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REVIEW

The yin and yang of nitric oxide in cancer progression

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Nitric oxide (NO) is a short-lived, pleiotropic molecule that affects numerous critical functions in the body. Presently, there are markedly conflicting findings in the literature regarding NO and its role in carcinogenesis and tumor progression. NO has been shown to have dichotomous effects on cellular proliferation, apoptosis, migration, invasion, angiogenesis and many other important processes in cancer biology. It has been shown to be both pro- and antitumorigenic, depending on the concentration and the tumor microenvironment in question. NO is generated by three isoforms of NO synthase (NOS) that are widely expressed and sometimes upregulated in human tumors. Due to its vast array of physiological functions, it presents a huge challenge to researchers to discover its true potential in cancer biology and consequently, its use in anticancer therapies. In this study, we review the current knowledge in this area, with an emphasis placed on NO modulation as an anticancer therapy, focusing on NO-donating drugs and NOS inhibitors.

Introduction

Over 25 years ago, studies revealed that the free radical nitric oxide (NO) was in fact the previously described endothelium-derived relaxation factor (EDRF) when it was observed that both molecules had extremely similar biochemical, chemical and pharmacological properties (1). NO is highly unstable and has a half-life of 1–5 s *in vivo* (2). NOs actions are typically mediated through cyclic guanosine monophosphate (cGMP)-dependent or cGMP-independent pathways. In the presence of cGMP, NO targets the heme component of soluble guanylyl cyclase and couples with cGMP-dependent protein kinase-G, phosphodiesterases and cyclic nucleotide-gated channels (3). Alternatively, it can function independently of cGMP at low concentrations by (i) interacting with transition metal-containing proteins (3), (ii) interacting with proteins without attachment of the NO group (4) and (iii) modulating cell signaling through posttranslational protein modification, typically through formation of *S*-nitrosothiol (SNO) by coupling of a nitroso moiety to a reactive thiol group in specific cysteine residues, namely, *S*-nitrosylation (5).

Abbreviations: CaM, calmodulin; cGMP, cyclic guanosine monophosphate; DETA/NO, diethylenetriamine nitric oxide adduct; EDRF, endothelium-derived relaxation factor; EMT, epithelial–mesenchymal transition; ER, extrogen receptor; ERK, extracellular signal-regulated kinase; GSNO, S-nitrosogluthione; HCC, hepatocellular carcinoma; L-NAME, Nω-nitro-L-arginine methylester; L-NNA, NG-nitro-L-arginine; MMP, matrix metalloproteinases; NO, nitric oxide; NOS, NO synthase; NF-κB, nuclear factor-kappaB; RKIP, Raf-1 kinase inhibitor protein; SNAP, S-nitroso-N-acetyl-DL-penicillamine; SNO, S-nitrosothiol; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor.

NO is generated by three isoforms of NOS: neuronal (nNOS/NOS1), inducible (iNOS/NOS2) and endothelial (eNOS/NOS3). NOSs are dimeric enzymes, each monomer consisting of two distinct catalytic domains: N-terminal oxygenase domain and C-terminal reductase domain. Heme-5,6,7,8-tetrahydrobiopterin (BH4), oxygen and L-arginine bind to the N-terminal. The C-terminal region binds reduced nicotinamide adenine dinucleotide phosphate, flavin mononucleotide and flavin adenine dinucleotide (6). NOS catalyzes NO synthesis from L-arginine using nicotinamide adenine dinucleotide phosphate and molecular oxygen as cosubstrates (7,8). NO synthesis takes place in two steps: (i) NOS hydroxylates L-arginine to Nω-hydroxy-L-arginine and. (2ii) Nω-hydroxy-L-arginine is oxidized to L-citrulline and NO (9,10). NOs unpaired electron enables reactions with inorganic molecules (i.e. oxygen, superoxide or transition metals), structures in deoxyribonucleic acid (DNA), prosthetic groups (i.e. heme) or proteins, thus explaining its extensive biological activity (11).

NOS1 and NOS3 generate nanomolar concentrations of NO for very short-time periods (seconds/minutes), whereas NOS2 produces micromolar concentrations of NO over longer time periods (hours/days) (12). NO synthesis is reliant on calmodulin (CaM) binding to NOS, a calcium regulatory protein. NOS1 and NOS3 activation require high levels of resting intracellular calcium [Ca2+i] to bind with CaM (13). NO is synthesized by NOS1 and NOS3 following activation by CaM, through a conformational change of the flavin mononucleotide domain from its shielded electron-accepting (input) state to a new electron-donating (output) state. CaM is necessary for proper alignment of the domains (14). NOS2 binds to CaM with extremely high affinity even at the low [Ca2+i] characteristic of resting cells. Thus, the intracellular activity of NOS3 and NOS1 may be closely modulated by transient changes in [Ca2+i], whereas the activity of NOS2 is not (13).

At low concentrations, NO acts as a signal transducer and affects many physiological processes including blood flow regulation, iron homeostasis and neurotransmission, whereas it exerts a cytotoxic protective effect at high concentrations, e.g. against pathogens and perhaps tumors (15). Other bodily systems effected by NO are respiration, cardiovascular system (16), wound healing (17) and nervous system (18). Observations show that neurotransmitter responses are mediated by L-arginine-dependent NO generation in neurons and some neurons depend on NO for signal transduction (18,19). NO is expressed throughout the brain and is involved in many brain functions including pain perception, memory, relaxation of the jejunum, colon and rectum and the rewarding effects of addictive substances (20,21). In addition to its direct biological effects, NO can interact with reactive oxygen species such as superoxide radicals, to generate reactive nitrogen species (RNS), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$) (3). Peroxynitrite promotes cellular transformation by functioning as a powerful antioxidant and interacting with or oxidizing kinases and transcription factors, perturbing the cellular signaling network (22). Nitrites, nitrates, *S*-nitrosothiols and nitrosamines are metabolites of NO and mediators of its cytotoxic/cytoprotective effects, namely inhibition of mitochondrial respiration, protein and DNA damage leading to gene mutation, loss of protein function, necrosis and apoptosis (23).

