Review

Nitric Oxide and Cancer Development

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Abstract: The role of nitric oxide (NO) in cancer development has become a very active research area. This review presents an overview of many studies that implicate an important role of NO in the development of cancer. These include results in experimental models as well as many observations made on NO and inducible nitric oxide synthase (iNOS) in human cancers. Our survey of the literature has allowed us to conclude that in general large levels of NO will kill cancer cells but paradoxically at intermediate to lower levels NO prevents cancer cell apoptosis and enhances tumor growth. This basic tenet has been demonstrated in many experimental models. The mechanistic basis of how intermediate levels of NO mediate tumor development is an active area of research and is the subject of this review. NO mediated S-nitrosylation of key enzymes and regulatory proteins plays a critical role. Key proteins S-nitrosylated which enhance tumor development include several caspases involved in apoptosis, PTEN the tumor suppressor protein, Bcl-2 the mitochondrial protein which protects from apoptosis, OGG1 the DNA repair protein and methionine adenosyl transferase the liver protein which synthesizes adenosylmethionine. The S-nitrosylation of these proteins enhances DNA mutations, prevents cancer cell apoptosis and enhances oncogenic cell growth. (*J Toxicol Pathol* 2007; **20**: 77–92)

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Introduction and Overview

Since the discovery in 1987 that nitric oxide was equivalent to endothelial relaxing factor (EDRF), this gaseous free radical as well as its reaction products (peroxynitrite, nitronium ion, N₂O₃ and others) have been shown to be important in many biological processes in addition to the control of blood pressure. Nearly simultaneous with the discovery that NO was EDRF, independently Hibbs and coworkers showed that NO was the active factor in the macrophage mediated killing of tumor cells in model systems¹. Research in the area of NO mediated killing of tumor cells by macrophages has continued since the early observations, and as anticipated, observations since then have demonstrated that the processes and cell biology involved is more complex than originally conceived². This area will not be covered in this review.

Many observations over the last several years clearly implicate an important role for nitric oxide (NO) in the development of many human cancers as well as experimental models. Despite these observations it must be recognized that the role of NO in cancer development is a controversial area of research. This appears to be due to many reasons including the fact that NO is an important signaling molecule and has the capacity to alter many cellular processes depending upon its rate of production. In general, at very high levels of NO, cancer cell killing occurs and at very low levels there seems to be very little effect, however at more intermediate levels the results clearly indicate that NO protects cancer cells from apoptosis. Therefore NO has been shown to be both pro-apoptotic or anti-apoptotic depending upon many factors, including not only the flux and dose of NO, but on the specific cells involved, as well as on the redox state of these cells. The molecular mechanism as to how NO enhances cancer development has become an active area of research. In this review we will provide a survey of many observations in the literature. Our goal is to help synthesize a coherent view of the possible molecular processes involved on the role of NO in mediating cancer development. We present a rationale

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based on the literature along with a brief summary of some of our primary observations in an experimental hepatocellular carcinoma model. The rationale implicates NO mediated S-nitrosylation of critical cysteines in specific proteins play a major role in cancer development. This review presents the historical observations summarizing the discovery of NO in cancer cell killing. We then summarize some of the most important observations both in humans as well as experimental animal models demonstrating the importance of NO in cancer development. We then summarize NO reactions that may be the basis of why NO enhances cancer development. Finally we present some of the observations we have made that supports the notion that NO plays an important role in cancer development.

Historical Observations

The discovery that NO was the toxic agent produced by macrophages in their killing of tumor cells was made by Hibbs and colleagues in 1988¹. This conclusion was the result of over an eight year span of research before nailing down the definitive identity of NO. Hibbs and colleagues demonstrated several important facts; A) that macrophage-mediated killing of tumor cells was dependent on L-arginine³, B) that L-arginine was metabolized to L-citrulline in the process, and that one of the guanidino nitrogens of arginine was oxidized to produce nitrite⁴. They also demonstrated C) that macrophages mediated the loss of aconitase activity⁵, caused the depletion of Fe⁶, and mediated the inhibition of mitochondrial respiration in tumor cells⁷. The occurrence of this research effort is remarkably near the same time (from early 1980 up to late 1987) that extensive research activity was also occurring independently by many groups especially those led by Ignarro, Moncada and Furchgott that led to the discovery that EDRF was equivalent to NO⁸⁻¹⁰. Paradoxically it was Moncada who had devoted much effort to the elucidation that NO was equivalent to EDRF, then later on, first definitely showed that NO generated from iNOS within cancer cells mediated the enhanced development of cancer in an experimental model¹¹.

Studies Demonstrating Importance of iNOS and NO in Cancer

Many observations on human subjects having various cancers have demonstrated that high levels of iNOS and NO are present in the tumors per se. In general a positive correlation of tumor aggressiveness with enhanced levels of iNOS/NO has been demonstrated for several different tumors. Many cancers have been studied with generally similar conclusions, see the review by Kirk *et al.*¹². In fact Kirk *et al.*¹² strongly suggest that therapeutic intervention of iNOS would impede tumor progression. A brief summary of the observations on liver cancer, prostate cancer, melanoma, colon cancer, gastric cancer, esophageal cancer and pancreatic cancers are presented here.

