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The Metabolomics of Nitric Oxide and Reactive Nitrogen Species in Immune Editing Tumor Milieu: Influence of Nitric Oxide-Modulating Therapies

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Abstract

Nitric oxide (NO), reactive nitrogen and oxygen species, hypoxia, L-arginine metabolites and peroxynitrite are contributing agents during various stages of carcinogenesis. Among these, NO contributes to various functions in the tumor microenvironment such as augmenting immune suppression, maintaining anergy to tumor associated antigens, stimulating angiogenesis, enhancing tumor growth and invasiveness, and modulating autophagy. Most cells in the tumor milieu either release NO and/or have altered physiological functions due to the influence of NO. Cancer cells evolve into a metabolic state to tolerate and reduce reactive nitrogen and oxygen species to elude oxidative damage. These cancer cells in the tumor microenvironment inhibit cellular infiltration of effector immune cells into the cancer milieu. Increased levels of CCAAT-enhancer-binding proteins such as C/EBP α and β are required for inducible Nitric Oxide Synthase (iNOS) gene expression and other transcripts for sustaining an immunosuppressive environment in growing tumors. The immunoediting property of reactive nitrogen and oxygen-modified metabolites change the functions of: intratumoral chemokines, T cell receptors, antigen specific tumor infiltrating lymphocytes (TILs), cytotoxic T lymphocyte, myeloid derived suppressive cells (MDSCs), gene expression, tumor suppressor genes, and Interferon responses which together countersignal tumor immunity. Recent studies show that reactive nitrogen species that promote tumor-mediated immune evasion can be reversed by gene therapy, immunotherapy, and chemotherapy by targeting or co-targeting excessive nitric oxide accumulation.

Keywords: Nitric oxide; Free radicals; Cancer; Metabolomics; Immunotherapy; Chemotherapy

Abbreviations: Nitric oxide: NO; Nitric oxide synthase: NOS; Inducible Nitric Oxide Synthase: iNOS; Peroxynitrite: ($ONOO^-$); Superoxide: ($O2^-$); Reactive nitrogen species: RNS; Reactive oxygen species: ROS; Tumor infiltrating lymphocytes: TILs; Myeloid derived suppressive cells: MDSCs; L-arginine: L-Arg; L-Citrulline: L-Cit; Cytotoxic T cells: CTL; T regulatory cells: Tregs; Immature myeloid cells: IMCs; L- Arginine: L-Arg; Cytoplasmic arginase: Arginase 1; Mitochondrial arginase: Arginase2; Non-steroidal anti-inflammatory drugs: NSAIDS; Superoxide dismutase: SOD; Pentose Phosphate Pathway: PPP; Hypoxia-inducible factor-1α protein: HIF-1α

Cancer Immunoediting Come of Age

The development in immunology in the last two decades has given us a different level of appreciation of the immune system and the dual role it plays in cancer. The immune surveillance system suppresses tumor formation by destroying cancer cells or obstructing their outgrowth. The development of some cancers might be seen as a failure of immune surveillance and the ability of tumors to thwart the development of effective immune responses against their antigens. This resistance of tumors against the immune response is facilitated by immunoediting of immune signals induced by the tumors and their metabolites during the course of carcinogenesis [1,2]. One of the components of the immune system that initiates the mechanism of cancer immunoediting is inflammation [3-5]. Some of the tools (such as nitric oxide and reactive nitrogen and or oxygen species) utilized by the immune system during infections and host defense also function to promote immune suppression in the tumor microenvironment.

Nitric oxide in cancer, a compelling relationship in tumor progression

Nitric oxide is a gaseous signaling molecule in various biological systems [6]. It is highly reactive and diffuses freely across cell membranes. This property of NO makes it an ideal transient paracrine and autocrine signaling molecule in biological systems. Five chemical processes occur in the biological milieu upon exposure to NO: nitration and nitrosation, nitrosylation, oxidation and interactions with other free radicals, e.g., H_2O_2 [7]. All the five effects of NO have been reported to be involved in generation of modified metabolites in various cell signaling processes [3,8,9] (Figure1).