It is well established that NO operates in a bimodal fashion. The dichotomous effects of NO on cancer arise from its ability to regulate various cancer-related events including tumor growth, migration, invasion, survival, angiogenesis and metastasis, depending on the concentration involved (see table 1). Additionally, the outcome of the aforementioned processes is determined by several additional factors including, but not limited to, NO flux, the chemical redox environment and duration of NO exposure (24,25). Other important

factors that highly influence the effects of NO are current cell cycle status and the tumor microenvironment (26,27). Additionally, studies investigating the effect of NO donation and deprivation on malignant cells show that redox status is crucial. Reactive nitrogen species and *S*-nitrosogluthione (GSNO) formation are affected by the relative abundance of oxidizing (reactive oxygen species) and reducing (glutathione) agents. This in turn can significantly influence the cytoprotective/cytotoxic effects of NO (3).

Nitric oxide and cancer

NO and iNOS are associated with numerous tumor types including lung (28,29), colon (30,31), breast (32–34), melanoma (35) and pancreatic cancers (36). Mechanisms by which NO and its derivative peroxynitrite induce inflammation-associated carcinogenesis include induction of DNA damage, suppression of DNA repair enzymes, post-translational modification of proteins, enhancement of cell proliferation, angiogenesis, metastasis, inhibition of apoptosis and antitumor immunity (22,37). Peroxynitrite can form DNA damaging 8-nitroguanine, a biomarker of inflammation-associated cancers (22). During inflammation, reactive nitrogen species and reactive oxygen species are released by activated inflammatory cells, attacking neighboring epithelial and stromal cells, altering function and initiating carcinogenesis (38). NOS, with nicotinamide adenine dinucleotide phosphate oxidase and cyclooxygenase, mediates hormone-induced oxidative/nitrosative stress in the rat, which leads to transformation of prostate epithelia into dysplasia (39). Another postulated mechanism is NO-mediated activation of cyclooxygenase-2 and the induction of CXCR4 (40). Clinical data show that NOS2 expression is an independent predictor of poor survival in women with estrogen receptor (ER)-negative breast tumors and correlated with tumor vascularization, accumulations of p53 mutations and activated epidermal growth factor receptor (32). NO has also been shown to induce CD44 and c-Myc, linked to stem cell-like phenotype in breast cancer (40). This mechanism of action by NO is mediated in part by the Ets-1 transcription factor in a Ras-dependent manner (41).

Nitric oxide and proliferation

The effect of NO on cellular proliferation clearly demonstrates NOs innate bimodality. In some cases, NO inhibits cellular proliferation and induces senescence; however, it can also stimulate cell growth, with similar effects seen on tumor growth and inhibition (23,42). Whether a cell is stimulated or inhibited to proliferate appears to be dictated by the concentration of NO involved (Figure 1). A number of studies have shown this bimodal effect. Low concentrations of NO donors (0.01–0.25 mM) increased proliferation of keratinocytes, whereas elevated concentrations (≥0.5 mM) induced cytostasis (43). Others assessed the cytotoxic effects of a series of novel furoxan-based NO-releasing derivatives of glycyrrhetinic acid at different concentrations on human hepatocellular carcinoma (HCC) and liver cells, demonstrating a cytotoxic effect on HCC cells (BEL-7402) at concentrations of 0.25–1.10 μM, whereas they had no cytotoxic effect on non-tumor liver cells. Further investigation using Greiss assay showed that the compounds generated 3- to 5-fold higher concentrations of NO in HCC cells compared with non-tumor liver cells cells. Use of hemoglobin, a NO scavenger reduced NO concentrations and inhibited cytotoxic effects (44). Concentration-dependent effects of NO on human leukemia line HL-60 was observed using the NO-donor diethylenetriamine nitric oxide adduct (DETA/NO), whereby 1–100 μM DETA/NO significantly stimulated proliferation and 250–1000 μM DETA/NO inhibited cell growth. This stimulation of growth was modulated by cyclin-dependent kinase 2 (Cdk2) activity and nitrosylation (45). Pheochromocytoma PC12 cells treated with DETA/NO for 24 h at low concentrations (25–50 μM) significantly stimulated proliferation, whereas concentrations of 150–250 μM produced between 200 and 600 nM NO and inhibited proliferation. It should be noted that the actual physiological concentration of NO in the cells is much lower, between 20 and 100 nM (46).

NO has been shown to inhibit the growth of gastric cancer cells (47), human endothelial cells at concentrations of ≥100 μM (48), breast cancer cells (MDA-MB-231) (49), neural precursor cells (50), human prostatic epithelial cell lines (51) and human bladder carcinoma cell lines (52). GIT-27NO, a novel NO donor, inhibited the growth of PC3 and LnCap prostate cancer cells xenografted into nude mice, in a concentration-dependent manner (53). Also, saquinavir (Saq-NO), a NO-derivative of the HIV protease inhibitor, induced apoptosis and production of proapoptotic BCL-2-interacting mediator of cell death (Bim), in PC3, whereas *in vivo* studies showed that Saq-NO inhibited PC3 xenotransplants to a greater extent than the parental compound (54). One mechanism described for the inhibition of cell proliferation by NO is the upregulation of the BRCA1/Chk1/p53/p21(Cip1/Waf1) pathway in human neuroblastoma cells, implicated in negative control of the cell cycle (55).

