killer cells. Intranasal administration of IM862 exhibits antitumor activity in patients with AIDS-associated Kaposi's sarcoma.[19] EMAP-II is a single chain polypeptide that inhibits tumor growth and has antiangiogenic properties.[20] EMAP-II induces apoptosis in growing capillary endothelial cells in both a time- and dose-

Cancer Research Alert, ISSN 1525-3333, is published monthly by American Health Consultants, 3525 Piedmont Rd., NE, Bldg. 6, Suite 400, Atlanta, GA 30305.

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Editorial Group Head: Valerie Loner.
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Marketing Manager: Schandale Kornegay.

GST Registration Number: R128870672.

Periodical postage paid at Atlanta GA 30304.
POSTMASTER: Send address changes to *Cancer Research Alert,* P.O. Box 740059, Atlanta, GA 30374.

Back issues: $48. One to nine additional copies, $230 each; 10 to 20 additional copies, $172 each.

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dependent manner. EMAP-II also prevents vessel ingrowth in experimental angiogenesis models and in primary tumors.

Both compounds appear to have minimal systemic toxicity. Cytotoxic effects have not been observed following administration of IM862 to patients or in experimental animals exposed to EMAP-II. BA mammary carcinomas growing in C3H mice were treated with PDT only, antiangiogenic therapy alone, or with PDT combined with an antiangiogenic agent. A PDT dose (5 mg/kg PH; 200 J/cm^2) that produced a moderate (39%) cure rate by itself was used to measure changes in tumor response when the PDT treatment was combined with 10 daily injections of IM862 (25 mg/kg) or EMAP-II (50 mcg/kg).[21] Antiangiogenic treatments significantly enhanced the tumoricidal action of PDT as measured by increased tumor cures rate from 39%, for PDT alone, to 78% and 89% when PDT was combined with IM862 or EMAP-II, respectively. Interestingly, these antiangiogenic compounds were observed to decrease PDT-induced VEGF levels in tumor samples indicating that potentiated PDT responsiveness may involve attenuating the angiogenic actions of VEGF.

Significance

The results of this study suggest that an adjunctive antiangiogenic approach for improving PDT responsiveness may have, because of minimal systemic toxicity, a positive clinical impact.[16] Optimization of antiangiogenic parameters and an examination of various methods to block angiogenesis currently are being performed. Combination procedures using antiangiogenic therapy may provide an efficient strategy for selectively enhancing PDT tumor responsiveness and possibly may improve PDT procedures for pathologies marked by neovascularization, including age-related macular degeneration.[4] *(Dr. Ferrario is a Research Specialist, Clayton Center for Ocular Oncology, Childrens Hospital Los Angeles; and Dr. Gomer is a Professor, Childrens Hospital Los Angeles, and Department of Pediatrics and Radiation Oncology, Keck School of Medicine, University of Southern California in Los Angeles.)* ❖

Acknowledgments

This investigation was performed in conjunction with the Clayton Foundation for Research and was supported in part by USPHS Grants CA-31230; United States Army Medical Research Grant BC981102 from the Department of Defense; Neil Bogart Memorial Fund of the T.J. Martell Foundation for Leukemia, Cancer and AIDS Research; and Las Madrinas Endowment for Experimental Therapeutics in Ophthalmology.

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Hypoxia-Inducible Factor in Prostate Cancer Progression

By Mikhail V. Blagosklonny, PhD and Konstantin Salnikow, PhD

PROSTATE CANCER IS THE SECOND LEADING CAUSE OF all cancer deaths in men in the United States. Between 1973 and 1992, the incidence rate in the United States increased more than 250%.[1] These numbers demonstrate the need for continued exploration of the molecular mechanisms of prostate carcinogenesis in order to estimate prognosis and develop new targets for treatment and prevention.