Nitric oxide is biosynthesized by three isoforms of nitric oxide synthases (NOSs). Endothelial NOS (eNOS), neuronal NOS (nNOS) and, inducible NOS (iNOS) are all involved in immune responses [3,7-9]. All the isoforms of NOS utilize L-arginine (L-Arg), oxygen, tetrahydrobiopterin and NADPH to generate NO and L-citrulline (L-Cit) [6,7]. Among these isoforms, iNOS exhibits high NO output and has been reported in various cell types, which include but not limited to M2 macrophages, MDSCs, dendritic cells, NK cells, tumor cells, endothelial cells, neuronal cells, and neutrophils which are involved in inflammation and cancer [10-16]. Cancer cells have a tendency to disregard oxygen availability, electing for less efficient anaerobic pathways of generating energy such as the pentose phosphate pathway and avoiding glycolysis [17]. The astuteness behind this is to manage the reactive oxygen species and or oxidative damage to itself in the tumor microenvironment. This process is specific for cancer cells, as

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it may damage other metabolic active cells such as liver and immune cells. Thus "cancer cells make sacrifices for survival" [17].

Low level of NO is essential for immune functions whereas high levels of NO are associated with immune suppression [18]. Several studies using $NOS^{-/-}$ mice and/or NOS inhibitors have demonstrated that NO mostly promotes tumor progression as reviewed by Fukumura et al. [19]. Cells of the tumor milieu (Figure 2) adapt to metabolic changes to limit the toxicity of free radicals, and acquire tumor immunity, resistance to apoptosis or other forms of cell death [7,17,20,21]. Thus NO is not only tightly regulated in the tumor environment, but it undergoes constant change and exhibits heterogenerous effects depending on the type of tumor, concentration of NO, NO's ability to interact with other free radicals, proteins, metal ions and genetic background of its target [18]. The iNOS activity can change during tumor progression. For example, in colon cancers, iNOS activity is at the highest in adenomas. This iNOS activity decreases with advancing tumor-stages and was found to be minimum in metastatic tumors [19]. We and others have reported the pleiotropic role of NO in normal and pathophysiological conditions [22] including cancers [23]. In the present review, we will focus on the role of NO, NO producing cells, the modification of metabolites by free radicals and their impact on other cells involved in tumor immunity, immunotherapy, and chemotherapy.

Free radical releasing cells in tumor milieu

The tumor milieu is a complex system of many cells [24]. They all participate in tumor progression, immunity, and immune suppression [5,25,26]. These cells include endothelial cells, pericytes, fibroblasts of various phenotypes, neutrophils and other granulocytes (eosinophils and basophils), cytotoxic T cells (CTL), mast cells, CD4 and CD8 T cells, B cells, natural killer (NK) lymphocytes, antigen-presenting cells such as macrophages, and dendritic cells [5,25,26]. Among these are cells with specialized functions such as T regulatory cells (Tregs) and MDSC [2,10,14,27]. The phenotypes of tumor-associated macrophages (TAMs) are distinct from normal macrophages [11]. Tumor-associated macrophages exhibit pro-tumoural functions and participate in suppression of adaptive immunity [11]. They also exhibit functional plasticity and also show reversible adaptation to changing environments [16,28].The TAMs, MDSCs, dendritic cells, NK cells, tumor cells, neutrophils, eosinophils, basophils, and endothelial cells have been reported to release NO and free radicals, which influence physiological functions in the tumor microenvironment [10,11,19,21,29-32].

