Review

Nitric Oxide and Aggressive Behavior of Lung Cancer Cells

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Abstract. *Nitric oxide (NO) is an important cell signaling molecule whose level is frequently elevated in many tumors including that of lung. Cellular phenotypes and behaviors are influenced by NO found in proximity to the tumor, namely the tumor microenvironment. In lung cancer, a high level of NO is linked to advanced stage and poor survival of patients. This review describes the promotory role of NO in aggressive behavior of lung cancer cells with a focus on apoptosis and anoikis resistance, cell migration and invasion and cancer stem cells, all of which are key determinants of cancer relapse and metastasis. We specifically address the effects of NO on the modulations of structure, stability, function and activity of key proteins, and discuss how these changes could affect aggressive behavior. Such knowledge will encourage additional experimental and clinical investigations that contribute to the understanding of the molecular basis of cancer pathophysiology which could lead to targeted cancer therapy.*

Lung cancer is one of the most common cancers and the leading cause of cancer-related death worldwide that kills more than one million people each year (1). Major hurdles for lung cancer treatment are the poor responses to chemotherapy and late diagnosis at locally advanced or metastatic stage (2-4). Increasing evidence has indicated that nitric oxide (NO) signaling is implicated in the pathophysiology of many types of cancer, particularly in tumorigenesis and cancer progression in various tissues, including the brain, breast, prostate, pancreas and lung (5, 6).

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A relatively short-lived free radical, NO is renowned for its role as a messenger or effector signaling molecule, is generated endogenously from the metabolism of L-arginine to L-citrulline through a complex reaction catalyzed by various NADPH-dependent enzymes called nitric oxide synthase (NOS) (7). NOS exists in three isoforms, each with a distinct function and working conditions: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) (8). Up-regulation of NOS and elevated NO activity is frequently detected in tumor microenvironments (9, 10). NO can be derived from tumor cells themselves and from neighboring cells. Due to its lipophilic nature, NO from neighboring cells, *e.g.* endothelial cells in the microvasculature or immune cells and stromal cells in tumors, can freely diffuse across cellular membranes and ultimately affect tumor phenotypes and behavior (9, 11). Depending mainly on the concentration and duration of NO exposure, NO appears to exert dichotomous roles in cancer (promotory or inhibitory), a relatively low (micromolar range), but sustained level of NO generally promotes tumors (12, 13).

In patients with lung cancer, a high level of exhaled NO and its metabolites nitrite and nitrotyrosine, as well as the serum nitrite/nitrate, an estimate of *in vivo* NO, were observed compared to healthy controls (14-16). It was further demonstrated that high levels of serum nitrite/nitrate were associated with advanced-stage lung cancer and poor survival rate of patients (16), suggesting that NO might be involved in aggressive tumor phenotypes and metastasis. In this review, we summarize the current findings and understanding of NO in aggressive phenotypes of human lung cancer and discuss its effect on key proteins in relation to chemotherapeutic resistance and each step of metastasis.

Chemotherapy Resistance in Lung Cancer

Chemotherapy is a major treatment modality for cancer, including lung cancer (2). Intrinsic chemotherapy resistance

