Molecular Pathways

Molecular Pathways: Inflammation-Associated Nitric-Oxide Production as a Cancer-Supporting Redox Mechanism and a Potential Therapeutic Target

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Abstract

It is widely accepted that many cancers express features of inflammation, driven by both microenvironmental cells and factors, and the intrinsic production of inflammation-associated mediators from malignant cells themselves. Inflammation results in intracellular oxidative stress with the ultimate biochemical oxidants composed of reactive nitrogens and oxygens. Although the role of inflammation in carcinogenesis is well accepted, we now present data showing that inflammatory processes are also active in the maintenance phase of many aggressive forms of cancer. The oxidative stress of inflammation is proposed to drive a continuous process of DNA adducts and crosslinks, as well as posttranslational modifications to lipids and proteins that we argue support growth and survival. In this perspective, we introduce data on the emerging science of inflammation-driven posttranslational modifications on proteins responsible for driving growth, angiogenesis, immunosuppression, and inhibition of apoptosis. Examples include data from human melanoma, breast, head and neck, lung, and colon cancers. Fortunately, numerous antioxidant agents are clinically available, and we further propose that the pharmacologic attenuation of these inflammatory processes, particularly the reactive nitrogen species, will restore the cancer cells to an apoptosis-permissive and growth-inhibitory state. Our mouse model data using an arginine antagonist that prevents enzymatic production of nitric oxide directly supports this view. We contend that selected antioxidants be considered as part of the cancer treatment approach, as they are likely to provide a novel and mechanistically justified addition for therapeutic benefit.

Clin Cancer Res; 19(20); 5557–63. ©2013 AACR.

Background

The most commonly recognized features of cancer-associated inflammation are those also expressed by the innate immune system, normally activated in response to stress or infection and which function teleologically during the initiation of wound control (1). The observed chronic inflammatory milieu in notable subsets of human cancers, and particularly in melanoma, is proposed to support tumor growth, plasticity, and resistance to therapy (2–6). Unfortunately, dysregulated persistent inflammatory contributions to the chronic phase of many diseases, including maintenance of many cancers. It is accepted that inflammation drives development of some cancers which adapt to and thrive in the oxidant-rich microenvironment, as described initially in the review by Cousens and Werb (5), by ‘co-opting’ expression of inflammatory mediators (7). From our view, this continues to provide a persistent and self-perpetuating oxidative stress composed of both reactive nitrogen species (RNS) and reactive oxygen species (ROS), and derived from proinflammatory interleukins (IL), chemokines, and NOSs (nitric oxide synthases) often via growth factor receptors (4). The critical oxidant sources are now realized to be more than reactive oxygen molecules, as the chronic production of another oxidant, NO (nitric oxide), also plays a major role in oxidative stress in melanoma and other cancers (4, 8–11) and the aberrant constitutive RNS is argued here as possibly a more important source of oxidative stress in many cancers. In comparison with the oxygen radicals, NO is a more stable oxidant, easily crosses lipid bilayers, and generates several types of posttranslational modifications with known ability to alter protein function and stability; these modifications are dependent on both NO concentration and temporal availability, resulting in a dynamic and reversible situation (12). A mathematical model of cutaneous melanoma predicted sufficient concentrations of NO at the periphery of a tumor to stimulate cell proliferation and lymphangiogenesis and to inhibit apoptosis (13).

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doi: 10.1158/1078-0432.CCR-12-1554
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Inflammation-Driven NO Supports Melanoma Growth and Apoptosis Resistance

In melanocytes, the precursor cell of melanoma, the pigment eumelanin provides a redox function supporting an antioxidant intracellular environment; however, in melanoma, a prooxidant status develops. The enzymatic production of NO is cell type specific, with cytokine-driven inducible NOS (iNOS) noted initially for the burst of higher levels as part of the pathogen defense system. Neuronal cells use nNOS to produce NO for signaling, and as melanocytes are of neuroectoderm origin, it is not surprising to find nNOS also expressed. The third NOS, endothelial NOS, regulates NO production in endothelia, is responsible for vascular relaxation, and has also been reported to be expressed in melanoma. In melanoma tumors, we (9, 10, 4, 11) and others (6, 8) document expression of NOS, particularly iNOS and nNOS. The iNOS protein was found in vivo in the melanoma tumor cytoplasm of approximately 60% of advanced patients and provided independent prognostic value by predicting decreased survival, so that the HR of iNOS-positive patients was 4.6 by multivariate analysis. This was an unexpected finding as the anti-iNOS antibody was used to identify activated macrophages, which were also often positive, but for which the positivity did not prove prognostic. Further evidence supporting intracellular NO production was by use of DAF-2DA staining as well as identification of irreversible protein nitration and the reversible thiol modifications known as “S-NO” (9). Using a human cell line model, in vitro experiments were carried out to scavenge endogenous NO, which resulted in melanoma cell growth inhibition; the growth was restored with an RNS donor, providing data to support a pivotal functional role of NO in cell growth and proliferation. Therefore, molecular analysis supports the hypothesis that NO can drive proliferation as well as resistance to apoptosis and the chemical quenching of NO resulted in G2 growth arrest followed by a gain of cisplatin-induced apoptosis, now confirmed in several reports.