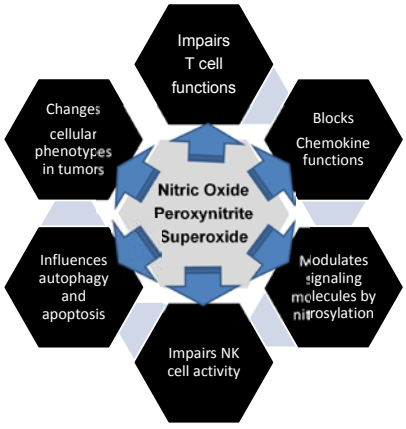

Figure 1: Nitric oxide, peroxynitrite, and superoxide promote immunosuppression in tumor milieu: Most tumors show increased levels of free radicals which include reactive nitrogen and or oxygen species; however, cancer cells disregard the availability of oxygen and choose a less efficient path for generating energy. Cancer cells operate via the pentose phosphate pathway (PPP) for their energy which generates fewer free radicals as compared to the glycolysis. The aim is to prevent the buildup of reactive nitrogen species and reactive oxygen species and oxidative damage. In the process, cancer cells show increased levels of superoxide dismutase (SOD) activity and hypoxia-inducible protein factors (HIFs). HIF-1α is a transcription factor which induces transcription of several genes in hypoxia and low oxygen tension for cell survival. SODs are responsible for eliminating free radicals. The reactive nitrogen and or oxygen species support conditions of immunosuppression in the tumor microenvironment. Some of the mechanisms include modification of metabolites of transformed cells and other cells in the vicinity such as impairing the activities of T and NK cells that are involved in tumor regression. Reactive nitrogen species modify chemokine that are required for signals that facilitate the entry of effectors cells into the tumor but allow myeloid cells which promote immunosuppression. Nitric oxide modifies various signal transduction molecules within the cells that alter the functions of various cells with respect to autophagy and apoptosis. It also modulates oncogenes like p53 which regulate apoptosis [7]. Reactive nitrogen and or oxygen species influence the changes in the cell-surface markers and modify cell phenotypes involved in carcinogenesis.

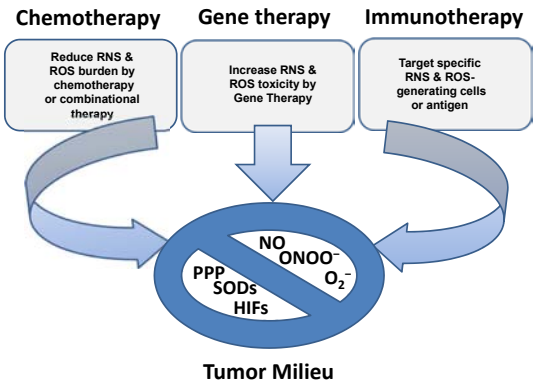

Figure 2: Strategies for modulating nitric oxide, peroxynitrite, and superoxide in tumor milieu for therapy: The figure shows the various targets in the tumor milieu for therapeutic intervention. Reactive nitrogen and oxygen species and peroxynitrite within the tumor milieu are necessary and collective contributors of tumor-mediated immune evasion.(3, 19). They may function independently or collaboratively depending on the biochemical event. The cancer cells have evolved into a metabolic state to not only tolerate, but reduce reactive free radicals and oxidative damage. This metabolic state is regulated by various biochemical pathways {e.g. pentose phosphate pathways (PPP)}, enzymes {e.g. superoxide dismutase (SOD)} and other factors {such as hypoxia-inducible factors (HIFs)} in the tumor milieu [17,56,57,71]. Several strategies have shown promising results in reversing immunosuppression by disrupting the roles of reactive nitrogen and oxygen species in tumors in animal studies and in man [56,57] These approaches include immunotherapy, gene therapy and chemotherapy [19,56,57,71]. Gene therapy targets the basic redox balancing processes. This approach is cancer-cell specific and it avoids collateral damage. Various chemotherapies and immune therapies reduce the levels of reactive nitrogen and/or oxygen species as secondary targets. Inhibitors of reactive nitrogen and or oxygen species have been used during Adoptive Cell Therapy or in vivo for preconditioning tumor microenvironment with other combinational therapies [19,46]. Another approach is by increasing the levels of endogenous nitric acid in tumors or introducing exogenous NO which sensitizes tumors to radiation therapy [19].