The subject of iNOS and liver tumor has not been studied as much as other tumors, however one recent study of 100 different HCC patients demonstrated that high expression of iNOS and COX-2 was correlated strongly with decreased length of patient survival¹³. It should be noted that surgical resection is the treatment of choice for patients having HCC but 80% of these patients will have regrowth of their tumor in 2 years¹⁴. Regarding prostate cancer there are about 230,000 new cases diagnosed each year and about 30,000 deaths per year from it occur in the US¹⁵. It has been established in several studies that the intensity of iNOS expression in the target tissue of subjects is correlated with the severity of prostate tumor development¹⁶⁻²⁰ and that it also has prognostic value¹⁹. The general relevant observations made in this field has lead to the belief that iNOS expression and NO formation generally contributes to tumor angiogenesis, tumor growth and metastasis, and tumor-related immune suppression¹⁷. A study with prostate tumor spheroids did show that enhanced NO formation was associated with a decrease in tumor cell death²¹. Concerning melanomas where 34,000 new cases are diagnosed annually²², several studies indicate that iNOS mediated-NO synthesis in melanoma cells is important in preventing their apoptosis²²⁻²⁴. One study of 52 primary malignant melanomas of the skin showed that iNOS was most frequently expressed in the "pure and invasive" radical growth phase, whereas in the vertical growth phase and metastatic phase it was less prevalently expressed^{25,26}. They concluded that NO played an important part in the vertical growth phase of metastatic melanoma²⁷. These general conclusions are supported by two other studies^{28,29}.

In the case of colon cancer the American Cancer Society estimates that about 147,000 new colorectal cancers were diagnosed in 2004, with about 57,000 deaths that resulted from them¹⁵. There is a large amount of evidence showing that both iNOS and COX-2 are very important in the development of colon cancer³⁰⁻⁴⁰. Many studies have indicated a direct linkage of iNOS with the induction of COX-2 in colon cancer^{33,37,38,40,41}. In the case of gastric cancer there are about 23,000 new cases of stomach cancer diagnosed per year¹⁵. The presence of iNOS in the target tissue of patients with gastric cancer has been known for some time⁴²⁻⁴⁶. Another major factor highly associated with gastric cancers is *Helicobacter pylori* infection⁴⁷. There are higher levels of iNOS expression and higher levels of nitrotyrosine staining in gastric mucosa of subjects infected with *H. pylori*⁴². Studies have shown that a specific C > T polymorphism in exon 16 of iNOS is related to higher incidences of gastric cancer^{44,48-50}. Transgenic mouse studies have shown that gastric mutation frequency is increased 4-fold in the mice infected with *H. pylori*⁵¹. *H. pylori* infection is known to increase iNOS expression⁵². It is likely that increased NO production may lead to DNA damage. Oxidative damage to DNA as well as iNOS expression and cellular apoptosis has been shown to increase in those subjects infected with *H. pylori*⁵³⁻⁵⁵. The inability to express iNOS decreased the number of mice with tumors

from 72.7% in wild type mice to 31.0% in those not expressing iNOS⁵⁶.

It is estimated that there are about 14,000 new cases of esophageal cancer and that over 13,000 deaths from this malady occurs in the United States per year¹⁵. iNOS expression is highly associated with esophageal squamous cell carcinoma⁵⁷⁻⁶¹. Barrett's esophageal is a premalignant condition arising from chronic reflux esophagitis and high levels of iNOS as well as COX-2 have been observed in this condition^{58,60-62}. This has led to the speculation that iNOS and COX-2 are involved in Barrett's-associated neoplastic progression^{58,62}. Animal models based on nitrosomethylbenzylamine induced esophageal cancer⁶³ have shown that catalytic inhibitors of COX-2⁶⁴ as well as iNOS⁶⁵ decreases cancer development. Pancreas cancer remains the fourth leading cause of death due to cancer in the Western World⁶⁶ and about 31,860 new cases were diagnosed in the US this last year and nearly that many deaths, i.e. 31,270, occurred due to this disease¹⁵. The 6 month survival is about 50% and the five-year survival after diagnosis is less than 5%⁶⁶. It has been known for several years that pancreatic cancer is associated with increased expression levels of iNOS and COX-2 when compared to normal pancreatic tissue⁶⁷⁻⁷¹. The most insightful study of the importance of iNOS and NO production on pancreatic carcinoma growth and their resistance to anti-cancer drugs was recently reported⁷². This study demonstrated that pancreatic fibroblasts caused increased IL-1 β secretion by the tumor cells, which then acted upon the fibroblasts to cause the upregulation of iNOS causing enhanced NO secretion, which then acted upon the tumor cells to mediate their resistance to etoposide-induced apoptosis. These results show that bystander stromal cells act to enhance the resistance of tumor cells to anti-cancer drugs. It also showed that the primary agent responsible is NO produced by the stromal fibroblast cells, which were induced to produce it by the IL-1 β cytokines produced by the tumor cells. Prevention of iNOS expression by the stromal fibroblasts may help break the paracrine cycle, thus making the tumors more susceptible to anti-cancer agents.