Role of Hypoxia in Prostate Tumor Progression

Fatal outcome in prostate cancer is linked to the development of hormone independency, and to the development of prostate cancer metastasis to bone and other tissues. Tumor progression toward aggressive and metastatic potential is a fundamental process in neoplasia, but stimuli that drive this progression are poorly understood. Although hypoxia limits tumor growth, and tumors with poor vascularization fail to grow and form metastases, hypoxia eventually selects a more aggressive, metastatic cancer phenotype that is associated with poor prognosis. We proposed that hypoxia is indeed a driving force for selection of aggressive, autonomous, and metastatic cancer cells.[2] Hypoxia already exists in primary prostate carcinomas, and highly metastatic human prostate cancers growing within the prostate of athymic mice overexpress vascular endothelial growth factor (VEGF).[3] Although hypoxia limits tumor growth, it is inevitably associated with tumor progression. We envision the ability of prostate cancer cells to survive hypoxia as a natural test, allowing further tumor progression.

Hypoxia, Cancer, and HIF-1

Hypoxia is an important pathological condition that accompanies tumor growth. Hypoxia develops early in tumors because of inadequate vascularization of the tumor. In response to hypoxia, cells increase synthesis of enzymes that metabolize glucose. Since hypoxia often is a result of inadequate angiogenesis, cells secrete VEGF that in turn stimulates endothelial cell proliferation and forms new blood vessels. Angiogenesis (formation of new blood vessels) is absolutely required for tumor growth. Numerous genes are important for cell survival under hypoxic conditions, including glucose transporters, glycolytic enzymes, VEGF, and nitric oxide (NO). Cells adapt to hypoxia by induction of hypoxia-inducible factor 1α (HIF-1α). HIF-1 is a transcription factor that stimulates expression of VEGF, glucose transporters, glycolytic enzymes, transferrin, NO, and other hypoxia-inducible genes.[4] In kidney, HIF-1 also transactivates the gene that increases whole body oxygen supply, namely erythropoietin, a growth and differentiation factor for red blood cells. The HIF-1 transcription factor activity is regulated by the stability of the HIF-1α protein, which is the limiting, hypoxia-inducible subunit of the HIF-1 transactivator. Under normal oxygen conditions, the HIF-1α protein is rapidly degraded, whereas under hypoxic conditions this degradation is prevented. HIF-1α accumulates, binds the HIF-1β subunit, and transactivates target genes.[4]

Cells deficient in either subunit of HIF-1 have impaired ability to form tumors.[5] Lack of HIF-1 retards solid tumor growth and vascularity because of the reduced capacity to produce VEGF during hypoxia. The HIF-1-dependent production of VEGF resolves hypoxia due to new blood vessel formation, although VEGF does not provide immediate protection against hypoxia. A switch from cellular metabolism to glycolysis may pro-

vide immediate protection to cells from hypoxia, and most glycolytic enzymes are HIF-1 inducible.

Hypoxia-Mimicking Metals Induce Malignant Transformation

Additional evidence that hypoxia and HIF-1 may play a role in tumor progression came from studies on nickel-induced carcinogenesis. Nickel is a potent non-mutagenic carcinogen. In vitro nickel compounds displayed incredible transforming capability in human and rodent cell systems.[6] In addition to high transforming potential, nickel compounds display tumor-promoting properties when added to polycyclic hydrocarbons.

Hypoxia and carcinogenic nickel exert almost identical effects on gene expression; furthermore, nickel induces gene expression, in part through the HIF-1 transcription factor.[7] Levels of HIF-1α, HIF-1-driven transcription, and ratio of HIF-driven transcription to p53-driven transcription are increased in the nickel-transformed cells.[8]

HIF Is Overexpressed in Highly Aggressive Prostate Cancer

Metastatic human prostate cancer cells exhibited enhanced VEGF production and tumor vascularity compared with prostate cancer cells of lower metastatic potential. HIF-1α protein was detected in PC-3 prostate cancer cells under normoxic conditions.[9,10] This prostate cancer cell line has lost dependence to testosterone and developed the ability to form metastases in nude mice. Metastasis-derived PC-3 cells were obtained and these cells display much higher metastatic potential than PC-3 cells. We observed that under hypoxic conditions, PC3-M cells produced lactate, indicating high levels of glycolysis. This may reflect high levels of HIF-1α and HIF-1-dependent transcription in PC-3-M cells. We found that levels of HIF-1 and its inducibility were higher in PC-3-M cells than in PC-3 cells.[2] In a panel of prostate cell lines ranging from normal prostate epithelial cells to the most aggressive PC-3-M cells, HIF-1-dependent transcription correlated with tumor progression.[2] The comparison of PC-3-M cells with normal prostate epithelial cells and LNCaP cells (lowly aggressive, testosterone-dependent, and non-metastatic prostate cancer cells) showed higher inducibility of such HIF-1-dependent genes as VEGF and Cap43, and higher levels of hypoxia-dependent transcription. Our data suggest that an increased inducibility of HIF-1-dependent genes may be a hallmark of hypoxia-driven selection.