In contrast, there are numerous examples of NO stimulating cell proliferation. Nanomolar concentrations of NO increased cellular proliferation in breast cancer cells MDA-MB-231 and MCF-7 (56), choriocarcinoma JEG-3 cells (57) and ovarian carcinoma HOC-7 cells (58). Glioma stem cell (GSC) proliferation and tumor growth are promoted by NOS2, depending on NOS2 activity for growth and tumorigenicity, distinguishing them from non-glioma stem cells and normal neural progenitors (59). Mechanisms of NO stimulation of cellular proliferation include increased endogenous basic fibroblast growth factor (60), mitogen-activated protein kinase pathway (61), NOS3 activation by the phosphatidylinositol-3-kinase(PI3K)/Akt pathway and/or recruitment of heat shock protein 90 (24,25) and protein modification (5). Low to intermediate concentrations found to stimulate cellular proliferation align with doses associated with chronic inflammatory disease, which might explain its role in carcinogenesis and tumor progression. In an animal model that allowed for the regulation of NOS2 levels, low levels were associated with tumor progression and high levels with tumor regression (62).

Nitric oxide and epithelial–mesenchymal transition

Epithelial–mesenchymal transition (EMT) encompasses a series of events during which epithelial cells lose their epithelial characteristics and assume properties typical of cells of mesenchymal lineage. This requires complex changes in cell architecture and behavior (63). During early carcinogenesis, the tumor remains encapsulated by the basement membrane. EMT enables transformed cells to disseminate through fragmented basement membrane and intravasate into lymph or blood vessels and be transported to other organs (64). Lone carcinoma cells can then extravasate at secondary sites and either remain solitary (micrometastasis) or form a new tumor through mesenchymal–epithelial transition (MET) (64). The role of NO in EMT is unclear and akin to cellular proliferation contradictory. NO can both promote (directly by induction of tumor cell migration and invasion and indirectly through expression of angiogenic and lymphangiogenic factors in tumor cells) and inhibit (through DNA damage, gene mutation and apoptosis) tumor metastasis (23), depending on the concentration involved.

EMT-6 cell growth *in vitro* was reduced 50% by NO, but when treated cells were injected into mice, tumor growth and pulmonary metastases increased 2-fold. The effects were reversed when the NOS2 inhibitor Nω-nitro-L-arginine methylester (L-NAME) was employed (65). B16-BL6 murine melanoma cells produced larger numbers of lung metastases in $NOS2^{+/+}$ than in $NOS2^{-/-}$ mice (66). Cell invasion is a critical event in metastasis. Constitutively, NOS2 expressing human colorectal adenocarcinoma cells HRT-18 cells were 3-fold more invasive than the non-NOS2 expressing HRT-29 cells. Treatment of HRT-29 cells with NO donor and inflammatory cytokines increased their invasiveness by 40 and 70%, respectively, whereas NOS inhibitor 1400W decreased invasiveness by 50% (67). A study performed using mammary adenocarcinoma cell lines C3L5 (highly metastatic) and C10 (weakly metastatic) showed that C3L5 cells expressed higher levels of NOS3 and produced more NO than the C10 cells. The C3L5 cells had a higher efficiency of spontaneous metastasis and were more invasive. L-NAME inhibits invasion of both cell lines (68). Matrix

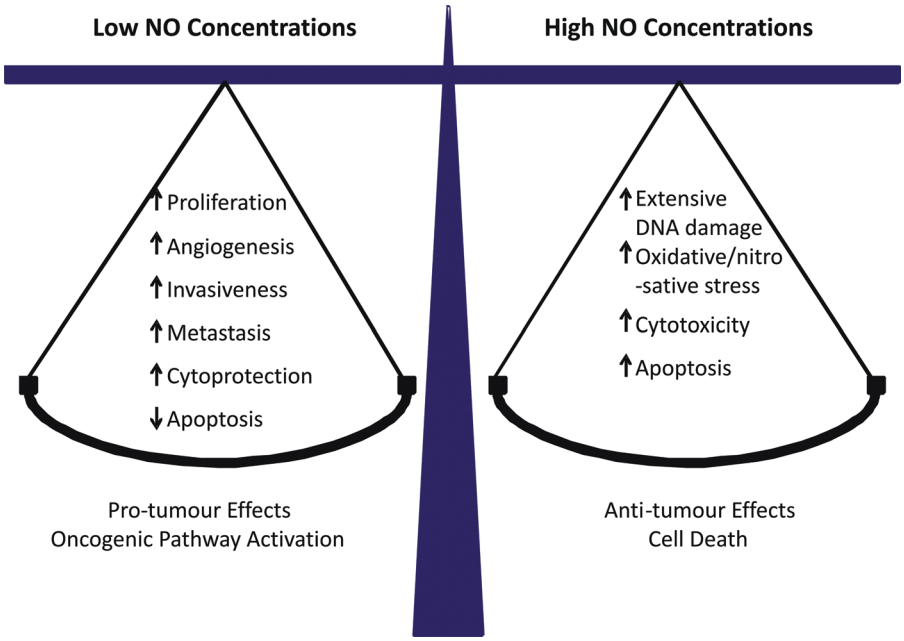

Fig. 1. Concentration-dependent effects of NO in cancer. Low levels of NO (<100 nM) promote increased proliferation and angiogenesis. Medium levels of NO (100–500 nM) promote increased invasiveness, metastasis, cytoprotection and repress apoptosis. High levels of NO (>500 nM) promote DNA damage, oxidative/nitrosative stress, cytotoxicity and apoptosis.