Animal Model Studies Evaluating iNOS/NO in Cancer Development

One of the most important early experiments conducted demonstrating the importance of iNOS and NO in cancer development was done by Moncada's group¹¹. They utilized DLD-1 human colon adenocarcinoma cells raised within nude mice from which they isolated a cell line (w/tR) which then was transfected with iNOS. They showed that this cell line (iNOS-19), which was shown to have functional iNOS, grew significantly slower in culture than the original cell line w/tR. Significantly however, they discovered that the iNOS-19 cells grown in nude mice produced tumors that yielded significantly higher mass than the tumors arising from w/tR cells lacking iNOS expression. They also noted that the blood vessel volume of the tumors from iNOS-19 cells was

5-fold higher than the blood vessel volume of the tumors from the w/tR cells. Therefore they showed that iNOS in tumor cells significantly enhanced tumor size and angiogenesis of the tumors when compared to tumor cells not having an extra level of functional iNOS.

Kisley *et al.*⁷³ examined the extent of urethane-induced lung tumorigenesis which developed in iNOS knock out mice, as compared to wild type controls capable of expressing iNOS. They noted a significant reduction in the number of tumors in the iNOS knockout mice compared to the wild-type mice. iNOS expression in the wild-type mice was in the lung tumors per se, as well as in clara cells, but there was very little in the uninvolved lung tissue, as well as in the non-urethane treated mice. Studies in several animal models have demonstrated the importance of NO and iNOS in the development of mammary gland carcinogenesis⁷⁴⁻⁷⁷. Mammary cancer development in C3H/HeJ mice utilizes implantation of a cell line (C3L5) that is highly metastatic in this model⁷⁴. C3L5 cells grown in Matrigel were implanted in the inguinal region on one side, and Matrigel containing no cells on the contralateral side. Mice were then given either L-NAME, a competitive inhibitor of iNOS, in the diet, or D-NAME, a non-competitive inhibitor, as a control. Invasiveness and angiogenesis of the tumor was measured using the CD31 marker for the presence of new blood vessels. The results clearly showed that tumor cell invasiveness was decreased significantly when L-NAME was given compared to D-NAME. This clearly implicates the importance of iNOS in angiogenesis and invasiveness of mammary tumors in the mouse model. Histologically they demonstrated localization of eNOS and iNOS in the tumor region. A different more definitive study of the role of iNOS in mammary gland carcinogenesis in mouse was done by another group⁷⁷. In this study endogenous mammary tumor development was mediated by the polyomavirus middle T antigen (PyV-mT) directed to the mammary gland in the C57BL/6 strain which either was capable of expressing iNOS or not. PyV-mT/iNOS^{-/-} mice and PyV-mT/iNOS^{+/+} mice were evaluated regarding the rate of mammary tumor growth and iNOS, as well as eNOS expression, was monitored using western blot analysis of the mammary tissue. Their results showed that mammary tumor development was significantly delayed in the PyV-mT/iNOS^{-/-} mice when compared to the PyV-mT/iNOS^{+/+} mice, thus clearly implicating the importance of iNOS in mammary tumor development.

Probably the most extensive studies demonstrating the importance of iNOS and NO in animal models have come from those done on colon cancer development. The studies by Rao and Reddy's groups have provided excellent data to support the notion that inhibition of iNOS production slows down colon cancer development in the azoxymethane-induced rat model, see for instance references^{37,78}. Another approach in another model has produced supportive data. Ahn and Ohshima⁷⁹ utilized the Apc^{min/+} mice that develop excessive amounts of intestinal adenomas. Administering amino guanidine, a specific iNOS inhibitor, in the drinking