NDRG-1/Cap43 in Prostate Cancer

NDRG-1/Cap43 is a new human gene recently cloned in a few laboratories, including ours, based on its high inducibility by nickel compounds.[11] The gene had different names, including RTP, DRG, and NDR; the latest nomenclature uses the name NDRG-1. The gene is highly induced by hypoxia and may be involved in cellular survival under hypoxic conditions. NDRG-1 was mapped on human chromosome 8q24. Although, the direct involvement of this gene in prostate cancer development was not shown, it is interesting to note that numerous studies reported 8q gain in advanced prostatic cancers. This gene is expressed at low levels in different tissues; however, because it is regulated by androgens, this gene is expressed at relatively high levels in normal prostate tissue.[12] In LNCaP cells that have androgen receptors, NDRG-1 is up-regulated markedly by testosterone and to a much lesser extent by hypoxia.[2,12] Other prostate cancer cells (e.g., PC-3, PC-3M, and DU145) have lost their androgen receptors and consequently are no longer regulated by testosterone. In these cells, hypoxic up-regulation of Cap43 was much stronger than in LNCaP or normal prostate epithelial cells.[2]

Is HIF-1 an Oncoprotein?

A tumor suppressor is a gene whose loss or inactivation increases cancer incidence. In von Hippel-Linday (VHL) syndrome, a familial cancer caused by germline mutations of the VHL tumor suppressor gene, inactivation of the pVHL protein was found in highly vascular tumors that overproduce angiogenic factors such as VEGF.[13] VHL-associated tumor cells express high levels of mRNA for all hypoxia-inducible genes in both normoxic and hypoxic conditions.[13]

VHL functions have recently been elucidated: VHL targets HIF-1α for degradation under normal oxygen conditions.[14] In the absence of VHL, HIF-1α is not degraded and, therefore, it is overexpressed in a cell. Such cells live under normal oxygen conditions as they do in deep hypoxia. This explains why all HIF-1-dependent genes are up-regulated when VHL is mutated or lost. Since the loss of the VHL tumor suppressor results in HIF-1 overexpression and that leads to cancerous phenotype, HIF-1 may be defined as an oncoprotein.

HIF, p53, and p21

Hypoxia represents severe stress that inhibits cell proliferation and induces apoptosis.[15] For example, rodent fibroblasts cease proliferation and eventually die following hypoxia.[16] We have shown that human metastatic prostate cancer cells neither arrest growth nor die under hypoxic conditions.[2] Such high tolerance of hypoxia in advanced prostate cancers is characterized by high basal and hypoxia-induced levels of HIF-1-dependent transcription, loss of p53 function, and inability of HIF-1

and p21WAF1/CIP1 to induce growth arrest. Hypoxia induces the p53 tumor suppressor, which in turn leads to growth arrest or cell death.[15,17] Growth arrest is largely mediated by the induction of several proteins, including p21, an inhibitor of cyclin-dependent kinases.

p53 mutations in primary prostate cancer are relatively infrequent. In contrast, they occur at high rates in metastatic disease, suggesting that prostate cancer progression involves p53 inactivation.[18] Hypoxia may select for the loss of p53, thus facilitating selection of a more malignant phenotype. Therefore, highly malignant cells can tolerate hypoxia and are characterized by a loss of p53 function and an increase in HIF-1 function. Literally, HIF-1 substitutes for p53 as a stress regulator in highly metastatic cells. Tumor-associated nitric oxide production that is under the control of HIF-1 may promote cancer progression by providing a selective growth advantage to tumor cells with mutant p53.