metalloproteinases (MMP) are a family of 'pro-metastatic' enzymes involved in the degradation of basement membrane proteins (69). A significant association was found between MMP-9 and NOS2 in hepatocellular carcinoma by immunohistochemistry, with MMP-9 and NOS2 strongly correlating with risk of recurrence (70). NO donated by *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) in colon adenocarcinoma cells increased MMP-2 and MMP-9 in a time-dependent manner through activation of extracellular signal-regulated kinase (ERK)-1/2 and activating protein 1 (AP-1) (71). MMP-1, MMP-3, MMP-10 and MMP-13 were transcriptionally enhanced by NO in the human melanoma cell line C32TG. Further investigation showed that NO-mediated MMP-1 was activated through ERK and p38 mitogen-activated protein kinase pathways, which are highly activated during tumor inflammation, resulting in tumor progression (72). Additionally, NO activated epidermal growth factor receptor and Src signaling via *S*-nitrosylation in ER-negative and basal-like breast cancer, leading to activation of β-catenin signaling (73). DETA/NO treatment led to decreased cell adhesion, decreased E-cadherin and concomitant increased expression of vimentin and β-catenin, indicating NO signaling results in ER-negative breast cancer EMT (73). The above studies demonstrate NOs ability to enhance tumor metastasis.

NO can also have anti-EMT effects. NOS2-transfected pancreatic cancer cells did not form tumors or metastases when injected into ectopic or orthotopic xenograft nude mouse models. Lack of tumorigenesis was attributed to NO-mediated apoptosis (74). Cells from three NOS2 null tumor cell lines, KX-dw1, KX-dw4 and KX-dw7, were injected into $NOS2^{+/+}$ and $NOS2^{-/-}$ mice. NOS2 null cells injected into $NOS2^{-/-}$ mice showed a higher proliferation rates and incidence of lung metastases than when transplanted into $NOS2^{+/+}$ mice (75). Transforming growth factor-β1-induced EMT in alveolar cells was reduced by treatment with NO donors (76). In another experiment, inhibition of NOS with L-NAME led to spontaneous EMT (76). Constitutively activated Snail, downstream of nuclear factor-kappaB (NF-κB), induces metastasis, whereas Raf-1 kinase inhibitor protein (RKIP) and E-cadherin repress metastasis. NO can inhibit Snail, thereby inducing RKIP, consequently inhibiting metastasis (77). RKIP overexpression mimics NO in tumor cell-induced sensitization to apoptosis. In resistant tumor cells, there appears to be a dysregulated NF-κB/Snail/YinYang1(YY1)/RKIP circuitry (78). Prostate metastatic cell lines treated with high levels of DETA/NO show decreased Snail, increased RKIP and E-cadherin, thereby inhibiting EMT. Additionally, tumor cells were sensitized to apoptotic stimuli (77). Topical treatment of NO-exisulind ultraviolet B-induced skin tumors in a murine model reduced EMT with decreased fibronectin, N-cadherin, SNAIL, Slug and Twist and increased E-cadherin (79). In contrast to the above studies, NO inhibited MMP-9 expression and activity in an orthotopic model of renal cell carcinoma (80). 12-*O*-tetradecanoylphorbol 13-acetate-induced MMP-9 is inhibited in an NO-dependent manner in MCF-7 breast cancer cells (81). Also, NO can destabilize MMP-9 mRNA (82). In HCC 1806 triple negative breast cancer cells, NO was found to upregulate N-Myc downstream-regulated gene 1 (NDRG1) in a dose- and time-dependent manner. NDRG1 expression in turn suppressed tumor cell migration (83).

Nitric oxide and angiogenesis

Angiogenesis is imperative for tumor progression, and NO is a key mediator of this process. Without the means to supply a tumor with nutrients and remove its waste products, tumor expansion and metastasis would be impossible. NO can promote or inhibit angiogenesis, depending on concentration and duration of exposure, intrinsic sensitivity of cells to NO and the activity and distribution of NO (23). NO acts as downstream mediator of multiple angiogenic effectors but its mechanisms are complex and involve multiple pathways (84). Angiogenic factors such as vascular endothelial growth factor (VEGF), sphingosine-1-phosphate, angiopoietins, estrogen, shear stress and metabolic stress stimulate the release of endothelium-derived NO by upregulation of NOS3 (85). Inhibition of NOS prevented prostaglandin E1-induced angiogenesis in the rabbit cornea *in vivo*, whereas angiogenesis was stimulated by NO-donor sodium nitroprusside (SNP) in these models (86). In vascular segments of rabbit thoracic aorta, VEGF stimulated a 2-fold NO release by pre-incubation with L-arginine (87). L-NAME blocked formation of capillary tubes induced by basic fibroblast growth factor and transforming growth factor-β on human umbilical venous endothelial cells in a 3-D gel, by terminating proliferative actions of growth factors and promoting differentiation of quiescent endothelial cells into vascular tubes (88). NO also exerts proangiogenic effects through inhibition of endogenous antiangiogenic factors. Thrombospondin 1 was reduced in vascular endothelial cells by addition of an NO donor

in a triphasic manner, with reduction of expression at 0.1 µM, increase at 100 µM and decrease at 1000 µM in an ERK phosphorylation-dependent fashion (89). L-NAME was found to increase angiostatin in vascular endothelial cells, thereby inhibiting angiogenesis (90). *In vivo*, the NOS2 inhibitor, L-nil, decreased the density of $CD31^+$ microvessels in immunodeficient mice injected with human melanoma or colon cancer cell lines (91). NOS inhibitor NG-nitro-L-arginine (L-NNA) induced acute and sustained reduction in human tumor blood volume, providing clinical evidence that inhibition of NOS has tumor antivascular effects (92). NO also promotes tumor vessel maturation and vessel dilation (23), in addition to recruitment of bone marrow-derived cells and perivascular cells, which enhance angiogenesis.