water caused suppression of adenoma formation in the small intestine but not the large intestine. They also utilized iNOS gene knockout methods in this mouse model and noted that $Apc^{min/+}iNOS^{-/-}$ mice as well as $Apc^{min/+}iNOS^{+/-}$ mice had significantly fewer adenomas than $Apc^{min/+}iNOS^{+/+}$ mice. This observation clearly implicates the importance of iNOS and NO in the development of intestinal adenomas. Recently it has been demonstrated that NO induces COX-2 in colorectal cancer cells⁴¹. The discovery that NO mediates the induction of COX-2 in colon carcinogenesis is very important for it makes it possible to establish the primary sequence of events where therapeutics could be developed to slow down or prevent the processes involved. Four critical points in the primary pathway are immediately apparent, i.e. A) catalytic inhibitors of COX-2 enzymes, B) inhibitors that prevent COX-2 gene induction, C) catalytic inhibitors of iNOS enzyme and D) inhibitors to prevent iNOS gene induction. Basic research activity in these four areas is presently ongoing. Rao and Reddy's groups have been the most active, primarily utilizing a rat model of colon cancer induced by azoxymethane^{33,34,36,37,39,80}. They have shown in the same rat model that an iNOS catalytic inhibitor, L-N⁶-(1-iminoethyl)lysine tetrazole-amide (SC-1), given in the diet decreased colon tumors in proportion to the amount of SC-1 present, and that this agent in combination with Celecoxib (a COX-2 enzyme inhibitor) was effective also in an additive manner⁸¹. Reddy summarized the work in this area, in the context of chemoprevention of colon cancer, and emphasized the importance of inhibition of iNOS and COX-2 as possible therapeutics³⁹. Another review drew a similar conclusion⁴⁰. Isoliquiritigenin, a natural flavonoid from licorice, decreased the expression of iNOS and COX-2, and inhibited preneoplastic aberrant crypt foci in the azoxymethane rat model of colon cancer³⁸.

Studies on a rat model of esophageal tumorigenesis have shown the importance of iNOS and NO^{63,65}. Observations in humans have shown a strong association of iNOS and enhanced NO production in esophageal cancers. Chen and Stoner conducted experiments with rats developing esophageal tumors caused by N-nitrosomethylbenzylamine. They showed that administering a diet containing a pheylenethiourea derivative referred to as PBIT, a selective iNOS inhibitor, significantly decreased the esophageal cancer incidence as well as tumor multiplicity^{63,65}. Very few definitive studies have been done on the importance of iNOS and NO in animal models of liver carcinogenesis. The best study thus far was conducted by Thorgierson's group with c-Myc/TGF α transgenic mice that develops liver cancer within 6 to 8 months. They noted that administering α -tocopherol in the diet caused a significant decrease in liver tumors and concomitantly a significantly decreased amount of iNOS was expressed as well as nitrotyrosine in livers of the mice⁸². There was a strong correlation of iNOS levels with the appearance of tumors in not only the c-Myc/TGF α transgenic mice but in the c-Myc as well as the TGF α transgenic mice also.

We have also detected increased NO in a choline-

Fig. 1. EPR spectra of MGD-FE-NO in rats treated with either choline deficient amino acid defined (CDAA) or choline sufficient amino acid defined (CSAA) diets for 7 days. D-N-Methylglucamine dithiocarbamate (MGD) (200 mg/kg)/iron sulfate (20 mg/kg) was injected intraperitoneally (ip) following cannulation of the bile duct under anesthesia (1.5–2% isoflurane).

deficient rat liver carcinogenesis model (see Figs. 1 and 2). A two-fold increase in NO was detected in the bile (bile duct cannulation) via electron paramagnetic resonance (EPR) spin trapping, as early as 1 week following dietary restriction of choline (Fig. 1). Flow cytometry analysis of isolated hepatocytes from CDAA diet-treated rats for 2 weeks, also indicated over a 2-fold increase in NO detection, compared to the CSAA controls (see Fig. 2). We had earlier demonstrated that isolated hepatocytes from rats placed on a choline-deficient diet for only 2 days had a population that produced enriched levels of NO which was suppressed by administering the synthetic antioxidant α -phenyl-tert-butyl nitron (PBN)⁸³. We also showed that PBN had anti-cancer activity in this model^{84–87}. We showed that administering PBN as well as the 3-hydroxy and 4-hydroxyl derivatives in the diet caused selective enhanced killing of the cells within the preneoplastic lesions at 16 weeks but had no effect of the cells in the surrounding liver tissue^{84,85}. We later surmised that the enhanced selective killing of the cells within the preneoplastic lesion by PBN and active derivatives was due to their suppression of NO formation therefore allowing the normal cellular stressors to enhance their apoptosis⁸⁸. Therefore we see our results as indicating that intermediate levels of NO suppressed the death of the cells within the preneoplastic lesions and therefore contributed to the development of hepatocellular carcinoma in the rat choline deficient model.

Nitric Oxide Formation and Reaction Chemistry

Nitric Oxide has an unpaired free electron and therefore is a gaseous free radical formed by nitric oxide synthase enzymes utilizing L-arginine as substrate. The oxygen atom of NO comes from molecular oxygen and the nitrogen atom arises from one of the guanidino nitrogens of L-arginine (Fig.

Fig. 2. Flow cytometry (FACS) expression of diaminofluorescein-2 diacetate (DAF-2DA) detected nitric oxide (NO) in isolated liver cells from CSAA and CDAA diet fed rats at 2 weeks.