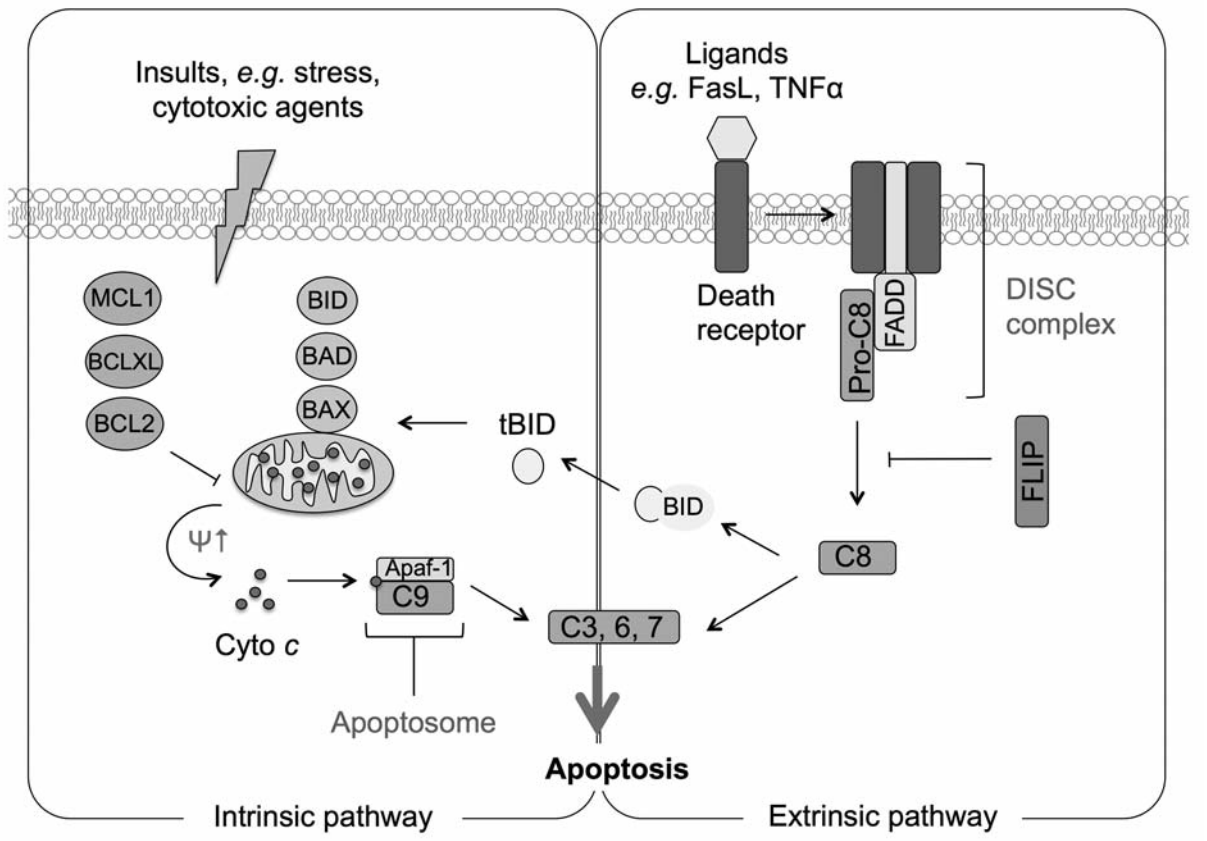

Figure 1. *Schematic overview of the major pathways of apoptosis. An intrinsic pathway of apoptosis is induced by various insults such as cytotoxic agents and cellular stress that lead to an alteration in mitochondrial membrane potential (ψ), which is regulated by B-cell lymphoma 2 (BCL2) family proteins, a release of cytochrome c (Cyto c) and an activation of caspase (C)-9. On the other hand, the extrinsic pathway of apoptosis involves the binding of death receptors to their respective ligands and an activation of caspase (C)-8, a process which can be inhibited by FLICE-inhibitory protein (FLIP). In addition, BH3 interacting-domain death agonist (BID) can be cleaved by caspase-8 and transduces these death signals to the intrinsic pathway.*

is often observed in non-small cell lung cancer (NSCLC) cells, while acquired resistance over the course of treatment is often reported in small-cell lung cancer (SCLC) (17). Most, if not all, chemotherapeutic agents induce cancer cell death through the induction of apoptosis (18, 19). Thus, the apoptotic machinery is important in dictating the success of chemotherapy, failure to induce apoptosis leads to chemotherapy resistance (20). NO has been shown to possess both pro- and anti-apoptotic activities, depending on the cellular context, redox status and dosage of NO (21). NO at high concentrations induces apoptosis *via* oxidative stress and caspase activation (22, 23). On the other hand, accumulating evidence has indicated that the physiological level of NO inhibits apoptosis in several cell types, including lung cancer cells, as detailed below.