Similar to cellular proliferation and EMT, NO also exerts inhibitory effects on angiogenesis. The NO-producing sodium nitroprusside (NaNP) and NOS substrate L-arginine, inhibited angiogenesis in an *in vivo* model of the chick embryo chorioallantoic membrane (CAM) (93), as did isosorbide mononitrate (94). Low SNAP concentrations (0.1–0.3 mM) caused an increase in angiogenesis in microvascular endothelial cells, whereas SNAP concentration of 0.5–4 mM inhibited angiogenesis in a dose-dependent manner, both effects mediated by PKC and ERK acting on activating protein 1 (95). RKO and SW480 colon cancer cells treated with a novel NO-NSAID (non-steroidal antiinflammatory drug), GT-094, had downregulation of VEGF and its receptors, coinciding with inhibition of cellular proliferation and induction of apoptosis (96). Another NO-releasing drug, JS-K, inhib ited human umbilical vein endothelial cells HUVEC proliferation and migration, decreased cord junction number and cord length and blocked vessel growth whollyin a chick aortic ring assay, at IC_{50} values of <0.7 µM, while also inhibiting tumor angiogenesis *in vivo* (97).

Nitric oxide and apoptosis

Apoptosis involves DNA damage-induced programmed cell death mediated through activation of caspases and is a safeguard against cellular transformation (98). NO can regulate many of the molecules and organelles involved in apoptotic pathways, including p53, Bcl-2, caspases, mitochondria and heat shock proteins (99).

High levels of extracellular NO can induce apoptosis by direct membrane damage, inhibition of ribonucleotide reductase and inhibition of cellular ATP generation by mitochondrial electron transport enzymes aconitase and mitochondrial glyceraldehydes-3-phosphate dehydrogenase (100). NO can induce apoptosis through *S*-nitrosylation of NF-κB, glyceraldehydes-3-phosphate dehydrogenase, Fas receptor and Bcl-2 (5,101). P53 accumulates post NO-mediated DNA damage and can lead to apoptosis. Therein lies a negative feedback loop as this leads to transrepression of NOS2 (40). Other NO-mediated proapoptotic mechanisms include induction of mitogen-activated protein kinase-phosphatase-1 (MKP-1) and survivin downregulation (3). Endogenous NO catalyzed by NOS1 induced *S*-nitrosylation of GLuR6 in ischemia-reperfusion, activating GluR6/PSD95/MLK3 and JNK apoptotic signaling (102). Treatment of A375 human melanoma cells with capsaicin and resveratrol inhibited cell growth and promoted apoptosis by increasing NO production leading to p53 activation (103).

NO can also inhibit apoptosis via cell death protective protein expression, radical–radical interferences (104) and *S*-nitrosylation of caspases at their active site cysteines and cGMP (105). Inhibition of apoptosis by NO has been observed in endothelial cells, lymphoma cells, ovarian follicles, cardiac myocytes, vascular smooth cells and hepatocytes (106). Both exogenous (NO donor and NOS transfection) and endogenous (proinflammatory mediators) NO inhibited transforming growth factor-β1-induced EMT and apoptosis in mouse hepatocytes (107). In primary B-cell cultures isolated from B-cell chronic lymphocytic leukemia patients, the introduction of L-NAME substantially increased apoptotic DNA fragmentation in B-cell chronic lymphocytic leukemia cells (108). Endothelial cells pretreated with proinflammatory cytokines or NO donor mediated an increase in Bcl-2 expression and inhibition of Bax protein and consequently protected cells from ultraviolet A-induced apoptosis. This effect was abrogated by addition of NOS2 inhibitor (109). Tumor necrosis factor-α and actinomycin-D-treated MCF-7 cells treated with NO donors showed an inhibition of Bcl-2 cleavage and cytochrome c release, leading to blockage of apoptosis and caspase-3-like activation (110). Apoptosis is induced and preneoplastic colonic lesions are prevented through the inhibition of NOS2 and NF-κB when dolastatin-15, a mollusk linear peptide, and celecoxib, a selective cyclooxygenase-2 inhibitor are used (111). Glycochenodeoxycholate-induced apoptosis of hepatocytes can be enhanced or abrogated by NO. The use of NO donors again demonstrated NOs dual role in apoptosis: low concentrations (0.1 mM SNAP/0.15 mM SNP) reduced apoptosis and high concentrations (0.8 mM SNAP/1.2 mM SNP) increased it, whereas glycochenodeoxycholate-induced apoptosis of hepatocytes was enhanced with NOS2 inhibitor 1400W. Therefore, in this case, endogenous iNOS inhibited apoptosis, but the exogenous NO played a dual role during the glycochenodeoxycholate-induced apoptosis (112).

Nitric oxide and radiotherapy

The effects of NO on radiation are not clearly understood. However, reports suggest that NO confers both radiosensitization and radioprotection to tumor cells (113). In human lung cancer, H1299 cells expressing wtp53, a range of NO concentrations induced opposing effects on radiosensitivity and chromosome aberrations, depending on cell cycle phase (114,115). Using NO donors and NOS inhibitors, NO extended significant radioprotection to mice receiving whole body irradiation (116). Radioprotection of soft tissue and prevention of apoptosis in irradiated mouse muscle *in vivo* were observed presumably by increasing NO levels through inhibition of CD-47 expression (117). The radiosensitivity of A549 and H3255 non-small cell lung cancer cells was enhanced by reduction in the levels of NO induced by radiation and N(G)-monomethyl-L-arginine-monoacetate (118). Tumor growth was enhanced by irradiation-induced increased NOS3, accompanied by endothelial cell migration, sprouting and formation of capillary-like structures on matrigel plugs implanted in mice, thereby demonstrating an increase in angiogenesis. Irradiation dose dependently induced the activation of the proangiogenic NO pathway in endothelial cells via NOS3 expression and phosphorylation (119). Use of NOS inhibitor L-NNA repressed irradiation-induced NO-mediated angiogenesis. Survival of mice in a model of squamous carcinoma that received L-NNA prior to ionizing radiation was significantly increased, with 80% reduced tumor blood flow and 82% cell death (120).