The hijacking of myeloid-derived suppressor cells (MDSCs) by tumors

The immune system is structured to protect itself from excessive immune stimulation induced by cancer and/or self-antigens released by trauma. In the process, a complex network of soluble factors stimulates the production of immature myeloid cells (IMC) from the bone marrow and into the blood circulation [33,34].

Increased amounts of IMCs are also associated with a state of immune suppression, dysfunctional dendritic cells, and T cells [35]. These IMCs can also generate a separate lineage of cells with distinct

immunoregulatory properties, which include MDSCs. While MDSCs have been reported to have a role in wound healing and tissue repair, tumors have learned to 'harness', these characteristics of MDSCs for antitumor immunity and tolerance, promote tumor development, and metastasis [16]. Myeloid derived suppressive cells are characterized by increased levels of arginase activity, reactive oxygen species and NO. In mice, their phenotype is $CD11b^{+}Gr1^{+}$ and in humans, it is $CD11b^{+}CD14\text{-}CD33^{+}$ or Lin-HLA-DR- $CD33^{+}$ [11,16,36]. The human cells do not express the Gr1 homologue. The human population of MDSCs in blood shows the $CD15^{+}$ marker [16]. Myeloid derived suppressive cells do not exhibit an immunosuppressive phenotype in the bone marrow, but that may change in the lymphoid organs and cancer due to the inflammatory environment, growth factors, cytokines and chemokines. In the tumor milieu, MDSC differentiates into a tumor associated macrophages like phenotype and retain their ability to generate NO and free radicals [16]. Kilinc et al. (2011) [1]suggested that iNOS+- myeloid cells in tumors have multiple effector functions. They are also phenotypically similar to TAMs. These cells could be immune stained for iNOS expression but could be separated into two populations (P1) $CD11b^{high}$, $Gr1^{low/-}$ $F4/80^{+}$ cells and (P2) $CD11b^{low/-}$, $Gr1^{low/-}$ F4/80. These populations of cells were also observed in the bone marrow and blood and their accumulation was dependent on tumor growth. Furthermore, In vitro experiments showed that these $iNOS^{+}$ myeloid cells in the tumors could inhibit proliferation of $CD8^{+}$ T cells and induce apoptosis which could be reversed by iNOS inhibitors [1]. These intra-tumoral MDSCs acquire strong immunosuppressive activity as described below by utilizing NO and other free radicals as one of the arsenals for their effector functions.

L-Arginine metabolism, reactive nitrogen species and peroxynitrites in immunoediting of immune signals

L-Arginine is a substrate to three enzymes involved in the regulation of free radicals. Cytoplasmic arginase (arginase 1) and mitochondrial arginase (arginase 2) hydrolyze L-Arg to urea and ornithine. Ornithine is converted to polyamines by ornithine decarboxylase [16,19]. iNOS oxidizes L-Arg to L-Cit and releases NO [37]. An increased level of arginase and iNOS activity has been documented in patients with melanomas, gliomas, sarcomas, prostate, breast, colon, and lung cancer [16,19,38]. Previous studies have proposed that the increased arginase enzymatic activity in tumors is required to sustain the high demand of polyamines necessary for tumor growth [39]. Furthermore, specific oncogenes and tumor-suppressor genes have also been reported to regulate polyamine metabolism [39].