In contrast to apoptosis, hypoxia-induced growth arrest is p53-independent. We showed that under hypoxic conditions, growth arrest may be mediated directly by HIF-1, which induces p21. It is noteworthy that HIF-1-null fibroblasts grow faster than normal fibroblasts, indicating that HIF-1 may inhibit proliferation. Additionally, it has been shown that hypoxia failed to induce p21 in cells lacking HIF-1, but induced p21 in normal cells,[19]making it plausible to link HIF-1 with p21 induction in normal cells. It has been proposed that p21 is regulated by an alternative signaling system in prostate tumors with p53 inactivation.[20] We demonstrated that HIF-1 transactivates the p21 promoter in cells that lack wild type p53. Furthermore, the p21 promoter contains the ACGTG sequence, which has been implicated in the regulation of lactate dehydrogenase A by hypoxia. Hypoxia slightly up-regulated the p21 mRNA in DU145 and PC-3-M prostate cancer cells. However, neither HIF-1α nor hypoxia induced growth arrest in these prostate cancer cells. Thus, induction of p21 is dissociated from growth arrest in the advanced prostate cancer cells. This situation results in uncontrolled growth without apoptosis that is associated with the most malignant phenotype.[21] In summary, increased HIF-1α expression, loss or mutation of p53, and resistance to p21-dependent growth arrest contribute to the aggressive metastatic phenotype in prostate cancer. *(Dr. Blagosklonny is an Investigator/Contractor, Medicine Branch, Division of Clinical Sciences, National Cancer Institute, National Institute of Health, Bethesda, MD; Dr. Salnikow is a Research Assistant Professor of Environmental Medicine, Nelson Institute of Environmental Medicine, and Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, NY.)* ❖

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Polyunsaturated Fatty Acids and Skin Cancer: Part I

By Anthony P. Albino, PhD
and Leonard A. Cohen, PhD

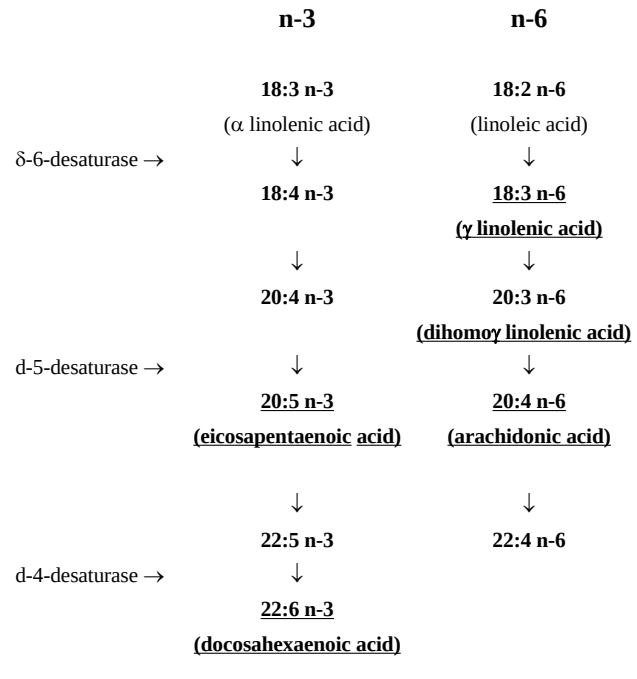
IN MANY CASES, THE LONG-TERM PROGNOSIS FOR INDIviduals with advanced cancers is dismal because of the lack of effective treatment options. New approaches are needed to control and suppress tumor progression. The stimulus for studies on the anticancer role of n-3 polyunsaturated fatty acids (PUFAs) comes from epidemiological studies conducted in the early 1980s of Greenland Eskimos and Japanese farmers, that confirm an association between low cancer incidence rates and a high intake of n-3 PUFAs.[1,2] These initial epidemiological observations, which suggested that dietary n-3 PUFAs play important roles in inhibiting the evolution and/or progression of a broad range of human cancers, simply tallied the total fish oil intake of individuals.[3] Subsequent studies using controlled model systems provided tantalizing evidence that n-3 PUFAs can moderate or reverse a diverse set of molecular mechanisms that are deranged during tumor progression of many cancer types and can increase the efficacy of various therapeutic modalities. Using dietary intervention to modify both the relative consumption of essential PUFAs and the n-3:n-6 ratio, PUFAs may be a potentially potent chemopreventive and adjuvant antitumor modality.[4]

