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Immunology Comes Full Circle in Melanoma While Specific Immunity Is Unleashed to Eliminate Metastatic Disease, Inflammatory Products of Innate Immunity Promote Resistance

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Abstract

Melanoma and many other cancers often express cells and molecular features of inflammation. Intrinsic to melanoma is the expression of a continuous cycle of cytokines and oxidative stress markers. The oxidative stress of inflammation is proposed to drive a metastatic process, not only of DNA adducts and crosslinks, but also of posttranslational oxidative modifications to lipids and proteins that we argue support growth and survival. Fortunately, numerous antioxidant agents are available clinically and we further propose that the pharmacological attenuation of these inflammatory processes, particularly the reactive nitrogen species, will restore the cancer cells to an apoptosis-permissive and growth-inhibitory state. Experimental model data using a small-molecule arginine antagonist that prevents enzymatic production of nitric oxide supports this view directly. I propose that the recognition, measurement, and regulation of such carcinogenic inflammation be considered as part of the approach to the treatment of cancer.

Keywords

nitric oxide; cytokines; MIF; CD74; iNOS

DEDICATION: Today, we are witnessing a remarkable and ever-increasing antitumor effect of immunotherapy, a dream and goal that Dr. Donald L. Morton worked so hard to realize. Dr. Morton was a most courageous pioneer in this area, with his focus clearly on activation of antigen-specific immunity, which is reflected by his early quest for melanoma antigens to his stimulation of immune responses with vaccines. My initial first-authored publication in 1976 was with Dr. Morton, reporting on serologically detectable antigens in the serum of melanoma patients,[1] and this experience and paper was a major stimulus for my own career. I remember that Dr. Morton was a proponent of adaptive-specific immunity and a firm believer in immune surveillance. I will forever be grateful for the inspiration of Dr. Morton, along with the vital pulse of the 1970's era in Los Angeles and the life-long friendships and opportunities that continue to come my way as a result of having had the privilege to work with him.

I continued to keep in contact with Dr. Morton, including as an EAB member on his P01 grants, and also became a collaborator. Eventually, I was asked by Dr. Morton to help establish the melanoma section of the Adelson Medical Research Foundation. I was most fortunate to discuss immunology with Dr. Morton at least several times a year and it was clear that he was up to date and most thoughtful.

Today, in this contribution, I present one side of how we have come full circle regarding melanoma and the immune system. Although an unprecedented number of immunotherapy agents have been approved for patient treatment in the past 2 years, unfortunately (to date), none of these new agents has proven to provide a consistent long-term disease control for any definable set of patients. Our work continues and mine especially addresses the failure to respond, which for this contribution is specifically defined as resistance mechanisms driven by nonspecific immunity. We now consider part of the protection of normal self from immune attack as a teological expression of a series of innate immune inflammatory processes; these appear to be expressed in many cancers and particularly in many melanomas. It is well worth considering what Dr. Morton might have said about this; we all miss him and his halting, yet clear and specific hard questions, always delivered with a smile and twinkle in his eye.

I. INTRODUCTION

Features of the innate immune system are often detected in cancers. Such features are normally activated in response to stress or infection and serve to protect “self” during the initiation of wound control. [2] In many human cancers, and particularly in melanoma, inflammatory cells and molecules have been proposed to support tumor growth, plasticity, and resistance to therapy.[3–7] It is accepted that inflammation drives the development of some cancers, which adapt to thrive in the oxidant-rich microenvironment, as described initially in the review by Coussens and Werb,[6] by “co-opting” expression of inflammatory mediators.[8] We put forth the hypothesis that this is the result of a perpetuating oxidative stress composed of both reactive nitrogen species (RNS) and reactive oxygen species (ROS), which are derived from numerous pro-inflammatory interleukins, chemokines, nitric oxide synthases (NOSs) often via growth factor receptors.[5] Although, initially, only oxygen molecule oxidants were considered as the critical oxidant source, today, we also recognize that the chronic production of another oxidant, nitric oxide (NO), also plays a major role in melanoma and other cancers[5,9,10,11,12] with aberrant constitutive RNS. NO is the more stable oxidant, easily crosses lipid bilayers, and generates several types of posttranslational modifications on cysteines. Such modifications are known to alter protein function and stability and are both dynamic and reversible.[13] One 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. [14]