NO and apoptosis resistance. Apoptosis is a process of programmed cell death that may be initiated either through the intrinsic mitochondrial pathway or extrinsic death receptor pathway (Figure 1). In general, the intrinsic pathway is activated in response to a variety of death signals, *e.g.* DNA damage, oxidative or nitrosative stress and cytotoxic agents, leading to the permeabilization of outer membrane of mitochondria, which is controlled by the balance of the pro-apoptotic (*e.g.* BCL2-associated X (BAX), Bcl-2 homologous killer (BAK), Bcl-2-associated death promoter (BAD) and BH3 interacting-domain death agonist (BID)) and anti-apoptotic (*e.g.* B-cell lymphoma 2 (BCL2), B-cell lymphoma-extra large (BCL-xL) and myeloid leukemia cell differentiation protein (MCL1)) BCL2 family proteins, and subsequent release of cytochrome *c* (24). The released cytochrome *c* binds to caspase adaptor molecule apoptotic protease activating factor 1 (APAF1) and recruits initiator caspase-9, forming a complex called the apoptosome that promotes activation of effector caspases (*e.g.* caspase-3, capase-6 and caspase-7) to induce apoptosis. The extrinsic

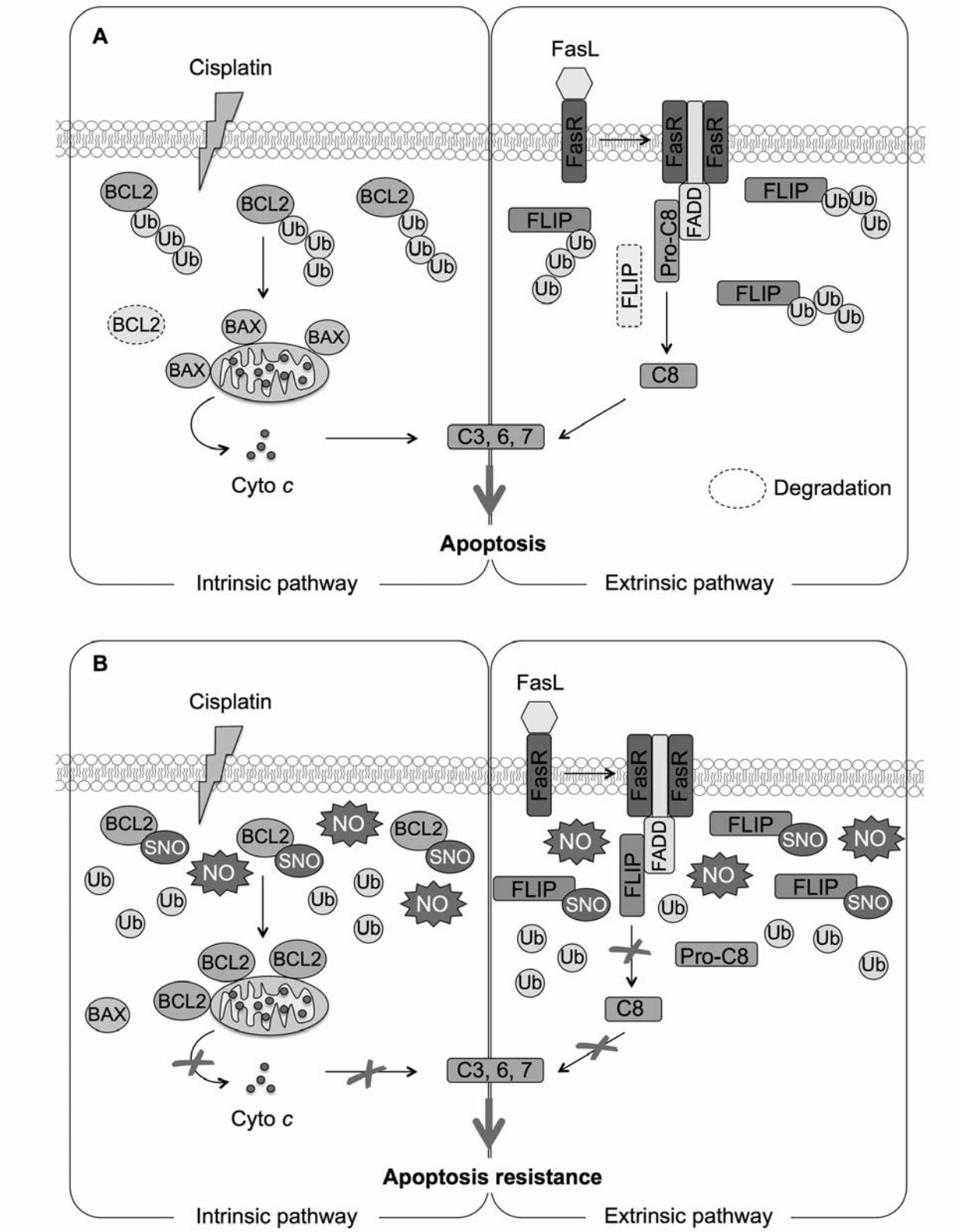

Figure 2. *Schematic representation of the regulatory effects of NO on intrinsic and extrinsic apoptosis pathways. A: Cisplatin and Fas ligand (FasL)-mediated apoptosis through the ubiquitin (Ub)-proteasomal degradation of B-cell lymphoma-2 (BCL2) and FLICE-inhibitory protein (FLIP), respectively. B: S-Nitrosylation (SNO) of BCL2 and FLIP by NO prevents its ubiquitin-proteasomal degradation and inhibits cellular apoptosis.*