Background

Among the four families of PUFAs (n-9, n-7, n-6, n-3; so termed because of the position of the first double bond from the methyl end of the molecules), the n-6 and n-3 (also called omega-6 and omega-3) are essential fatty acids that cannot be synthesized by humans and therefore, must be obtained from the diet. In Western diets the 18-carbon linoleic acid (c18:2, n-6) is the most abundant n-6 PUFA. Vegetable oils (e.g., sunflower, safflower, and corn oil) are particularly rich sources of n-6 PUFAs. In contrast, the most common n-3 PUFAs in the human diet are α-linolenic acid (αLA; c18:3, n-3; found in canola and soybean oils, some nuts, and flaxseed), and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (both found in high levels in cold water fish such as mackerel, menhaden, salmon, and tuna). In addition, αLA also can be desaturated and elongated to form EPA (20:5, n-3) and DHA (22:6, n-3) in the body (*see Figure 1*).

The rationale for using n-3 PUFAs as antitumor agents is based on epidemiological and experimental data that support both a suppressive role of dietary lipids in all phases of tumor progression, and a therapeutic role when used in combination with conventional treatment modalities. Epidemiological studies show that the stage-for-stage survival of patients with one of several different common cancers, including those of the skin, is consistently greater in countries with low dietary fat intake but high intake of n-3 PUFAs (e.g., Japan) vs. Western

Figure 1
Synthesis of Essential Fatty Acids



countries in which there is high fat intake but low n-3 PUFA intake.[2,5] A wide range of studies also support the conclusion that diets high in n-6 PUFAs play a key role in the growth and/or progression of many common human cancers, including breast, prostate, and skin (i.e., non-melanoma skin cancers), whereas diets high in n-3 PUFAs attenuate various aspects of these cancers.[6,7] Animal experiments clearly demonstrate the enhancing effects of diets high in n-6 PUFAs on ultraviolet radiation (UVR)-induced skin neoplasms and the inhibitory effects of diets high in n-3 PUFA.[8]

Both in vivo and in vitro experiments with a range of human cancers have demonstrated that specific n-3 PUFAs can increase the efficacy of chemotherapeutic treatment and radiation therapy.[9,10] In addition to non-melanoma skin cancers, a growing body of data indicate that dietary fat influences the development and progression of cutaneous malignant melanoma, with the n-6 PUFAs exerting stimulatory effects and n-3 PUFAs having inhibitory activity.[11-13] For example, a diet supplemented with DHA, or the closely related EPA, inhibits the cyclooxygenase- and lipoxygenase-catalyzed formation of downstream eicosanoids (*see Figures 1 and 2*) and can suppress the growth, invasiveness, and metastatic potential of melanoma cells in a dose-dependent manner.[14,15] Thus, n-3 PUFAs have the potential to attenuate several of the most relevant and aberrant characteristics of tumor cells (i.e., deregulated cell-cycle control, development of invasive and metastatic potential, and inhibition of apoptotic circuits).

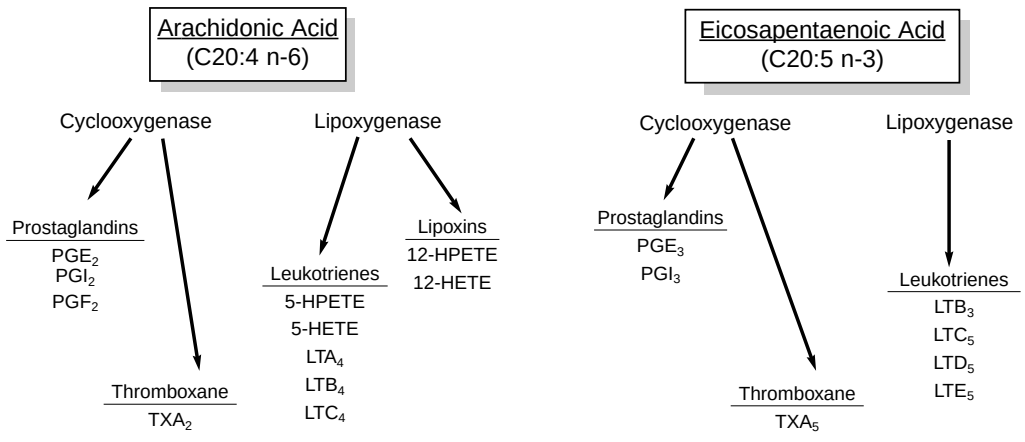
Role of Nutrition in Non-Melanoma Skin Cancers