Tumor infiltrating lymphocytes cells (TILs) migrate from the blood stream into the tumor. In most solid tumors, TILs are unable to kill autologous tumors and are predominantly in an anergic state [16,40]. Increased arginase activity in tumor infiltrating macrophages diminished synthesis of the CD3 zeta chain and antigen-specific T cell responses [16,40]. Macrophages stimulated with IL-4 plus IL-13 up-regulated arginase1 and amino acid transporter 2B, which triggered a rapid reduction in the levels of L-Arg in the extracellular compartment. This coincided with decreased expression of CD3 zeta chain in T lymphocytes and reduced proliferation of T lymphocytes [29]. Addition of excess L-Arg or competitive inhibitors of arginase 1 reversed the expression of CD3 zeta chain and recovered the proliferation of T lymphocytes. In contrast, inhibitors of iNOS inhibitors or arginase 2 failed to significantly reduce the extracellular levels of L-Arg or restore CD3 zeta chain function [29]. The loss of CD3 zeta chain was more significant for inhibition of $CD4^{+}$ T cell than of $CD8^{+}$ T cell function. This cancer related immunosuppressive activity is also observed in the spleen where splenic MDSCs also down-regulated of CD3 zeta chain expression in antigen-stimulated $CD4^{+}$but not $CD8^{+}$ T cells. Another mechanism where L-Arg metabolism augments T cell suppression is reported in individuals with prostate cancer [35]. Increased expression of arginase 2 and iNOS by cancer cells inhibited $CD8^{+}$ tumor-infiltrating lymphocytes. These $CD8^{+}$ tumor-infiltrating lymphocytes did not show any changes in the expression of CD3 zeta chain or other significant deficiencies in the TCR signaling mechanisms [29,41]. The third possible mechanism to inhibit T cell proliferation in tumors milieu is mediated by neutrophils, which showed increased levels of arginase 1 and depletion of extracellular L-Arg [15].The mechanism of immune suppression by neutrophils was associatedwith release of azurophil granules.

The upregulation of NO in several human cancers contribute to neoangiogenesis, tumor metastasis, tumor-related immune suppression, and autophagy by modification of signal transduction pathways [18,19].Unlike the increased activity of arginase, which leads to paralysis of T cell functions due to depletion of the CD3 zeta chain, increased levels of NO inhibit JAK-1 and -3, Erk, Akt, STAT5 phosphorylation, and blocks IL-2 signaling In T cells [12,19]. Nitric oxide modulates autophagy by S-nitrosylation of JNK1 and IKKβ. In the process, inhibition of JNK1 decreases Bcl-2 phosphorylation, and blocks the formation of the hVps34/Beclin 1 complex and related signaling pathway. Nitric Oxide induces inhibition of IKKβ, decreases AMPK phosphorylation and activates TSC2 and mTORC1 [42]. Overexpression of all isoforms of NOS impairs autophagosome formation through the JNK1–Bcl-2 pathway and in the process facilitates tumor growth [42]. S-nitrosylation of caspase 3 (at Cys163) results in decreased apoptosis of cells, where S-nitrosylation of p21Ras (at Cys118) results in activation of Ras and increased proliferation and migration of endothelial cells [19,43,44]. Increased levels of NO is associated with G:C to A:T mutation in oncogene p53 at 5-methylcytosine site in breast, brain and stomach cancer [3]. Increased levels of nitric oxide in MDSCs correlated with nitration of STAT1 (Ni-STAT1) and inhibition of phosphorylation of Try 701. The Ni-STAT1 coincided with reduced IFN-response in immune cells in animal models of adenocarcinomas which could be reversed in $iNOS^{-/-}$ mice [19].

Post-translational modification of DNA bases, amino acids, and chemokines have been reported in tumor micro environments with increased levels of reactive nitrogen species and peroxynitrite [3,45]. The production of reactive nitrogen species induce nitration of Chemokine (C-C motif) ligand 2- (CCL2) leading to formation of N-CCL2, which deters T cell infiltration into the tumor. Although N-CCL2 trapped tumor-specific T cells in the stroma that surrounded cancer cells, N-CCL2 attracted myeloid cells into the tumor [46-48]. The action of N-CCL2 was reversible by preconditioning of the tumor milieu with drugs (with peroxynitrite–scavenging activity) that block CCL2 nitration, which also facilitated CTL invasion of the tumor. Furthermore, NO induces changes in cellular phenotypes that promote immune suppression. For example, NO facilitated transformation of CD4+CD25+ Foxp3+ regulatory T cells from $CD4^{+}CD25^{-}$ T cells via p53 and IL-2 [16,19]. Recent studies have also shown a close association between NO and NK cell-mediated target-cell-killing. For instance, OX40 NK cell's cytotoxic activity is dependent on NO synthesis and inhibitors of NO synthesis impair NK cell-mediated target cell killing [49]. Several studies have shown that Th1 and Th2 cytokines competitively regulate arginase and iNOS within the intracellular biochemical pathways, negative feed-back loops, and competition for the same substrate [16]. The dual activation of arginase and iNOS in