pathway is activated through the binding of death-inducing ligands such as Fas (CD95), tumor necrosis factor-alpha (TNFα) or TNF-related apoptosis-inducing ligand (TRAIL) to their respective cell surface death receptors, resulting in the recruitment and activation of initiator caspase-8 to death-inducing signaling complex (DISC), which subsequently activates effector caspases to cleave cellular substrates (25).

NO was shown to suppress apoptosis induced by various agents through both intrinsic and extrinsic pathways. In the intrinsic pathway, NO was shown to render lung cancer cells to resistance to apoptosis induced by cisplatin chemotherapy through mechanisms that involve BCL2 up-regulation by preventing its degradation through ubiquitin-proteasome pathway that is mediated by *S*-nitrosylation a major post-translational protein modification resulting from a coupling of nitroso moiety to reactive cysteine thiol (SH), of BCL2 (Figure 2) (26). In the extrinsic pathway, *S*-nitrosylation of FLIP (FLICE-inhibitory protein) is an important mechanism rendering FLIP resistant to ubiquitination and proteasomal degradation by Fas death ligand and ultimately inhibits apoptosis (Figure 2) (27). It has been shown that defects in Fas-mediated apoptosis affects not only immune cells, but also the responses of tumor cells to chemotherapy and irradiation. Recently, *S*-nitrosylation of FLIP was additionally revealed to link to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation through the disruption of FLIP/retinoic acid inducible gene-1 (RIP1) complex and RIP1 redistribution to cell membrane. Impairment of RIP1 translocation impaired anti-apoptotic function of FLIP, indicating that *S*-nitrosylation of FLIP is necessary to confer apoptosis resistance (28). Since increased expression of BCL2, FLIP and NO are frequently observed in chemotherapy-resistant lung cells, *S*-nitrosylation of BCL2 and FLIP could be a key mechanism of chemotherapy resistance.