Fig. 3. Reaction demonstrating the formation of NO from L-arginine catalyzed by nitric oxide synthase (NOS). List of the three types of NOS and their properties as well as the occupancy of the π^*2p orbitals demonstrating the properties of NO different than O_2 and that NO is a free radical. Listing of the reaction products of NO with biological molecules and summarizing some of the consequences.

3). There are three nitric oxide synthase (NOS) enzymes formally referred to as NOSI, NOSII and NOSIII for eNOS, iNOS and nNOS or so-called endothelial NOS, inducible NOS and neuronal NOS, respectively. Both eNOS and nNOS are dependent on the presence of calcium to trigger NO synthesis but iNOS synthesizes NO independent of the presence of calcium and therefore produces NO in much larger sustained amounts than are formed by the other two enzymes. The cancer development literature is more focused on the role of iNOS than the other two NOS isoforms. NO and the various products originating from

iNOS react with proteins, nucleic acids and lipids thus mediating biological effects the consequences of which are still being elucidated.

Some of the reactions NO may undergo readily are shown in Fig. 4. NO reacts at diffusion limited rates with superoxide, O_2^- , to yield peroxynitrite which then will react with many biological molecules. NO reacts via its oxidation species, nitronium ion, with glutathione (GSH) and specific protein cysteine residues to form S-nitrosoglutathione and proteins S-NO adducts respectively. The formation of cysteine S-nitrosylation adducts are commonly referred to as

Fig. 5. Mechanisms of the formation of S-nitrosylation products with acidic cysteine residues in proteins. The thiolate anion of cysteine is very reactive with NO which exists as N₂O₃ or [NO⁺---NO₂] in a hydrophobic environment or as an active species when associated with a transition metal. Reaction taken from Hess *et al.*⁹⁸.

compounds such as nitroso glutathione⁹⁸.

The S-nitrosylation of some protein tyrosine phosphatases (PTPs) has been demonstrated^{102,104–106}. It should be noted that even though NO mediates inactivation of PTP1B^{104,106,107}, it can be reactivated by chemical treatment with dithiothreitol, but there is nothing known about possible natural occurring systems that may reactivate the SNO modified proteins. Gaston *et al.*¹⁰⁸ recently presented an excellent review with pertinent information related to this issue. With regard to nitrosoglutathione (GSNO) there are several pathways whereby it has been shown to be metabolized. GSNO is degraded in a NADH/NADPH-dependent process by a rat liver cytosolic enzyme that has been identified as alcohol dehydrogenase (ADH) class III enzyme¹⁰⁹. This enzyme has GSH-dependent formaldehyde dehydrogenase (GDFDH) activity also. However it has a K_m for GSNO of ~20 μM and a K_{cat}/K_m of 94,300 mM⁻¹ min⁻¹, which is much higher than the other ADHIII substrates^{108,109}. Based on this and all the other evidence¹⁰⁹ it is considered that ADHIII is the primary enzyme responsible for metabolizing GSNO^{109,110}, and that the two enzymes, ADHIII and GDFDH, are the same¹⁰⁸. In addition to ADHIII, the Cu/Zn superoxide dismutase enzyme (Cu/Zn SOD) has been shown to effectively degrade GSNO in the presence of GSH with a K_m of 5.6 μM¹¹¹. Additionally thioredoxin in the presence of NADPH will degrade GSNO to GSH and NO with a K_m value of 60 μM¹¹². Xanthine Oxidase in the presence of hypoxanthine will metabolize GSNO as well as nitrosocysteine (CysNO) with a K_m in the range of 300–700 μM respectively¹¹³.

The above points highlight another area of considerable uncertainty; namely what are the exact molecular events and

the mechanisms dictating why specific cysteines in specific proteins become nitrosated within a cell where there are so many other potential available thiol groups. First it is quite clear that the reason why specific cysteines are S-nitrosated is dictated by the amino acids spatially positioned very close to the target thiol group such that it assumes acidic character making it labile to nitrosation. However the most uncertainty arises regarding the nitrosating entity. The K_m for reaction of GSNO to inactivate papain is 60 μM⁸⁹. As noted above the K_m of GSNO reaction with ADHIII is 20 μM and with CuZn SOD is 5.6 μM, therefore it is unlikely that GSNO within a cell exists long making it unlikely that it is an important protein S-nitrosylating species. Supporting this notion are the observations that S-nitrosylated proteins are present in biological samples even though GSNO is undetectable. Whereas in ADHIII knockout cells S-nitrosylated proteins increase dramatically but GSNO is only then detectable^{108,110}. Based on the above logic then, if the target cysteine exists in a hydrophobic domain of the protein it is highly likely that N₂O₃ is the reacting species simply because NO and O₂ are much more soluble in hydrophobic domains¹⁰³. N₂O₃ can be written as [NO⁺---NO₂]⁻ thus making it clear that the thiolate sulfur reacts with the nitrosonium cation.