As iNOS is downstream of Toll-like receptors, recently described in an earlier CCR Molecular Pathways article and results in NO production leading to a feed–forward loop and tumor progression, we asked whether we could identify a signature of the downstream common inflammatory markers. Although many markers have been noted in an extensive literature, in melanoma we have identified inflammatory cytokines IL-1α and β, IL-6, and IL-8; and in a more recent study, we have observed that an MIF-CD74 autocrine interaction was upregulated by IFN-γ, promoting the phosphorylation of AKT Ser473 and driving the expression of IL-6, IL-8, BCL-2, and BCL-xl (unpublished data). IFN-γ also regulates iNOS gene expression via interferon regulatory factors (IRF). IRFs are nuclear transcription factors that respond to IFN-γ via the JAK–STAT signaling pathway. The importance of IRF-1 and STAT-1 for the induction of iNOS gene expression in response to IFN-γ has been well documented. Our earlier study showed that IL-24 (also known as melanoma differentiation antigen 7) signaling modules the IRF transcriptional system to the extent that IL-24–treated melanoma cells exhibit a decline in IRF-1 and an increase in IRF-2, which blocks the IRF-1 pathway. This alteration in the IRF balance predicts the result in inhibition of iNOS expression. We have also used gene array studies, followed by validation of protein in patient tumor samples, and identified iNOS, arginase, VEGFα, CXCL-10, IL-8, IL-1α/β, and TNFSF9 as produced constitutively. Using melanoma patients’ tumors, recent results from our laboratory continue to support iNOS protein associating with NT, COX2, pSTAT3, and arginase, consistent with the report of Johansson and colleagues and with other recent studies.

Biochemical and Molecular Mechanisms of NO-Mediated Dysregulation of Cancer Signaling

NO in the presence of equimolar O2− forms ONOO− (peroxynitrite), which under physiologic conditions rapidly reacts with available tyrosine- or thiol-containing proteins to form nitrotrosine or reversible nitrosylation of thiols (S-nitrosylation also known as S-NO). Nitrination of tyrosines is considered a surrogate marker for higher but generally nontoxic μmol/L concentrations of NO. Global analysis of melanoma patient samples for tyrosine nitration has been used by several groups as a biomarker of NO exposure and iNOS expression in melanoma tumors. S-nitrosylation is considered irreversible in eukaryotes, and once modified, nitrosative stress, but based on the specific molecule modified, also serves functionally to activate oncoproteins, inhibit apoptosis, drive growth and angiogenesis, and alter immunosuppressors. In a recent study reported by Switzer and colleagues, oncogene activation includes S-NO of Ras in estrogen receptor (ER)-negative breast cancer. Using a model ER-negative breast cancer cell line, it was shown that NO not only induces S-nitrosoylation of wild-type (WT) Ras, but led to phosphorylation and activation of Erk-1 through the Ras/MEK/ERK pathway. Erk-1 is a key transcriptional mediator of oncogenic NO signaling, promoting the development of an aggressive disease phenotype in ER breast cancer.
Inflammation-Associated Nitric-Oxide Supporting Melanoma

Figure 1. Simplified examples of molecular pathways known to induce NO production in cancer cells and examples of proteins directly modified by reactive nitrogens. Numerous mediators of chronic inflammation are produced by melanoma and microenvironment cells including growth factors, transcription factors, adhesion molecules, signaling molecules and ECM proteins. Melanoma tumor cells also are known to produce NO from NOS, and each of the four major NOS-inducing pathways is shown in different colors (green, purple, blue, and white). Once produced, NO combines chemically with oxygen radicals to form ONOO⁻ (peroxynitrite) resulting in direct nitrosylation/nitrosation or nitration on many important signaling proteins (in red with •) in the tumor cells. The concentration of NO also influences the antitumor immune response, as shown in the box on the left, with increased NO associated with immune suppression. Immune system cells are functionally affected including the recruitment and/or activation of myeloid-derived suppressor cells (MDSC), Treg, tumor-associated macrophages, and Th2 lymphocytes. Once activated, MDSCs also produce high amounts of NO in the tumor microenvironment, which further contributes to the inhibition of antitumor responses and nitrosative stress. "Posttranslational confirmation of nitration or nitrosylation affecting function of each protein consistent with supporting growth, inhibiting apoptosis, and/or driving angiogenesis and growth.