NO can radiosensitize mammalian cells and especially hypoxic cells (121). Oxygen concentration is critical. At anoxic (<0.01% O_2) conditions, increased NOS expression induced by cytokines did not correlate with NO production, most probably due to lack of O_2 availability for NO generation, and HT-1080 and MDA-MB-231 tumor cells used in the study were not sensitive to radiation. However, when O_2 concentration was increased to 1%, the cells were sensitized with increased NO production (122). This illustrates that NO can confer radiosensitivity to tumor cells. Stewart *et al.* (123) radiated PC3 prostate stromal and cancer cells with and without the addition of NO-sulindac, demonstrating that addition of NO radiosensitized the prostate cancer cells, via inhibition of the hypoxia response and increasing DNA double-strand breaks, whereas the stromal cells were not affected. This is promising for adjuvant therapy to radiation for prostate cancer patients. In a range of tumor cells pretreated with DETA/NO followed by irradiation, the level of radiosensitization observed correlated with the degree of malignancy, suggesting NO specifically targets tumor tissue (124). In colorectal cancers, NO and ionizing radiation work together to activate p53, inducing apoptosis and increasing radiosensitivity (125). Also radiosensitization of tumor cells by NOS2 endogenous production of NO (via, proinflammatory cytokines) was transcriptionally controlled by hypoxia and NF-κB (126).

Nitric oxide and epigenetics

The involvement of NO in the field of epigenetics has recently emerged. Epigenetics is defined as heritable changes to chromatin, which regulate gene expression without altering the underlying DNA sequence (127). Epigenetic mechanisms include DNA methylation, small RNA activity and histone modifications, each of which can be modulated by NO (128). One mechanism of epigenetic contribution

Table I. Summary of effects of NO donors, NOS inhibitors and other methods of NO modulation on proliferation, EMT, angiogenesis and apoptosis*

	Cell type	NO modulation	Effect of NO on function	Reference
Proliferation	Keratinocytes	0.01–0.25 mM DETA/NO	Increase	(43)
	HL-60 human leukemia	1–100 μM DETA/NO	Increase	(45)
	PC12	25–50 μM DETA/NO (20–100 nM NO)	Increase	(46)
	MDA-MB-231 and MCF-7 breast cancer cells	10–60 μM/l DETA/NO (2–52 nM/l NO)	Increase	(56)
	JEG-3 choriocarcinoma cells	10 μM SNP decreased proliferation	Increase	(57)
	Ovarian carcinoma cells	Progesterone-induced NO	Increase	(58)
	Glioma stem cells	NOS2-directed shRNAs and 1400W decreased proliferation	Increase	(59)
	Keratinocytes	≥0.5 mM DETA/NO	Inhibit	(43)
	BEL-7402 HCC Cells	0.2–1.10 μM furoxan/glycyrrhetinic acid (GA) (<20 μM NO)	Decrease	(44)
	HL-60 human leukemia	250–1000 μM DETA/NO	Inhibit	(45)
	PC12	150–250 μM DETA/NO (200–600 nM NO)	Inhibit	(46)
	Gastric cancer	1 mM sodium nitroprusside (SNP)	Inhibit	(47)
	Human endothelial cells	≥100 μM SNP, GSNO and SNAP	Inhibit	(48)
	Breast cancer	1 mM DETA/NO (0.5 μM NO)	Inhibit	(49)
	DAOY medullablastoma cells	100 μM DETA/NO	Inhibit	(50)
	Human prostatic epithelial cells	4.6–12 μM nitrosulindac	Inhibit	(51)
	Human bladder carcinoma cells	14.5–23.1 μM NO-NSAID	Inhibit	(52)
	PC3 and LnCap	150–300 μM GIT-27NO (>10 μM NO)	Inhibit	(53)
EMT	EMT-6 (injected into mice)	Lipopolysaccharide/interferon stimulation (25 μM nitrite)	Increase in metastases	(65)
	Murine melanoma cells	$NOS2^{+/+}$ mice	Increase in metastases	(66)
	HT-29 human colorectal adenocarcinoma cells	50 nM DETA/NO	Increase in invasiveness	(67)
	C3L5 mammary adenocarcinoma cells	Endogenous NO (80 μM nitrite/nitrate)	Increase in metastases and invasiveness	(68)
	WiDr colon adenocarcinoma cells	15.6 μM SNAP (~175 nM NO)	Increase in MMP-2 and MMP-9	(71)
	Human melanoma cells	0.25 mM SNAP	Increase in MMP-1, MMP-3, MMP-10 and MMP-13	(72)
	MDA-MB-468 breast cancer cells	0.5 mM/l DETA/NO (>300 nM/l NO)	Increase EMT	(73)
	PC3, AGS, DLD-1, HT-1080, MDA-MB-453, 253J BV, SKOV3.jp1 and SN12PM6	Ad.NOS2 (120 μM nitrite/nitrate generated in *in vitro* system)	Inhibit metastases	(74)
	Fibrosarcoma cell ($NOS2^{-/-}$))	$NOS2^{+/+}$ mice, (20–60 μM nitrate/nitrite)	Decrease metastases	(75)
	Alveolar cells	2 mM L-NAME induced EMT	Decrease EMT	(76)
	Prostate metastatic cells	1000 μM DETA/NO	Inhibit EMT	(77)
	Skin tumors (murine)	NO-exisulind (5 mg per mouse)	Decrease EMT	(79)
	Renal cell carcinoma	JS-K (4 μM/kg)	Decrease metastases and inhibit MMP-9	(80)
	MCF-7 breast cancer cells	500 μM DETA/NO	Inhibit MMP-9	(81)
	HCC 1806 breast cancer cells	500 μM DETA/NO (~80–750 nm NO)	Inhibit cell migration	(83)
Angiogenesis	Rabbit cornea model	SNP	Increase	(86)
	Vascular endothelial cells	0.1–10 μM DETA/NO (~2 nM NO) decreased thrombospondin 1, increased cellular outgrowth	Increase	(89)
		L-NAME decreased tube formation and increasing angiostatin	Promote	(90)
	Human melanoma and colon cancer cells	L-nil decreased CD31 + microvessel density	Promote	(91)
	Non-small cell lung cancer, prostate cancer and cervical cancer	0.1–0.9 mg/kg L-NNA reduced human tumor blood volume	Promote	(92)
	Microvascular endothelial cells	SNAP 0.1–0.3 mM (12–26 μM nitrite/nitrate)	Increase	(95)
	Chick embryo chorioallantoic membrane	25–210 nM/disc isosorbide mononitrate	Inhibit	(93,94)
	Microvascular endothelial cells	0.5–4 mM (36–280 μM nitrite/nitrate)	Inhibit	(95)
	Colon cancer cells	NO-NSAID 50 μM	Decrease VEGF and MMP	(96)
	HUVEC	JS-K <0.7 μM	Inhibit	(97)