Regarding the adaptive responses of lung cancer cells to chemotherapy, our group found that relatively long-term (7-14 days) exposure of the cells to NO resulted in significant up-regulation of survival-related proteins including ATP-dependent tyrosine kinase (AKT), caveolin-1 (CAV1) and anti-apoptotic BCL2 (29). Up-regulation of AKT and BCL2 was further found to render lung cancer cells to apoptosis resistance induced by various chemotherapeutic agents, including cisplatin, etoposide and doxorubicin, while up-regulation of CAV1 was related only to doxorubicin and etoposide resistance.

Cancer Metastasis

Cancer metastasis is a multi-step process in which a restricted proportion of tumor cells spread from the primary tumor to form a secondary tumor at distant sites. It is the leading cause of cancer-related death and is a prime target for cancer therapy (30). To metastasize, tumor cells must sequentially follow the principal steps: (i) cell detachment from the primary tumor; (ii) local invasion of host stroma into the lymphatic and blood circulation (intravasation); (iii) survival of cells in the circulation (anoikis resistance); (iv) adhesion of cells to capillary walls; (v) cell invasion and penetration out of the circulation (extravasation); and colonization and formation of secondary tumors (31, 32). NO has been shown to be involved in all the essential steps of lung cancer metastasis as detailed below.

NO and anoikis. During metastasis, the loss of cell interaction with neighboring cells and the extracellular matrix (ECM) triggers apoptotic cell death called anoikis, which is an important biological mechanism inhibiting cancer cell dissemination (33). Thus, anoikis resistance may facilitate distant metastasis. This consensus is supported by clinical evidence demonstrating a strong correlation between circulating tumor cells with anoikis resistance in advanced cancer and poor survival of patients (34, 35).

Numerous direct and indirect studies suggested that increased NO production suppresses anoikis through the alteration of pro-survival signals, apoptosis-regulatory signals, certain membrane microdomains, and oncogenes. With regard to pro-survival signals, NO regulates PI3K/AKT and suppresses anoikis through mechanisms that likely involve *S*-nitrosylation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (36). *S*-Nitrosylation of PTEN also induce its ubiquitination and subsequent degradation, leading to the loss of PTEN that is known to confer anoikis resistance on lung carcinoma (37).

As a form of apoptosis, anoikis is regulated through the common pathways of apoptosis, which include the intrinsic mitochondrial and extrinsic death receptor pathways. As mentioned above, NO affected anti-apoptotic protein FLIP and BCL2 stability through its *S*-nitrosylation. Overexpression of FLIP and BCL2 was reported to render cells resistant to anoikis (38, 39) and is commonly observed in lung carcinoma (40, 41), suggesting the role of NO in anoikis resistance through these proteins.

CAV1, an essential constituent of cell membrane invaginations, has been implicated in metastasis and poor prognosis of lung cancer (42). Our studies have shown that the level of CAV1 in lung carcinoma H460 cells gradually decreased in a time-dependent manner after cell detachment and that ectopic expression of CAV1 prevented anoikis through stabilization of anti-apoptotic protein MCL1 (43). CAV1 interacts with MCL1 and interferes with its degradation, which occurs during anoikis, through ubiquitin-proteasomal pathway. NO is a critical regulator of CAV1 stability and anoikis resistance. We showed that NO induced *S*-nitrosylation of CAV1, which subsequently prevented its proteasomal degradation and induction of anoikis under cell-detachment conditions (Figure 3) (44).