myeloid cells unleash a powerful inhibitory signals preventing antigen-specific T lymphocytes to penetrate the tumor and eventually leading to apoptotic death of these antigen-specific T lymphocytes [35]. When iNOS and arginase 1 are induced together, peroxynitrite generated by iNOS under conditions of limiting L-arginine, causes activated T lymphocytes to undergo apoptosis [35]. Thus iNOS and arginase 1 may act separately or synergistically in vivo in cancer milieu. Cancer cells depend on intricate antioxidative reactions which supply large amounts of reducing equivalents that neutralize reactive oxygen species [48]. The isoforms of Pyruvate Kinase (PKMs) regulate anti-oxidative metabolism in cancer cells. Lung cancer cells which show oxidation of PKM2 (on Cys 358) exhibited decreased PKM2 activity shifting glucose-6-phosphate to the pentose phosphate pathway [17]. The activation of this pathway in cancer cells is essential for limiting reactive oxygen species accumulation, reducing oxidative stress, and tumor growth [17].

CCAAT-enhancer-binding proteins (C/EBP) are common regulators for iNOS gene expression, tumor induced tolerance, and immune suppression [50]. The regulation of MDSCs is influenced by the cytokines such as GM-CSF, G-CSF and IL-6, which are also dependent on CCAAT-enhancer-binding proteins. These cytokines promote the production of MDSCs from precursors present in bone marrow (BM). The BM-MDSCs were dependent on the transcription factor C/EBPβ for some of their effector functions [51]. Adoptive transfer of tumor antigen-specific CD8+ T cells in mice devoid of C/EBPβ in myeloid cells gave rise to promising therapy in established tumors [49-51]. These observations suggested that C/EBPβ is not only a key regulator of iNOS expression [52,53], but also regulates several other transcripts (such as GM-CSF, G-CSF and IL-6) that foster the immunosuppressive milieu structured by growing cancers [49-53].

Challenges and promises of targeting NO, reactive nitrogen and oxygen species -modified metabolites for immunotherapy and chemotherapy.

Small molecules and biologics are being developed to create isozyme-specific inhibitors for both nitric oxide synthases and arginases [19,54,55]. It should be noted that inhibitors of arginase 1 may interfere in the final cytosolic step of the urea cycle and the clearance of nitrogenous waste in the liver [19,39]. Thus arginase inhibitors may cause hyperammonemia. Selective iNOS antagonists such as N-nitro-L-arginine methyl ester have also been reported to inhibit arginase activity [12]. Another iNOS-selective inhibitor (1400W), inhibited growth of iNOS expressing tumors [19].

Co-activation of arginase and iNOS within the same environment can generate the production of several types of reactive nitrogen and oxygen species. For example, solid tumor environments which are in a hypoxic state with increased arginase 1 activity deplete L-arginine concentrations. The uncoupling reaction by the NOS reductase domain at low L-arginine concentrations release superoxides (O_2^-) [12], which reacts immediately with residual NO, leading to the formation of peroxinitrite [3,12]. Superoxide dismutases (SODs) which are unregulated in cancer cells and MDSCs are vital enzymes that eliminate superoxide radical (O_2^-) and therefore, protect cancer cells from injury induced by free radicals [56]. Several reports in the literate describe SOD as a potential target for immunotherapy [57]. Recent studies suggest that therapeutic failure of cancer correlated with high contents of free radicals and peroxynitrite in tumors [19,46,47]. Peroxynitrite has been described to react with several amino acids by directly modifying tryptophan, methionine, cysteine, phenylalanine, histidine and typtophan [45]. Peroxynitrite that is generated by tumor conditioned- MDSCs {by their iNOS and NADPH oxidases} can modify tyrosine residues on the T cell receptors and CD8 receptors, resulting in a decreased recognition of peptide–MHC complexes [58]. Since modifications of CCR2 chemokines in the tumor environment can block the entry of effector cells into the tumor environments, therapeutic intervention to avert the formation of N-CCR2 expression may be one approach for effective active immunotherapy [46].