S-nitrosylation of Critical Proteins Enhance Cancer Development

Our survey of the existing literature provides us the perspective that there are at least 4 to 5 critical proteins that can help explain on a mechanistic basis how NO, at intermediate levels, mitigates tumor development. The critical proteins and a brief description of how they enhance tumor development are discussed briefly here and summarized in Fig. 6. A) NO mediates S-nitrosylation of specific caspases that are involved in apoptosis processes, thus preventing processes which would normally cause the death of cancer cells, and therefore allows continued growth and development of tumors. B) NO mediates the S-nitrosylation of the DNA repair enzyme OGG1¹¹⁴, thereby preventing the repair of oxidative adducts such as 8-hydroxy-2'-deoxyguanosine, which then allows mutagenic lesions to be incorporated into DNA therefore leading to mutational events enhancing tumor development. C) NO mediates the S-nitrosylation of the PTEN protein¹¹⁵ that acts as a tumor suppressor in several tumors. The results of PTEN inactivation cause enhanced AKT signal transduction processes which is known to enhance cancer development. D) NO mediates the S-nitrosylation of Bcl-2, thus preventing the proteosomal degradation of this protein, and thus resulting in higher levels of Bcl-2¹¹⁶ which acts to prevent apoptosis. E) NO mediates the S-nitrosylation of methionine adenosyltransferase (MAT 1/111) leading to decreased levels of S-adenosyl-methionine which enhances cell proliferation. Inactivation of MAT 1/111 is most important in the case of hepatocellular carcinoma development. F) NO mediates the activation of the Ras pathway which is

Proteins S-Nitrosylated which Enhance Cancer Development

<u>Proteins S-Nitrosylated</u>	<u>Consequences of Inactivation</u>
Specific Caspases	Inactivation of apoptosis mechanisms prevent cellular apoptosis
Bcl-2	Prevents proteosomal loss of Bcl-2. Bcl-2 buildup, prevents apoptosis
OGG1 DNA Repair Enzyme	Causes buildup of 8-OHdG enhances mutations
PTEN Tumor Suppressor Protein	Enhances Akt-mediated signaling enhances oncogenesis
Liver Methionine Adenosyl Transferase	Decreases S-adenosyl methionine levels causes enhanced cell proliferation

Fig. 6. List of the possible proteins which if they are S-nitrosylated by NO enhance the development of cancer by the mechanisms listed.

important in the development of many tumors. In the sections below we amplify the background rationale for some of these six key notions noted above.

S-nitrosylation inhibition of apoptosis in cancer cells

Several careful studies have shown that apoptosis of tumor cells is controlled by S-Nitrosylation of specific proteins. The most definitive studies have been done with human colon carcinoma cells¹¹⁷, human cholangiocarcinoma cells¹¹⁸, human breast carcinoma cells¹¹⁹, and human melanoma cells²³. Kim and Tannenbaum utilized HT-29 colon carcinoma cells to show that S-nitrosylation of procaspase-9 was the key event that protected these cells from TNF α plus interferon γ (TNF α /Ifn γ) induced apoptosis¹¹⁷. When the cells were exposed to the nitric oxide synthase inhibitor N^G-methyl-L-arginine prior to TNF α /Ifn γ treatment then apoptosis occurred. Utilizing a biotin switch method¹²⁰ specific S-nitrosylation of procaspase-9 was demonstrated to be directly correlated with decreased apoptosis. It should be noted that plated tumor cells may respond to nitrosative stress in a different manner than those undergoing fluid shear stress similar to that expected when malignant cells are traveling through the blood stream, i.e. see for example the studies conducted by Laguinge *et al.* on 3-D rotated colorectal carcinoma cells¹²¹.

As with the studies on colon carcinoma cells noted above, Torok *et al.*¹¹⁸ showed that S-nitrosylation of caspase-9 in cholangiocarcinoma cells is directly associated with protecting them from apoptosis induced by etoposide. The source of NO in the cells was either from transfected iNOS or from an exogenous donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP). The arguments presented is that external sources of NO are meaningful in the context of cholangiocarcinoma because primary sclerosing cholangitis, a chronic inflammatory condition, is the main identifiable risk factor for this tumor and that enhanced NO production is associated with inflammation. Therefore exogenous sources

are meaningful under this condition¹¹⁸.

Utilizing T46D human breast carcinoma cells Radisavljevic¹¹⁹ demonstrated that inhibition of iNOS using N^G-monomethyl-L-arginine caused enhanced apoptosis. Apoptosis signaling was shown to be independent of caspase-3 as well as the PI3K/AKT pathway. In another study using T47D cells Kampa *et al.*¹²² also showed that inhibition of iNOS induction enhanced apoptosis in these cells. Growth of MCF-7 cells, another human breast cancer cell line, was shown to be dependent on arginine¹²³ and that cell growth, not protein synthesis per se, but due to its role as a substrate for nitric oxide synthase to produce NO. Several studies in humans^{124–126} have shown a general positive correlation between iNOS expression with severity of breast cancer. The conclusions were that iNOS was involved in angiogenesis, invasion and metastasis of the tumor growth^{124–127}. These predictions have been born out in animal studies also^{75,76}. Additionally, studies with an iNOS negative mouse model compared to the normal iNOS positive control mice have shown that mammary tumor development and invasiveness was more rapid in the normal iNOS expressing animals⁷⁷.