Table I. *Continued*

	Cell type	NO modulation	Effect of NO on function	Reference
Apoptosis	A549 and Jurkat T cells	*S*-nitrosocysteine (1 mM) – (30 pM/mg SNO)	Increase	(113)
	Ischemia model (rat)	Endogenous NO	Increase	(102)
	A375 human melanoma cells	Capsaicin and resveratrol no access to paper	Induce	(103)
	Hepatocytes (rat)	SNAP/SNP 0.8 mM/1.2 mM	Increase	(112)
	Mouse hepatocytes	NOS2 transfection (0.07 μM/μg nitrite)	Inhibit	(107)
	B-cell chronic lymphocytic leukemia cells	Induced by CD23 ligation	Inhibit	(108)
	Endothelial cells	DETA/NO 50–1000 μM (4–5 μM NO)	Inhibit	(109)
	MCF-7 breast cancer cells	SNAP (140 μM)	Inhibit	(110)
	Hepatocytes (rat)	SNAP/SNP 0.1 mM/0.15 mM	Inhibit	(112)

*Care should be taken when comparing concentrations of NO donors as the amount of NO produced and its half-life differs greatly. In addition, the efficacy of NOS inhibitors also differs substantially, and effects of NO donors and inhibitors are dependent on experimental conditions, in particular duration of exposure and method of quantification of proliferative, EMT, angiogenic and apoptotic response.

to oncogenesis is via DNA hypermethylation, leading to gene silencing and downregulation of tumor suppressor gene expression (127). NO regulates epigenetic effects both directly and indirectly. NO can be synthesized in the nucleus, thereby enabling its direct effects, by impacting functional activity of histone-modifying enzymes, e.g. histone deacetylase 2 in neurons (5). NO-dependent histone modification includes hyperacetylation of histone H3 in oral cancer, correlating with upregulated nucleophosmin and glyceraldehydes-3-phosphate dehydrogenase levels (129). Epigenetic regulation is important in cellular reprogramming. An epigenetically active cocktail of all-trans retinoic acid, phenyl butyrate and DETA/NO reprogrammed human cardiac mesenchymal stromal cells into functionally competent cardiovascular precursors (130), via increased H3K4Me3 and H4K16Ac and reduced H4K20Me3 and H3s10P. This led to reduced proliferation and chromatin relaxation, and increased the expression of miR-133a, miR-210 and miR-34a. Whether NO has similar effects on cancer stem cells is unknown.

Indirect epigenetics includes *S*-nitrosylation and regulation of transcription factors e.g. NF-κB, HIF-1 and activating protein 1 (131). Also tyrosine-nitration can mediate NO epigenetic effects. Yakovlev *et al*. (132) showed that MCF-7 and Saos-2 cells treated with low concentrations of NO increased p53 activation due to p53 nitration at tyrosine 327. In contrast, tyrosine-nitration of p53 in human glioblastoma led to protein inactivation (133). In prostate cancer, ER-β/eNOS complex silenced glutathione *S*-transferase P (GSTP1) via local chromatin remodeling. Introduction of eNOS inhibitor reversed GSTP1 gene silencing (134). Colorectal cancer carcinogenesis may be influenced by NO-mediated epigenetic regulation. Colonic inflammation induction in a rat model using 2,4,6-trinitrobenzene sulfonic led to intercellular adhesion molecule-1 expression by translocation of NF-κB to the nucleus. Using the NO donor GSNO, NF-κB DNA binding could be blocked, via transcriptional downregulation of global histone deacetylase 3 and decreased DNA interaction at the intercellular adhesion molecule-1 promoter containing the binding motifs of NF-κB, and suppression of H4K12 acetylation, thus suppressing inflammation (135). Conversely, NO promotes cellular senescence and upregulation of miR-21 in Crohn's disease (42). Crohn's disease patients are at increased risk of colon cancer, and miR-21 is associated with aggressive colon cancer (136,137). Deletion of NOS2 in a murine lung cancer model decreased tumor growth, mir-21 expression and inflammatory responses initiated by oncogenic KRAS, suggesting cooperation between v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog and NOS2 in lung tumorigenesis and inflammation (28).