Several immunization protocols have shifted the function of NO in immunotherapy. For example, Ribavirin (RBV) has been reported to change an immune response from Th2 toward a Th1 cytokine profile and also inhibit tetrahydrobiopterin synthesis: an essential cofactor for the generation of NO by NOS. Ribavirin decreases NO production and can be useful for sensitizing the treatment of melanomas, which are known to overexpress NOS [59]. Similarly, Kahn et al. [60] have shown that adjuvant based immunotherapy required functional iNOS synthesis before procuring immunoprotective effects of immunotherapy. These adjuvant immunized mice showed high expression of iNOS expression in spleen and lymph nodes [60]. Weiss et al. [61] showed that iNOS expressed in the macrophages controlled metastasis of renal carcinomas to the lung during IL-2/anti-CD40 immunotherapy [62]. These outcomes highlight the feasibility of using NO-modulating agents in combination-immunotherapy to build new strategies for cancer therapy.

Tumor cells can be targeted by increasing NO toxicity in the tumor microenvironment. Several studies have shown that targeted overloading of reactive oxygen and nitrogen species render cancer cells susceptible to oxidative damage by neutralizing the protective shield of the Warburg's effects [17]. For example, this can be achieved by transfection of iNOS gene which inhibited growth of several cancers [19]. Similarly, retroviral vector-expression iNOS and an anti-carcino embryonic antigen (CEA) antibody chain targeted tumor cells and induced apoptosis of malignant cells [62]. Injection of NO-releasing agents such as iNOS-expressing-microencapsulated cells amplified the levels of FAS and FASL protein in tumors and inhibited growth of colon and ovarian cancers [19]. These approaches also improved vascular density and radio-sensitivity in human colorectal cancer. Nitric oxide–producing hypoxic cells are sensitive to radiation due to impaired DNA damage repair mechanisms [63]. iNOS expression in colon cancer potentiates radiation treatment [19]. Activation of eNOS by low dose radiation increased blood flow into tumors and also increased radiation sensitivity in fibrosarcomas, liver, and lung cancers [19].

Non-steroidal anti-inflammatory drugs (NSAIDS) have been reported to play a protective role in various tumors [64]. We and others have previously reported the ability of Aspirin to inhibit iNOS at clinically relevant concentrations by acetylating NOS [65]. NSAIDS in combination with NO donors seem to be more effective therapeutic agents. Nitro-aspirin (NO-ASA) inhibits both arginase1 and iNOS activities in lymphoid organs, spleen, and tumor-related myeloid cells [66,67]. Administration of a mixture of NO-ASA in colon cancer models showed additive effects and strong synergism with increased reduction in tumor growth than single-drug treatments [68]. Nitro-aspirin may sensitize cancer cells for the effects of other antitumor drugs. Bronte et al., designed a NO donor designated as AT38. AT38 promoted a massive T cell infiltration into the tumor milieu in prostate cancer to augment tumor regression by immunotherapy [46]. Michaud et al. showed that reducing the activity of two genetic determinants: ATG5 and ATG7 (which regulate autophagy) by chemotherapy-induced autophagy in mouse tumor cells [69]. A preclinical study with

Hydroxychloroquine (HCQ) which also regulates autophagy (and iNOS expression) increases the efficacy of chemotherapy. Treatment of patients with metastatic melanomas in Phase I studies with Temsirolimus plus HCQ showed stabilization of tumors in 73% of the patients as compared to none with Temsirolimus alone [70].