A careful study of human melanoma cells done in comparison to normal melanocytes demonstrated that both cells expressed iNOS at about equal levels²³. However addition of aminoguanidine, a selective inhibition of iNOS, to both cells demonstrated that this led to apoptosis of melanoma cells but not to the normal melanocytes. They show that apoptosis in melanoma cells involved caspase 3 activation, alteration of mitochondrial membrane potential and down regulation of Bcl-2 and the upregulation of a series of genes involved in apoptosis including Bax, caspase-1, caspase-3, caspase-6, gadd 45 β , mdm2 and TRAIL²³. The data in this study clearly showed that iNOS activity is important in melanoma cell survival. Another more recent study, where several melanoma cell lines were examined in comparison to normal melanocytes, showed that in contrast

to the normal cells which responded to $\text{TNF}\alpha$, $\text{Inf}\gamma$ and LPS stimulation by showing a strong positive iNOS induction, the melanoma cells did not²⁴. However, contrary to normal melanocytes the melanoma cell lines already showed weak iNOS expression. In a careful study evaluating iNOS and MDA-7 (melanoma differentiation association gene, considered a novel tumor suppressor gene) in 81 tumor samples consisting of 38 primary melanomas and 43 metastatic melanomas demonstrated that there was a significant negative correlation ($P = 0.03$) of iNOS expression with MDA-7 expression²². In other words when MDA-7 was expressed iNOS was not expressed. This study was followed up with a companion series of experiments with melanoma cell lines having adenoviral vector transfected MDA-7 gene. In these studies they demonstrated MDA-7 expression caused a decreased expression of iNOS²². They further showed that STAT-3 modulated expression of interferon regulating factors 1 and 2 is regulated by MDA-7 and that this may mediate iNOS expression in these cells.

PTEN inactivation by S-nitrosylation

Phosphatase/tension homolog on human chromosome ten (PTEN) has emerged over the last few years as one of the most important tumor suppressor genes. The tumor suppressor function of PTEN is largely associated with suppression of cell growth and survival signaling through the phosphatidylinositol (PI) 3-kinase/Akt pathway^{128–136}. It was discovered in 1997 as a multiple mutated gene in many advanced cancers including those of breast, prostate, endometrial and brain tissue^{137–141}. Mutation of PTEN is a marker for glioblastoma and is associated with Cowden's disease and Bannayan-Zonana syndromes, which are characterized by multiple benign tumors, and a risk for breast and thyroid. PTEN cleaves the phosphate group from the 3'-OH position of the inositol head group of PIP_3 and therefore opposes PI 3-kinase signaling¹³⁵. PI 3-kinase is a ubiquitous intracellular kinase upstream of many signaling pathways related to several cell functions including growth and survival. Like PTP1B, the PTEN active site thiolate group has been shown to be susceptible to oxidation by H_2O_2 ¹⁴². Unlike PTP1B, but similar to some related phosphatases, this oxidation results in the formation of an intramolecular disulfide bond with a "back-door" cysteine residue (Cys71) on another domain. We have postulated that the PTEN active site cysteine could be S-nitrosylated and therefore cause inactivation of the enzyme. This has subsequently been demonstrated¹¹⁵.

Bcl-2 S-nitrosylation and cancer cell apoptosis

B-cell lymphoma-2 (Bcl-2) is a key protein that regulates apoptosis by the mitochondrial dependent pathway. Bcl-2 prevents apoptosis by A) forming heterodimers with the proapoptosis protein Bax, B) by inhibition of cytochrome C release from mitochondria and C) by regulating mitochondria membrane potential, and therefore increased levels of Bcl-2 are antiapoptotic. Bcl-2

levels are controlled by its rate of synthesis, as well as by its rate of degradation by the ubiquitin-proteasomal pathway. Azad *et al.*¹¹⁶ have demonstrated that NO causes S-nitrosylation of Bcl-2 in the human lung cancer cell line NIH-H460 and this prevents the ubiquitin-proteasomal degradation of Bcl-2, thus increasing its level and preventing Cr(VI)-mediated apoptosis of these cells. Bcl-2 S-nitrosylation occurs on cys¹⁵⁸ and cys²²⁹ of this protein and is mediated by either endogenous production of NO or by addition using external donors. They also discovered that treatment of H460 cells with the stress inducers Fas ligand or buthionine sulfoxide also induced Bcl-2 S-nitrosylation. Several observations clearly demonstrate that nitric oxide from iNOS involving Bcl-2 is important in preventing normal B-cell lymphocyte apoptosis¹⁴³, as in human B-cell lymphomas^{144–146}. In the case of the treatment of B-cell lymphomas with resveratrol, it was shown to prevent iNOS expression, to cause decreased expression levels of Bcl-2, and to enhance cellular apoptosis^{145,146}. Intravenous administration of natural polyphenols to mice bearing metastatic B16 melanomas enhanced apoptosis as well as decreased Bcl-2 expression in the metastatic cells involving nitric oxide dependent processes¹⁴⁷. Although not formally proven that S-nitrosylation of Bcl-2 was involved in all the cases, this probably explains the mechanistic basis of these observations. Growth of spontaneous B-cell lymphoma in SJL mice is also highly correlated with iNOS expression¹⁴⁸.