A. NO Supports Melanoma Growth and Apoptosis Resistance

In melanocytes, the precursor cells of melanoma, the eumelanin pigment supplies an antioxidant redox function; however, in melanoma, a pro-oxidant status is reported to develop.[9] The enzymatic production of NO is cell-type specific with cytokine-driven inducible NOS (iNOS) noted mainly for the burst of high toxic levels as part of the pathogen defense system, but lower levels being temporarily cytoprotective. Neuronal cells use neuronal NOS (nNOS) to produce NO for signaling and, because melanocytes are of neuroectodermal origin, it is not surprising to find that nNOSs are also expressed. The third NOS, endothelial NOS, regulates NO production in endothelia and, at lower levels, is toxic, responsible for vascular relaxation, and has also been reported to be expressed in melanoma. [15]

In melanoma we[5,10–12] and others[7,9] have documented expression of both *iNOS* and *nNOS*. The iNOS protein was found *in vivo* in the melanoma tumor cells of ~60% of advanced patients and provided independent prognostic value by predicting decreased survival, so that the hazard ratio of iNOS-positive patients was 4.6 by multivariate analysis.[10] This was an unexpected finding because the anti-iNOS antibody was used initially to identify activated macrophages, which were also often positive, but for which the positivity did not prove prognostic. Only tumor expression was prognostic for poor survival. Further evidence supporting intracellular NO production was by the use of DAF-2DA staining,[5] as well as the identification of irreversible protein nitration and the reversible thiol modifications known as “S-nitrosylation” (S-NO). [5,10–12] Using a human cell line model, *in vitro* experiments scavenging endogenous NO showed results of 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.[16] Therefore, molecular analysis supports the hypothesis that NO can drive proliferation and 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. [12,16,17]

Because iNOS is downstream of Toll-like receptors, recently described as resulting in a feedforward loop and tumor progression,[18] we asked whether we could identify common inflammatory markers. In melanoma, we have identified inflammatory cytokines IL-1α and β,[19] IL-6, and IL-8 and, in a currently unpublished study, we have observed a macrophage migration inhibitory factor–CD74 autocrine interaction upregulated by IFN-γ. IFNγ also regulates iNOS gene expression via interferon regulatory factors (IRFs), nuclear transcription factors that respond to IFN-γ via the JAK-STAT signaling pathway.[21–22] Because an earlier study showed that IL-24 (also known as Melanoma Differentiation Antigen-7, MDA-7) signaling modulates 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 that blocks the IRF1 pathway, this alteration in the IRF balance predicts the result in inhibition of iNOS expression. [23] Gene array studies, followed by validation of protein in patient tumor samples, has identified iNOS, arginase, VEGFα, CXCL-10, IL-8, IL1α/β, and TNFSF9 as being produced constitutively.[24,25] More recent results from our laboratory continue to support iNOS protein associating with NT, COX2, pSTAT3, and arginase, which is consistent with the report of Johansson et al.,[7] and with other recent studies.[26,27]

B. NO-Mediated Dysregulation of Cancer Signaling

NO in the presence of equimolar O_2^- forms $ONOO^-$ (peroxynitrite), which under physiological conditions reacts rapidly with available tyrosine or thiol-containing proteins to form the irreversible nitrotyrosine (NT) or reversible nitrosylation of thiols (S-NO). S-NO is not only a marker of nitrosative stress, but based on the specific molecules modified, may also activate oncogenes, inhibit apoptosis, drive growth and angiogenesis, and inhibit tumor suppressor functions. In a report by Switzer et al.,[28] oncogene activation includes S-NO of Ras in ER-negative breast cancer. S-NO-modified apoptotic proteins include bcl-2 in lung carcinoma, [29] the death receptor FAS in colon and breast cancer cells[30] and the associated FLICE inhibitory protein (FLIP),[31] and caspase 9 in cholangiocarcinoma cells.[32] Other publications supporting a role for NO in apoptosis resistance is the classic *in vitro* work of Mitchell of NO-driven inhibition of caspase 3.[33] In head ad neck cancers, nitrosylation has also been shown to stabilize mitogen-activated protein kinase phosphatase-1 (MKP-1), thus decreasing radiosensitivity by inhibiting apoptosis.[34] Publications of tumor growth regulation by NO in a variety of models has been noted via S-NO of Ras[35] and S-NO of the epidermal growth factor receptor. [36]