proteins involved in the apoptotic machinery include bcl-2 in lung carcinoma (30), the death receptor FAS in colon and breast cancer cells (31) and the associated FLICE inhibitory protein (FLIP; 32), and caspase-9 in cholangiocarcinoma cells (33). Other early notable evidence supporting a role for NO in apoptosis resistance is the classic in vitro work of Mitchell and Marletta (34) with caspase-3 inactivation by reversible S-NO modification. Earlier in vivo data also indicate the NO-driven inhibition of caspase-3 (35). Nitrosylation has also been shown to stabilize mitogen-activated protein kinase phosphatase-1 in head and neck cancer, thus decreasing radiosensitivity by inhibiting apoptosis (36).

Tumor growth was also noted to be regulated by NO in a variety of tumor models via S-NO of Ras (37) and S-NO of the EGFR receptor (38). Cellular migration in prostate cancer is enhanced by S-NO on the integrin-α chain (39), and breast cancer invasiveness is enhanced by nitrosylation of c-Src (40). In addition to the functional effects of nitrosylation of caspase-3 and -9, Ras, p53, PTEN, iNOS, COX2, and Akt referenced above, other molecules that may have altered function as a result of S-NO include Bcl-2 (41, 42), HIF-1α (43), PI3K (44), NF-κB, and AP-1 (45, 46).

S-nitrosylation of a number of proteins can be linked to tumor proliferation. Many GTPases within the Ras superfamily contain redox-sensitive Cys residues that are susceptible to S-nitrosylation. Ras S-nitrosylation is associated with the initiation of tumorigenesis and maintenance of established tumors. In tumor cells, oncogenic K-Ras activates eNOS by PI3K–Akt-dependent phosphorylation of eNOS at S1117, which results in S-NO of WT N- and H-Ras; S1117-phosphorylated eNOS is elevated in tumors isolated from patients with pancreatic cancer, compared
with matched and unmatched normal tissue controls from these patients. (37).

Our additional data are consistent with the model that p53 in melanoma is inactivated by NO (15), and the quenching of NO in melanoma led to cisplatin-mediated apoptosis, which was blocked with biochemical donation of NO, indicating that this was not due to toxicity of the quencher. Tang and Grimm further reported that the apoptosis was dependent on p53 via use of siRNA. In melanoma, p53 does not express the usual driver mutations and is considered as rarely mutated yet functionally inactivated, and the NO is somehow responsible for p53 inactivation (15). We have preliminary data of S-NO modification of WT p53 (Qin: manuscript in preparation), which likely adds another major protein to the list above.

From the molecular view, we hypothesize that endogenous NO in the presence of stoichiometric availability of O₂ chemically nitrosylates accessible thiols to activate onco-
genases, inhibit multiple apoptosis and growth pathway proteins, and support enhanced growth. Local production of NO as a plausible mechanism for tumor resistance to therapy was elegantly presented by Dedon and Tannenbaum (47) almost a decade ago, and while still accepted to be provided by microenvironmental macrophages as in that first report, we now extend this understanding that NO is produced by tumor cells including melanoma. Although studies of S-NO modifications were carried out initially by redox chemists, more accessible methods are becoming available to cancer biologists such as the biotin-switch method for direct S-NO protein characterization (48), and even more recently the mass-spec/peptide sequencing methods for biochemical validation of the specific site and protein verification such as the SNO-Flo (49–51). The S-NO modifications are dynamic, can be identified biochemically, and can be reversed with various antioxidant approaches, some of which may prove useful in melanoma for inhibition of growth and restoration of apoptosis pathway function.

A second mechanism resulting in S-NO on signaling proteins in cancers is called trans-nitrosation, as reported with NO transferred from PTEN to AKT (52) or from iNOS to COX2 (53), as iNOS and PTEN are both themselves reported to have S-NO as well in those reports; the actual transfer of NO is based on differential nucleophilic profiles of the cysteine donor and receptor sites on each molecule. Therefore, after NO is generated and proteins are S-NO modified, the transfer of NO radicals can also regulate the dynamic and reversible posttranslational modifications.