A direct relationship between UVR-induced cancers of the skin and diet has long been recognized. In 1939, Baumann and Rusch first observed that rodents fed high levels of dietary fat formed UVR-induced tumors more rapidly than animals receiving less fat.[16] More recently, it has been shown that high dietary fat influences both the initiation and promotion stages of UVR-induced tumors. Using UV-irradiated hairless mice, Black and colleagues observed a convincing linear relationship between increased lipid level intake and the numbers of UVR-induced skin tumors per mouse.[17] Increased lipid intake also resulted in a decrease in the tumor latency in these mice. Moreover, after UVR-initiation, switching to a low-fat diet abrogated the exacerbating effects seen with high dietary fat intake.[8]

The observation that dietary fatty acids can directly influence the development and progression of UVR-induced skin cancers provided a strong rationale for a clinical intervention trial to determine the potential of dietary fat modification as a prevention strategy for both skin cancers and clinically identifiable precursor lesions.[6,8] Several clinical trials have confirmed that a reduction in fat intake plays an important role in reducing the incidence of premalignant and malignant skin lesions. For example, in a two-year clinical intervention trial, 76 patients were randomized to either a control group (approximately 38% of calories from fat) or to a low-fat dietary intervention group (20% calories from fat).[18] The patients on the low-fat diet had a 66% reduction in actinic keratosis, a premalignant lesion with a high conversion rate to squamous cell carcinoma. In a second trial, 101 patients with either squamous or basal cell carcinoma experienced a large decrease in occurrence of new skin cancers in the low-fat intervention group (20% of calories from fat) as compared to the control group which remained on a high-fat diet (35-40% of calories from fat).[6] Thus, these clinical studies support the conclusion that a reduction in premalignant and

Figure 2

Eicosanoid Pathways



malignant skin lesions can be achieved by reducing dietary fat, and that dietary manipulation can play an important role in the management and prevention of the most common forms of skin cancers (i.e., basal and squamous cell carcinomas).

Role of Nutrition in Cutaneous Malignant Melanoma

Similar to non-melanoma skin cancers, there is a growing body of data indicating that nutrition, dietary fat, and specific PUFAs influence the development, progression, and treatment of cutaneous malignant melanoma. In 1987, Mackie et al analyzed 100 melanoma patients and matched controls for constituent fatty acids in samples of subcutaneous adipose tissue.[12] Compared to the controls, melanoma patients had substantially increased percentages of n-6 PUFAs. The supposition of this study was that increased consumption of dietary n-6 PUFAs had a contributory effect in the etiology of melanoma. A recent epidemiology study of more than 50,000 men and women with cutaneous malignant melanoma indicated that n-6 PUFA intake was associated with a significantly increased risk of melanoma in women.[11] A case-control study showed that obesity was significantly related to melanoma risk, indicating that a high proportion of calories from fat may facilitate melanoma development.[19] A population-based control study suggested that high intake of fish oils and n-3 fatty acids reduces the risk of melanoma.[13] A study comparing five-year survival rates of patients with stage I, II, III, or IV melanoma who consumed a low-fat diet (in addition to other nutrients) to those on a standard diet found a considerable stage-for-stage survival increase for melanoma patients on a low-fat diet.[20]

Conclusion

These studies, though not dismissing excessive UVR exposure as the major risk factor, suggest a complicated and, as yet unclear, contributory role for dietary fat in the evolution and progression of melanoma. Moreover, these studies also suggest that dietary fat can play a significant role in the clinical management of melanoma. To date, however, no studies similar to those examining the role of dietary fat and specific PUFAs in non-melanoma skin cancers have been performed in individuals with melanoma or at high risk for developing melanoma by virtue of having increased numbers of normal or atypical nevi. *(Dr. Albino is the Director of Research and Dr. Cohen is the Section Head, Nutrition and Endocrinology, The American Health Institute, Valhalla, NY.)* ❖

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The Tumor Cell Surface-Associated Urokinase-Type Plasminogen Activator as a Maspin Target