Nitric oxide as an anticancer agent

It comes as no surprise that NO has been exploited as an anticancer target for some time. Various approaches have been investigated, including NO as radiotherapy and chemotherapy sensitizers, NOS inhibitors and novel NO-donating drugs (5). In a phase II study, low doses of NO releasing glyceryl trinitrate were administered to prostate cancer patients following primary treatment failure. NO significantly reduced hypoxia-induced cancer progression, as measured by prostate-specific antigen doubling time (138). Various forms of NO-donor drugs have been investigated on a range of cancer cell lines, including NONOates DEA/NO and PAPA/NO, *S*-nitrosothiols (i.e. SNAP and GSNO), which are effective antiproliferative agents against many cancer cell lines (5). Investigation of the inhibitory effect of JS-K on androgen receptor signaling in castration resistant 22Rv1 prostate cancer cells, showed attenuation of intracellular functional androgen receptor, due to generation of high NO levels, coupled with significant growth inhibition (139). NO-NSAID donor drugs are potential anticancer drugs derived from traditional NSAIDs, modified to include NO-releasing moiety via a linking spacer (140). NO-NSAIDs were originally developed to overcome side effects of NSAIDs, such as gastrointestinal complications, while maintaining the positive effects of the parental NSAID in addition to the anticancer properties of NO (141). NO-NSAIDs exert their anticancer function through inhibition of proliferation and cell cycle, induction of apoptosis and modulation of Wnt and NF-κB signaling pathways (141).

NO-NSAIDs inhibited HT-29 colon adenocarcinoma cells substantially more so than the parental NSAID alone (142). F344 rats with azoxymethane-induced colon cancer subjected to NO-Aspirin at 40 and 80% of the maximum tolerated dose for two weeks, demonstrated significantly reduced tumor incidence, multiplicity and reduced tumor NOS2 activity (143). It remains unclear, however, whether it is in fact the NO moiety that infers its biological effects, as opposed to the spacer (144). NOS inhibitors have also been studied extensively. In a KC mouse model of preinvasive pancreatic cancer, treatment with L-NAME, and use of NOS3$^{-/-}$ mice, attenuated development of pancreatic lesions (145). NOS2 inhibitor 1400W inhibited tumor growth by 54 and 52% (in combination with CXCR4 antagonist) in a xenograft mouse model of human adenoid cystic carcinoma. Significant reduction in lung metastasis, correlating with a reduction in microvessel density and an increase in tumor stroma and parenchyma, was also noted (146). The weakly tumorigenic and non-metastatic fibrosarcoma (QR-32) assume a highly malignant tumor phenotype once transplanted *in vivo* along with gelatin sponge into C57BL6 mice. Mice were treated with NOS2 inhibitor amino guanidine, before and after inoculation. After 4 weeks, cells derived from the arising tumors were transplanted into normal mice. Cells derived from the amino guanidine-treated mice tumors transplanted 4 weeks later had a significantly reduced incidence of metastases compared with controls (147). L-NAME fed C57BL/6 mice transplanted with C26GM colon carcinoma and RMA T lymphoma had a significantly reduced tumor volume compared with controls (148). A study investigated the effect of NO scavengers, non-isoform-selective NOS inhibitors and NOS2 selective inhibitors, on the growth and vascularization of rat carcinomasarcoma. Results showed that the NO scavengers and the L-NAME caused a 60–75% reduction in tumor growth, whereas the NOS2-specific inhibitors had no effect. This would suggest that a complete inhibition of NO is required for antitumor effects, rather than NOS2 alone (149). Overexpression of NO by human osteocalcin in PC3 xenografts yielded tumor growth delays of up to 52.2 days along with the upregulation of NOS2 and cleaved poly (ADP-ribose) polymerase protein expression (150). ZR-75-1 breast cancer cells transfected with the NOS2 gene delivered by the novel designer biomimetic vector, underwent 62% cytotoxicity and <20% clonogenicity (151).

Conclusions

Extensive investigation has been carried out on the effects of NO on cancer biology. At first glance, the data appear conflicting and inconclusive. This has led to difficulty in deciphering its role in tumor biology. However, upon closer examination of the available literature, it becomes quickly apparent that these conflicting results are in reality due to the biphasic nature of NO-mediated cellular effects, which are dependent on NO concentration experienced by the cells, NO flux, the chemical redox environment and the duration of NO exposure (24,25). Consequently, NO can have both pro- and antitumorigenic effects (23), which in terms of its potential as a therapeutic target provides us with multiple options. With respect to tumors dependent on NO for their growth 'NO-addicted', we can either use NOS inhibitors to inhibit tumor promoting NO fluxes or NO donors to increase NO levels in the tumor to inhibitory cytotoxic levels (152). In tumors that are not NO-dependent, it is likely that NO donors be cytotoxic to the tumor, as these cells are not adapted to a NO-rich environment, this may be particularly useful for radiosensitization. Although the role of NO on cellular proliferation, EMT, angiogenesis, apoptosis and radiotherapy is well understood, its role in epigenetic regulation of cancer is an emerging area of interest. Further research is needed to decipher its impact on epigenetic mechanisms including DNA methylation regulation, chromatin remodeling, modulation of miRNAs and also new emerging non-coding RNAs such as lncRNA, snoRNA and piRNA. In conclusion, the multifaceted nature of NO in tumor biology demonstrates its role as a master regulator of tumor progression, with the ability to regulate multiple cellular processes in a dynamic fashion.

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