Tang and Grimm[16] presented data consistent with the observation that p53 in melanoma is inactivated by NO and that 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. In this same study, the investigators further reported that the apoptosis was dependent on p53 via use of siRNA. In melanoma, p53 does not express the

usual driver mutations, is considered as rarely mutated yet functionally inactivated, and the NO is thought to be somehow responsible for p53 inactivation.[16] We have preliminary data of S-NO modification of wt p53, which likely adds another major protein to the list above.

At present, 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[37] and, in another report, antisense *BRAF* inhibited iNOS expression. [38] More analysis is needed to understand the driving forces for NO production and the associated effects.

C. Association of Inflammation- Associated Molecules in the Pro- Tumor Cancer Microenvironment

As introduced above, iNOS, COX2, and proinflammatory cytokines and chemokines are associated with a chronic inflammatory state in melanoma, which also induces tumor-supporting myeloid cells such as tumor-associated macrophages and myeloid-derived suppressor cells (MDSCs) and drives their infiltration of the tumor microenvironment. [39,40] Although many host cell types, including subsets of T cells, are involved in creating an inflammatory pro-tumor microenvironment, we present data for NO being involved with recruitment of MDSC and macrophage polarization. As tumors progress, the macrophage population is thought to switch from a tumoricidal M1 to a pro-tumor M2 phenotype under the influence of IL-4, IL-10, IL-13, and TGFβ. M2 macrophages are associated with immunosuppression via arginase expression, the release of TGFβ and IL-10,[41] and Treg recruitment.[42] M2 macrophages are also involved in promoting angiogenesis.[43] MDSCs are a myeloid cell type known to mediate a critical role in establishing a cancer-supporting microenvironment, [44] including suppression of antitumor immune responses.[45] Lu and Gabriolvich[46] described MDSCs producing NO and $ONOO^-$, leading to protein modifications *in vivo* and *vitro*. Jayaraman et al.[47] also reported a major role for iNOS and ROS as mediators of MDSC recruitment and immunosuppression because *in vivo* melanoma-tumor-expressed iNOS regulated MDSCs by modulating vascular endothelial growth factor (VEGF) release. Jayaraman et al.[47] further reported that pharmacologic iNOS inhibition depleted intratumoral MDSCs and unmasked antitumor immunity. 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.[24] We also showed that iNOS inhibition blocks the 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 MDSCs.[47] Nagaraj et al.[48] also reported that down-regulation of ROS with the antioxidant CDDO-Me triterpenoid compound resulted in a dramatic reduction of $ONOO^-$ and suppressed MDSC function while boosting immunity in tumor-bearing mice and in cancer patients receiving the drug in a clinical trial.[48] Together, these studies argue in favor of pursuing therapeutic strategies for reversing tumor-mediated immunosuppression by blocking inflammation-induced MDSCs, particularly by inhibition of NO activity.

II. CONCLUSION

Many human cancers, particularly the most aggressive human melanomas, express inflammatory molecules that lead to an intrinsic pro-oxidant environment in the cancer cell and potentiate 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 RNSs 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 as part of a therapeutic consideration that may be useful when combined as part of targeted and immune therapy approaches.

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ABBREVIATIONS

RNS	reactive nitrogen species
NOS	nitric oxide synthase
NO	nitric oxide
iNOS	inducible NOS
S-NO	S-nitrosylation

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