By Shijie Sheng, PhD

In a search for tumor suppressor genes, the mammary serine protease inhibitor (maspin) gene was identified by subtractive hybridization on the basis of its expression at the mRNA level in normal, but not tumor-derived, breast epithelial cells.[1]

Maspin protein has an overall sequence homology with members of the serine protease inhibitor (serpin) superfamily.[1] Subsequent studies show an inverse correlation between maspin expression and breast cancer progression and oral squamous cell carcinoma, as well as head and neck cancer.[1-5] In addition, maspin expression is down-regulated in prostate carcinoma cells compared with that in immortalized normal prostate epithelial cells.[6] Thus, the down-regulation of maspin expression appears to be an important attribute to tumor progression toward more aggressive phenotypes. Biological evidence further suggests the potential therapeutic application of maspin in treating human malignancies. Maspin specifically inhibits the invasion and motility of an array of breast and prostate carcinoma cells in vitro, and inhibits the growth and metastasis of human breast and prostate cancer cells in xenograft mouse models.[1,6-9]

The Mechanism of Maspin

To develop maspin-based, therapeutic anticancer strategies, it is crucial to understand the molecular mechanism of maspin. Previous in vitro studies using both endogenously expressed maspin or purified recombinant maspin protein have demonstrated that maspin's inhibitory effect on tumor invasion and metastasis is, at least in part, due to its localized action at the interphase between cell membrane and the extracellular matrix (ECM).[6,10,11] As reported earlier by Seftor and associates, one of the downstream events following maspin treatment of breast carcinoma cells MDA-MB-435 was an increased cell surface expression of $\alpha_3\beta_1$ integrin, which in turn led to increased cell adhesion to fibronectin.[11] However, to date, the direct molecular target of maspin remains elusive. Central to this issue, maspin does not act as a classical serpin in cell-free solutions; i.e., it does not inhibit a series of serine proteases including tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).[12]

In 1998, Sheng and colleagues reported that maspin specifically inhibits tPA associated with fibrinogen or polylysine.[12] Based on the complex interaction between maspin and the fibrinogen-associated tPA, it was hypothesized that maspin may specifically target plasminogen activators that are bound to a biological surface such as the plasma membrane. It is well documented that uPA, but not tPA, binds specifically to its cell surface-anchored receptor, uPAR.[13,14] Furthermore, uPA along with uPAR is causatively involved in the invasive and metastatic phenotypes, and the poor prognoses of many types of carcinomas.[15,16] Thus, it is important to determine whether a potential inhibitory interaction between maspin and the tumor cell-associated uPA underlies the molecular mechanism of maspin.

Maspin and Tumor Cell Surface-Associated uPA

A recent report by McGowen coworkers provided the first evidence that the tumor cell surface-associated uPA is a maspin target.[17] To address the specific interaction between maspin and the cell surface-associated uPA, the authors took advantage of the established prostatic carcinoma cell line DU145 that produces uPA as the predominant plasminogen activator, but does not express detectable levels of endogenous maspin. In addition, the authors developed a coupled enzymatic assay to detect the plasminogen activation mediated by viable DU145 cells in monolayer culture. It was shown that recombinant human maspin protein specifically inhibited DU145 cell-mediated uPA activity. The inhibitory effect of maspin was similar to that of a specific uPA-neutralizing antibody, and was reversed by the polyclonal antibody made against the reactive site loop (RSL) sequence of maspin. Based on a current paradigm, an inhibitory serpin uses its RSL to dock into the catalytic site of the target enzyme. This initial docking induces a massive β-sheet rearrangement of the serpin molecule, leading to a

stabilized enzyme/inhibitor complex.[18,19] The essential role of the intact RSL of maspin in its proteolytic inhibitory activity toward cell-associated uPA suggests that maspin may act as an inhibitory serpin.

Interestingly, as compared to several serpin inhibitors of plasminogen activators, such as PAI-1 and PAI-2, maspin exhibited a novel cell surface-dependent interaction with uPA. First, under a non-permeabilizing condition, maspin bound to the surface of DU145 cells in a saturable manner, suggesting that it may interact specifically with a cell surface-associated molecule. Second, maspin inhibited only the cell surface-associated uPA, not the secreted uPA or purified uPA in cell-free biochemical reactions. Third, maspin formed a stable complex only with the uPA in the cell lysate fraction of DU145 cells, not with the purified uPA or the uPA secreted by DU145 cells into the conditioned culture medium. These data demonstrated an important role for the epithelial cell surface in mediating the inhibitory interaction between maspin and uPA. The authors postulated that since the activation and the activities of uPA on the cell surface are mediated by its receptor uPAR, the association with uPAR may render uPA prone to inhibition by maspin.[13,14] On the other hand, the cell surface microenvironment also may provide additional cofactors that further increase the inhibitory potency of maspin by facilitating its critical transition from a latent to an active conformation.