S-nitrosylation of liver methionine adenosyltransferase

It is highly likely that NO-mediated inactivation of liver methionine adenosyltransferase (MAT I/III) plays a major role in choline deficiency-mediated HCC development. The processes are involved in methylation of biological molecules is expected to be considerably altered in the choline deficiency (CD) liver because a major dietary supply of methyl groups (choline) is absent and another one (methionine) is significantly decreased. About 85% of the total methylation¹⁴⁹ and 48% of total methionine metabolism occurs in the liver¹⁵⁰. S-adenosylmethionine (SAME, a product of MAT I/III), is the primary methyl donor substrate. Mato's lab, over the last 10 years, demonstrated that NO mediates inactivation of the MAT I/III expressed in the adult liver by S-nitrosylation^{149,151–156}. Hepatic MAT exists in an oligomeric state, and both MAT I as well as MAT III have been shown to be S-nitrosylated, thus inhibiting enzymatic activity^{149,152–155}. Nitric Oxide has been demonstrated to also inactivate MAT I/III *in vivo*¹⁵⁷. Adult MAT (MAT I/III) is coded by the MAT1A gene. Mato's group demonstrated that knockout mice lacking this gene were predisposed to liver injury, exhibited increased expression of genes involved in cellular proliferation¹⁵⁸, had impaired liver regeneration, had lower levels of SAME, and spontaneously developed hepatocellular carcinomas^{159,160}. S-nitrosylation of MAT I/III significantly decreased the levels of SAME, and sensitized rat hepatocytes to enhanced proliferation^{150,161,162}, which is mediated by hepatocyte growth factor scatter factor (HGF), and probably involves

IGF-1 priming¹⁶³. HGF is turned on by lower levels of SAME¹⁵⁰. Liver cell proliferation caused by MAT I/III inactivation requires another MAT enzyme, MAT II, that is encoded by the constitutive MAT2A gene^{150,164}. MAT II is expressed at low levels in the liver except in the case of pathological conditions where it is upregulated^{149,150}. NO mediated S-nitrosylation of MAT I/III lowers SAME levels that triggers the enhanced expression of MAT II, which is insensitive to NO-mediated S-nitrosylation. MAT II produces much lower levels of SAME. Normal higher levels of SAME are required to maintain the normal liver metabolic state but lower levels of SAME produced by MAT II mediates a control switch that regulates liver function¹⁵⁰ that results in cell proliferation. It is known that choline deficiency causes liver fibrosis and cirrhosis as well as decreased levels of S-adenosylmethionine^{165,166}. It is also known that enhanced iNOS expression occurs in liver cirrhosis^{167–170}. S-nitrosylation of MAT I/III in the CD livers probably accounts for the decreased SAME levels that have been observed¹⁶⁵.

Ras pathway in cancer development

The Ras pathway is frequently activated in human cancer¹⁷¹ and is considered to play a critical role in many aspects of tumorigenesis¹⁷². Activation of Ras can occur by signal transduction processes triggered by specific growth factor binding to receptors that become tyrosine phosphorylated¹⁷³ setting up signal transduction processes. In about 30% of human cancers, mutations occur in the Ras gene protein (P₂₁^{Ras}) that causes it to be in a continuously activated state¹⁷³. The Ras activation pathway primarily involves the following activation sequence Ras/Raf/Mek/Erk^{172,173}, however Ras activation can also influence other pathways, including Akt^{171,173}, which is also important in cancer development. It has been known for many years that NO activates Ras to enhance guanine nucleotide exchange^{174–179}. The first experiments demonstrating that NO enhances guanine nucleotide exchange was done by exposing jurkat cells to NO gas¹⁷⁴. Subsequent studies have been done using isolated proteins inactivated with chemical NO donors. NO-mediated Ras activation has been shown to mediate signal transduction processes in cells including Raf¹⁸⁰ and Erk activation^{181,182} leading to many different effects depending on the specific cells^{180,182–185}. It appears that Ras mutations do not normally occur in human hepatocellular carcinoma development¹⁸⁶ but it is quite clear that the Ras pathway is activated, however as evidenced by significantly enhanced Raf overexpression and enhanced activation^{187,188}. The Raf kinase is an important target for anticancer therapeutics¹⁷².

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