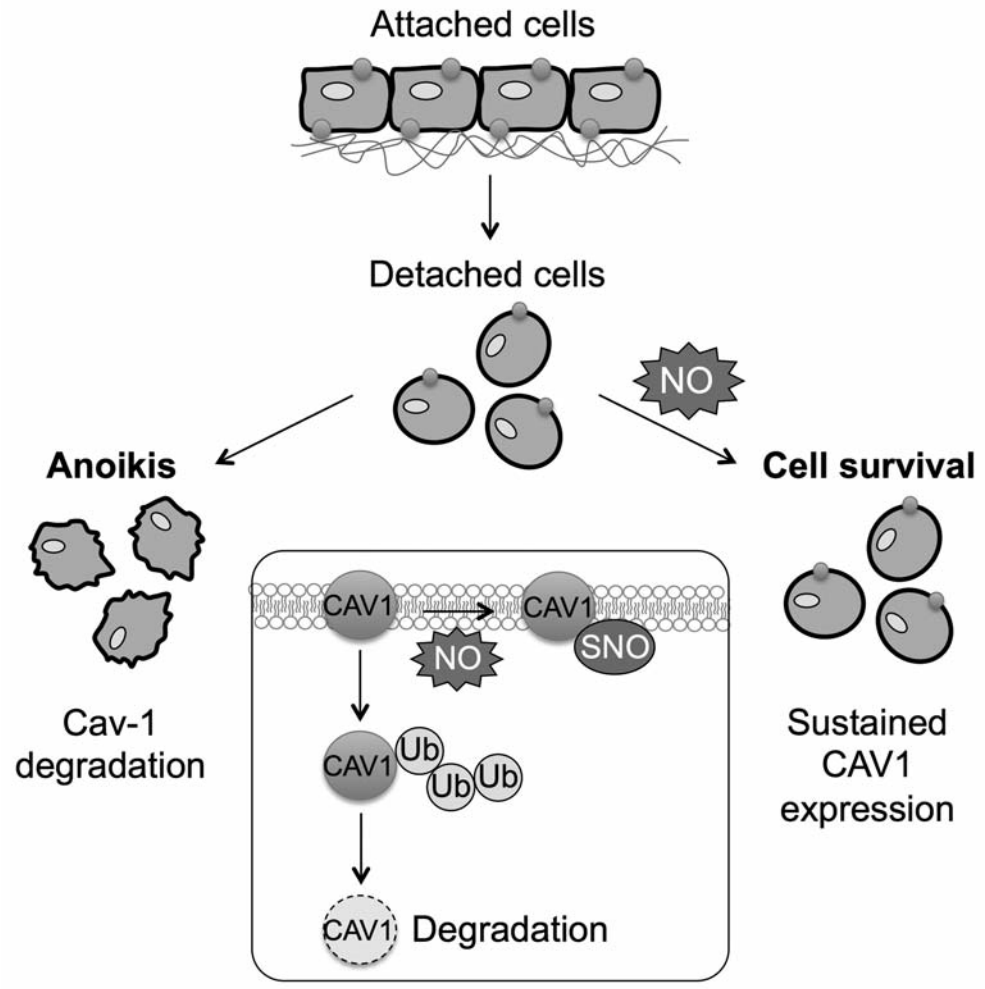

Figure 3. *Schematic representation of the regulatory role of NO in resistance to anoikis. The majority of tumor cells undergo detachment-induced apoptosis (anoikis). S-Nitrosylation (SNO) of caveolin-1 (CAV1) by NO prevents its ubiquitin-proteasomal degradation and renders cells anoikis-resistant.*

NO and cell migration and invasion. The ability of lung cancer cells to migrate and invade allows them to change their position within tissues and into other tissues. Cell migration and invasion are thereby critical steps of cancer metastasis. To intravasate into and extravasate out of lymphatic and blood circulation, primary tumor cells must migrate and invade through the epithelial basement membrane, surrounding stromal structures consisting of the ECM, and vascular basement membrane (45). Only a small fraction of primary cells become invasive and eventually metastasize at any given time, and NO has been shown to affect such fraction through regulation of multiple proteins. We focus on the promoting effects of micromolar levels of NO on cell motility.

An elevated level of CAV1 expression is reported to correlate well with enhanced invasive ability of lung carcinoma. Re-distribution of CAV1 to lung cancer cells led to the formation of filopodia, a spike-like projection enriched with F-actin filaments at the leading edge of cells that facilitates cell movement (42). We found that prolonged exposure of lung cancer H460 cells to NO altered cell morphology, *e.g.* cell enlargement, and cell migration in accordance with an increase in filopodia formation and CAV1 expression (46). We further revealed that CAV1 mediates focal adhesion kinase (FAK) and its downstream AKT pathways, affecting lung cancer cell protrusion and migration.