Currently, there is no evidence that inflammation is uniquely linked to a specific driver mutation in melanoma; however, in some recent data testing melanoma tumors containing mutated BRAF, it was reported that the mutation may actually support expression of IL-1β expression (54), and in another study antisense BRAF inhibited iNOS expression (55). More analysis is needed to understand the driving forces for NO production and associated effects.

**Inflammation-Associated Molecules Drive Formation of the Protumor Cancer Microenvironment and Tumor-Mediated Immunosuppression**

By upregulating key inflammatory molecules, including iNOS, COX2, and proinflammatory cytokines and chemokines, tumor cells invoke a chronic inflammatory state that also induces tumor-supporting myeloid cells such as tumor-associated macrophages and myeloid-derived suppressor cells (MDSC), and drives their infiltration of the tumor microenvironment (56, 57). Although many host cell types including T cells are involved in creating an inflammatory protumor microenvironment, herein we focus on inflammation-directed recruitment of MDSC and macrophage polarization. As tumors progress, the macrophage population is thought to switch from a tumoricidal M1 to a protumor M2 phenotype, under the influence of IL-4, IL-10, IL-13, and TGF-β. M2 macrophages are associated with immunosuppression via arginase expression, the release of TGF-β and IL-10 (58), and regulatory T-cell (Treg) recruitment (59). M2 macrophages also contribute to formation of the protumor microenvironment and tumor progression by promoting angiogenesis (60).

MDSC are a myeloid cell type shown to play a critical role in establishing a cancer-supporting microenvironment (61) and implicated in suppression of antitumor immune responses (62). The role of MDSC has been the focus of a recent Molecular Pathways article by Lu and Gabrilovich (27) describing MDSC producing NO and ONOO⁻ leading to protein modifications in vivo and vitro. A recent article by us also identified a pivotal role for iNOS and ROS as mediators of MDSC recruitment and cancer-mediated immunosuppression, as in vivo melanoma tumor-expressed iNOS regulated MDSC induction by modulating VEGF release, and that pharmacologic iNOS inhibition depletes intratumoral MDSC and unmasks antitumor immunity (63). We have further investigated the role of iNOS in orchestrating MDSC migration in response to iNOS or NO inhibition, leading to upregulation of CXCL-10 (23), as well as that iNOS inhibition blocks release of a number of inflammatory mediators by melanoma cells, including VEGF, which we subsequently showed to be required for accumulation and functional activation of MDSC (63). Another interesting article shows in several preclinical cancer models that nitration of the chemokine CCL2 abolished intratumoral infiltration of antitumor CD8⁺ T cells, and in clinical specimens that tumor-infiltrating T cells are depleted in NO-expressing tumors (64, 65). COX2 can also play a role in MDSC induction via the direct MDSC-stimulating effects of its enzymatic product prostaglandin E 2 (PGE2; 66–68). Nagaraj and colleagues also showed that pharmacologic modulation of ROS with an antioxidant CDDO-Me triterpenoid compound resulted in dramatic reduction of ONOO⁻ and suppressed MDSC function and boosted immunity in tumor-bearing mice and in patients with cancer receiving the drug in a clinical trial (69). Each of these articles suggests potential therapeutic strategies for reversing tumor-mediated immunosuppression by
blocking inflammation-induced MDSC, particularly involving NO regulation.

Clinical–Translational Advances

On the basis of the contention that a cancer’s “inflammatory niche” drives alterations in critical protein function via identifiable S-NO posttranslational modifications as proposed above, two clinical-translational advances are in progress. The first is development of a marker set or predictive signature of inflammation that would prove useful for targeting. This work is active currently with a clinically useful melanoma protein signature around iNOS, IL-1s, IL-6, MIF, pSTAT3, and at a global level of total nitrated tyrosines and total S-NO at both the cellular and microenvironmental levels still being resolved. As our detection methods become more sensitive and specific, it is likely the modified proteins such as SNO-PTEN or AKT will also be useful markers. Another important complementary marker in the tumor microenvironment was MIF itself also from both melanoma cells and infiltrating immune cells, for which its presence negatively associated with CD74 expression. Our work in progress as well as that of others continues to support the growing evidence that “an inflammatory protein signature” is likely to continue as a consideration of an independent prediction model for clinical outcome, still to be validated. A large set of markers is undergoing analysis on both blinded patient tumor samples and prospective testing on tumor samples from ongoing clinical trials. Ideally, a blood test and specific anti S-NO–protein epitope are envisioned and under way using a new method called the SNO-Flo system (49). Direct measurements of SNOed proteins and their loss of this adduct after use of an anti-inflammatory agent are a future consideration as a set of predictive markers.