Hypoxia is known to induce iNOS gene expression and up-regulate peroxynitrite production during malignant transformation [71]. HIF1α was shown to drive the sequence of events leading to upregulation of arginase 1 and iNOS in tumor macrophages [72]. Thus inhibition of HIF-1α can be a potential therapeutic target against cancer. HIF-1α inhibitors such as farnesyl transferase inhibitors and PI3K inhibitors are currently in clinical studies as anti-cancer drugs [19].

Summary and Conclusions

The role of NO in cancer like other biological systems is dependent on the effects and concentration of NO in the microenvironment. Nitric oxide participates in both direct and indirect reactions in cancer.Nitric oxide may directly interact with its molecular targets suchas metal ions, orit may indirectly react with oxygen and / or superoxide generating free nitrogen species which promote nitrosative and / or oxidative stress [6, 8, 66]. The tumor milieu in most cancers adapts itself to utilize NOto its advantage. Nitric oxide is part of an inflammatory process [4,12,22,23,65]. Chronic inflammation is associated with several cancers including infection-induced cancers [4,9]. The $iNOS^{-/-}$ animals showed impaired or delayed wound healing whereas arginine and NO administration improved healing [73]. Wound repair and tumor growth are analogous with respect to tissue remodeling, cell proliferation, angiogenesis, infiltration of inflammatory cells, regulation of cytokines and growth factors [73]. The inflammatory process which is involved in wound healing evades the normal wound healing process bysubtle modifications in the cancer milieu. In cancer, the Tregs, MDSCs and TAMs exhibit pro-tumoural functions and participate in suppression of adaptive immunity [2,10,14,27,28,29-32]. The immature myeloid cells from the bone marrow that are normally involved in woundhealing exhibit a state of immune suppression during carcinogenesis [33-35]. Furthermore, the loss of T cell receptor chain prevents antigen specific T cells response in tumors [29, 41]. Another mechanism to refocus the role of T cells by tumor generating NO is bynitration of chemokineswhich deters T cell infiltration into the tumor [46-48]. The dual actions of arginase and iNOS modulate innate and adaptive immunity to further promotetumors survival and growth [1, 2, 3]. The metabolic activity of tumor cells alsotransforms from normoxia to hypoxia by shifting to the utilization of the pentose phosphate pathway, limiting reactive oxygen species accumulation, reducing oxidative stress and tumor growth [17].

The role of NO in cancer is like a "double edge sword" since its differential actions are dependent on the concentration of NO in the microenvironment. Low levels of nitric oxide (<10nM) is normally associated with homeostatic physiological functions in the muscle and endothelial cells [73]. Hundred to three hundred nM of nitric oxide is sufficient for HIF-1expression, p53-phosphoration, DNA damage, anti-apoptotic response and MMP 9 expression in tumor cells [73]. More than1000nMof NO are associated withphagocytosis, apoptosis and cell death [73]. Thus, higher levels of NO promote anti-tumor activity and cell death, and sustained fluxes of low levels of NO promote tumor growth. Indeed, five years survival rate of cancer patients decrease with enhanced iNOS expression in the tumor [73].

In summary, the future of using NO and/or other free-radicals modulating agents with standard anti-cancer therapies appear promising. These approaches will require the consideration of immunization or therapeutic timetable and suppressive networks present in a particular tumor. The complete inhibition of iNOS activity and or other free radicals (which are also required for housekeeping activity) may not be necessary to reverse immune tolerance in cancer. These modulators may be effective in combination with other therapies: For example, Chemically Modified Tetracyclines with reduced anti-microbial activity such as ORACEA™ whose pharmacological properties include decreasing free radicals, inflammation, and nitric oxide production [3,19,73] can be a promising FDA-approved agents to sensitize cancer cells when combined with other anti-cancer drugs and therapies.

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Footnote

Identification and characterization of a particular subset of MDSC with possible multiple effector roles in tumorgenesis. Lauren P. Virtuoso, Jamie L. Harden, Paula Sotomayor, Fuminobu Yoshimura, Nejat K. Egilmez and Mehmet O. Kilinc. Presented at Society for Immunotherapy of Cancer. North Bethesda, November, 2011.

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