McGowen et al also performed detailed kinetic analyses, showing that maspin acted as a competitive inhibitor of uPA with an apparent Ki value of 20 nM. These kinetic characteristics are comparable to those of PAI-1 and PAI-2 in similar cell-based biochemical analyses. It is important to point out, however, that different plasminogen-activator inhibitors may play distinct roles in tumor progression. For example, PAI-1, along with uPA and uPAR, is causatively involved in the progression of breast cancer.[20] In contrast, maspin, which is down-regulated in several types of carcinomas, has tumor-suppressing activity. In their report, McGowen et al further demonstrated that the proteolytic inhibitory effect of maspin was quantitatively consistent with its inhibitory effect on the motility of DU145 cells in vitro.

Conclusion

Taken together, the results of McGowen et al suggest that maspin may inhibit tumor cell invasion and motility by blocking the uPA-mediated, ECM-degrading proteolytic cascade. While future studies are needed to test whether endogenous maspin protein has a similar proteolytic inhibitory effect on the cell surface-associated uPA, and whether uPA produced by other types of carcinoma cells is inhibited by maspin, it is intriguing to hypothesize that novel maspin-based, therapeutic strategies may prove useful to target specific human malignancies that are associated with markedly elevated uPA. *(Dr. Sheng is an Assistant Professor of Pathology in the Department of Pathology, Wayne State University School of Medicine, Detroit, MI.)* ❖

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Funding News

The National Institute of Environmental Health Science Toxicogenomics Research Consortium

The National Institute of Environmental Health Science (NIEHS) has issued a Request for Application (RFA) to develop a national Toxicogenomics Research Consortium (TRC). The development of the consortium is intended to increase the scientific community's ability to apply microarray gene expression profiling to biological responses to environmental stress. The goals of establishing the TRC are to accelerate research in this field using microarray gene expression profiling; develop standards for the analysis of gene expression with intra- and inter-laboratory validation; develop a database of gene expression information in this area; and define how the entire genetic complement of an organism responds to environmental agents, including chemicals, physical agents, and physiological changes. Applicants are eligible if they perform research in this area within the United States at a non-profit or for-profit institution. The mechanism of support is through the National Institutes of Health U19 Cooperative Agreement, which can be used to support individual research projects or core facilities. The NIEHS has budgeted as much as $5.5 million to support five or six first-year awards. It is expected that the size of the award will vary with each award recipient. Letters of intent responding to this RFA are due on Feb. 15, 2001, with the final application due on March 15, 2001. More information on this RFA can be found at http:\\grants.nih.gov\grants\guide\rfa-files\RFA-ES-01-002.html. ❖

CME Questions

1. **Which component of hypoxia-inducible factor is rapidly degraded by the ubiquitin-proteasome system under normoxic condition?**
   a. VEGF
   b. HIF-1α
   c. HIF-1β
   d. HIF-1δ

2. **Hypoxia-inducible factor transactivates which of the following genes?**
   a. Angiostatin
   b. Erythropoietin
   c. Endostatin
   d. The multi-drug resistant gene

3. **The HIF-1 transcription factor activity is regulated by the stability of which of the following proteins?**
   a. VEGF
   b. HIF-1α
   c. HIF-1β
   d. HIF-1δ

4. **Common n-3 polyunsaturated fatty acids in the human diet include which of the following?**
   a. α-linolenic acid
   b. Eicosapentaenoic acid
   c. Docosahexaenoic acid
   d. All of the above

5. **Maspin protein has an overall sequence homology with:**
   a. serine protease inhibitors.
   b. HIF-1α.
   c. angiostatin.
   d. erythropoietin.

In Future Issues: Polyunsaturated Fatty Acids and Skin Cancer: Part II