A second advancement we view is the development of clinically useful pharmacologic approaches with carefully selected antioxidants and/or small-molecule inhibitors of RNS, possibly combined with inhibitors of other prooxidant enzymes to permit the restoration of susceptibility to immune or targeted therapy in patients with melanoma. The known reversibility of SNO posttranslational modifications suggests a novel paradigm for attempting to restore the apoptotic pathway in the inflammatory-driven tumors of patients for therapeutic goals. We now propose pharmacologic approaches with carefully selected antioxidants that will inhibit the RNS of oxidants to permit the restoration of susceptibility to immune or targeted therapy in patients with melanoma, possibly predicted by reversal of SNO on selected markers, for which much research is currently underway.

Although nonsteroidal anti-inflammatory drugs have been popularized and continue to be assessed for prevention of cancers, their application to inhibiting inflammation supporting metastatic disease remains to be considered. The in vivo use of other classes of antioxidants in treatment of established cancers has been explored. As one of the most investigated antioxidants, resveratrol has been shown to inhibit growth and induce apoptosis in cell lines, potentially by regulating p53 expression, cyclin-dependent kinases, and COX-2 (71, 72). Another much studied agent, curcumin (Curcuma longa), has been shown by many groups, including our own, as an apoptosis inducer in human melanoma cells in most known pathways through the inhibition of NK-kB, which drives iNOS (73, 74). Other approaches include clinically applicable arginine antagonists such as L-NIL, which was developed for treatment of asthma and used in our recent mouse model (11) and for which an entire class of agents is currently available. Other drugs with anticancer potential known to downregulate iNOS include WP-760 (75), which was found to inhibit iNOS production and also localize to the mitochondria and activate caspase-3. As aberrant expression of inflammatory molecules is common to many cancers, it stands to reason that therapeutic approaches targeting these molecules have the potential for antitumor efficacy across a broad range of tumor types. In fact, much of the preclinical literature describing these approaches describes targeted inhibition of inflammatory molecules to simultaneously prove their relevance to cancer and invoke therapeutic efficacy. As discussed above, we have shown that pharmacologic inhibition of iNOS has antitumor effects in various melanoma models which may be directly related to tumor cell-intrinsic growth and survival pathways (11, 15) or indirect and mediated by effects on the tumor-promoting microenvironment including vascular (11) or immunoregulatory cells (63). The role of MDSC is particularly relevant here, as Nagaraj and colleagues also showed that an antioxidant triterpenoid compound can suppress MDSC function and boost immunity in tumor-bearing mice and in patients with cancer receiving the drug in a clinical trial (69).

In conclusion, many human cancers express inflammatory molecules that lead to an intrinsic pro-oxidant environment in the cancer cell, as well as potentiating a microenvironment that drives immune escape and resistance to apoptosis. We contend that by attenuating the NO production resulting from this inflammation, possibly by repurposing currently available agents, the prospects for cancer control will be greatly increased. Our data and those of others provide an emerging molecular model by which the RNS drive growth, immunosuppression, angiogenesis, and apoptosis resistance by specifically altering the function of signaling molecules by nitration and/or nitrosylation. We propose that the NO–contributed oxidants and the pathways that drive them may be useful targets for therapeutic consideration when combined as part of targeted and immune therapy approaches.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Development of methodology: E.A. Grimm
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.A. Grimm, S. Ekmekcioglu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E.A. Grimm, A.G. Sikora, S. Ekmekcioglu

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Clin Cancer Res; 19(20) October 15, 2013
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Grant Support
This work was partially supported by the grants from MD Anderson Cancer Center SPORE in Melanoma P01 CA093459; Cancer Center Support Grant CCGS P30 CA016672; Miriam and James Mulva Foundation; and Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (to E.A. Grimm and S. Ekmekcioglu), and also supported by the grants from the NIH National Institute of Dental and Craniofacial Research (5R03DE021741-02) and the NIH National Cancer Institute (1K08CA154963-01A1; to A.G. Sikora)

Received April 30, 2013; revised July 8, 2013; accepted July 10, 2013; published OnlineFirst July 18, 2013.

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