Prolonged NO exposure also activated epithelial-mesenchymal transition (EMT) in various lung cancer cell lines, namely H292, H23, A549 and H460 (47). EMT is a multi-step cellular process that allows epithelial cells, which normally interact with the basement membrane, to undergo changes leading to a mesenchymal phenotype, which lacks membrane bounding and allows for enhanced cell migration

and invasion (48). EMT is characterized by the progressive loss of epithelial markers, such as vimentin, tight junction protein 1 (ZO1) and E-cadherin, and gain of mesenchymal markers, such as zinc finger protein SNAI2 (SLUG), zinc finger protein SNAI1 (SNAIL), N-cadherin and β-catenin. Prolonged NO exposure induced dramatic increase in vimentin and SNAIL, independently of CAV1, suggesting the additional mechanism of NO-mediated cell motility.

Cancer Stem Cells

Cancer stem cells (CSCs; also known as tumor-initiating cells) are defined as a rare sub-population of tumor cells that are capable of self-renewal and generate a progeny of differentiated cells that constitute a large majority of cells in the tumor (49). The existence of CSCs has been identified in many types of cancer, including that of the brain, breast, colon, prostate and lung (50). Currently, CSCs are being considered as the underlying causes of chemo/radioresistance, cancer relapse and high mortality rate of lung cancer, due to their reported role in aggressive behavior of human cancer cells and tumorigenesis (51, 52). Hallmarks of CSCs include unlimited cell proliferation, evasion of apoptosis, increased cell migration and invasion, and capability of initiating tumor formation.

NO and CSCs. The role of NO in CSCs has been suggested in breast cancer in that NO induced CD44 expression and signal transducer and activator of transcription 3 (STAT3) activation, indicative of breast CSCs (53). The previous findings that NO increased lung cancer aggressiveness, together with its suggested role in breast CSCs, led to the hypothesis that NO may mediate its lung procarcinogenic effects through CSCs. Our findings revealed that prolonged NO exposure induced two well-known lung markers of CSCs, namely CD133 and aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) in multiple lung cancer cell lines, *e.g.* H460 and H292, in a dose- and time-dependent manner in agreement with the greater anchorage-dependent growth, spheroid formation and anoikis resistance, indicating the induction of CSC-like behavior by NO (54). Such effects of NO, however, were reversible after cessation of NO treatment. Furthermore, CAV1 was found to be critical in NO-mediated aggressiveness of CSCs, although its level did not correlate well with CD133 and ALDH1A1. CAV1 has been shown to interact and regulate the transporter ATP-binding cassette sub-family G member 2 (ABCG2) another potential marker of lung CSCs (55, 56). Thus, it is plausible that CAV1 might regulate NO-mediated CSCs through ABCG2.

Concluding Remarks

Accumulating evidence has demonstrated the tumor-promoting role of NO in various types of cancer, including of the lung, spanning from tumor initiation of cellular transformation to tumor progression through the metastatic cascade and resistance to radio/chemotherapy. In the present review, we summarize the role of NO and its key target proteins in relation to chemotherapeutic and anoikis resistance, and cell migration and invasion of lung cancer cells. Understanding the roles of NO in such aggressive behavior of lung cancer cells is important because they are key determinants of cancer metastasis and relapse that remain therapeutic challenges. Ultimately, we summarize the relatively novel findings on the implication of NO in lung CSC regulation, which could be the underlying causes of all NO-mediated aggressive behavior. NO seems to manifest its effects through key target proteins through the post-translational protein modification of *S*-nitrosylation. *S*-Nitrosylation controls the function and activity of numerous tumor-associated proteins and its dysregulation could lead to cancer pathology, inspiring the new idea of targeted cancer treatment based on *S*-nitrosylation.

Conflicts of Interest

No potential competing financial interests were disclosed.

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