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ROLES OF NITRIC OXIDE IN CARCINOGENESIS. PROTUMORIGENIC EFFECTS

MACIEJ STĘPNIK

Department of Toxicology and Carcinogenesis
Nofer Institute of Occupational Medicine
Łódź, Poland

Abstract. Nitric oxide (NO) is a small gaseous molecule involved in many physiological and pathophysiological processes in the human organism. Reports published so far indicate its multiple and not fully understood role in tumor initiation and progression. The paper reviews basic mechanisms of protumorigenic effects of NO: genotoxicity and mutagenicity, the results of the studies on nitric oxide synthases activity and expression in human tumors of different origin, as well as the main mechanisms of NO-mediated processes responsible for tumor progression (tumor cell growth, angiogenesis, invasiveness and metastatic activity). Because the effects of NO in cancer development are not clearly defined after the review of protumorigenic effects, some data, which indicate the antitumorigenic effects of NO are also presented.

Key words:
Nitric oxide, Tumor growth, Angiogenesis, Invasiveness, Metastasis

INTRODUCTION

Nitric oxide (NO) is a molecule showing free-radical properties and playing very important roles in the human organism [1]. Endogenous nitric oxide is produced by nitric oxide synthase (NOS), of which three distinct isoforms have been identified in mammalian tissues. Although the enzymes do not have an absolutely tissue-specific pattern of expression, they are commonly referred to as endothelial NOS (eNOS), neuronal NOS (nNOS), and macrophage or inducible NOS (iNOS). They catalyze the oxidation of L-arginine to produce NO and L-citrulline via mechanism involving several cofactors or prosthetic groups. The NOS enzymes possess many common features, including 50–60% amino acid sequence homology and an $alfa_2$ quaternary structure.

Numerous studies have been performed so far to clarify the role of nitric oxide in tumor initiation/promotion and progression. Studies on tumor initiation point to genotoxic and mutagenic potential of endogenously overproduced and/or exogenously added NO in *in vitro* and *in vivo* systems. The results of experimental investigations on the role of NO in tumor progression seem to be more complicated, pointing to its dual pro- and antitumorigenic effects.

Role of NO in tumor initiation - genotoxic and mutagenic effects

Although NO plays important roles as a messenger in many physiological processes, there exists a potential danger that, in some circumstances, it can be toxic or even cause tumor initiation. Suggestions confirming the genotoxic potential of NO come both from *in vivo* and *in vitro* studies.

Chronic inflammatory processes may lead to local overproduction of NO which may cause DNA alterations in adjacent cells. It was suggested that, e.g. schistosomal eggs and bacteria of *Helicobacter pylori* species are involved in cancer initiation in bladder and stomach, respectively

Address reprint requests to Dr. M. Stępnik, Department of Toxicology and Carcinogenesis, Nofer Institute of Occupational Medicine, P.O. Box 199, 90-950 Łódź, Poland (e-mail: mstep@imp.lodz.pl).

[2,3]. Recently, Yadav and Seth [4] reported an increase in mitotic index, the frequency of sister chromatid exchanges and chromosomal aberrations in peripheral blood lymphocytes of goldsmiths exposed to NOx. This report confirms the existence of genotoxic effects in humans exposed to NO by inhalation.

The *in vitro* studies explain some of the possible mechanisms of genotoxic effects of NO. It was observed that NO led to deamination of nucleotide bases and DNA isolated from calf thymus [5], evoked mutagenic effects in *in vitro* tests with *Salmonella typhimurium* [6], pSP189 plasmid [7], human lymphoblastoid cells TK6 [8] and also in *in vivo* in rat lung tissue [9].

Although, NO has been shown to be mutagenic in a variety of experimental systems, no pattern of mutations that can be specifically attributed to NO has yet been characterized. It caused predominantly A to G transitions in plasmids [7] and C to T transitions in a bacterial system [5]. NO donor compounds have been shown to cause predominantly G to A transitions in plasmids replicated in bacteria [10], while peroxynitrite caused strand breaks, G to T transversions and G to C transitions [11] in the same system.

One of the possible mechanisms of DNA alteration may involve reaction of autooxidation products of NO with low molecular amines or amides and generation of N-nitroso-compounds which are able to alkylate DNA bases [12]. Procarcinogenic effects of NO can also depend on inhibition of DNA repair enzymes as a result of their S-nitrosylation. Such effects were shown in the case of O^6-methylguanine-DNA-methyl transferase, formamidopyrimidine glycosylase and ligase [13,14]. It is postulated that NO can react very rapidly with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), which is much more toxic than the parent compounds. NO and $ONOO^-$, both can induce DNA strand breaks which may lead to activation of poly(ADP-rybose) synthetase (PARS) and eventually to irreversible cell damage [15]. NO has been shown to exert multiple effects on p53 gene and protein. Stimulation of p53 gene expression was observed in mammalian cells exposed to NO [16]. On the other hand, it is known that product of this gene can inhibit iNOS expression [17]. The data indicate the existence of a feedback inhibition between these two genes. NO, through accumulation of p53 protein, can cause cell apoptosis [18]. It can also change conformation of p53 protein and significantly decrease its specific binding to DNA [19].

Role of NO in tumor progression

Although there are actually two conflicting views on the role of NO in tumor progression, more clinical and experimental data confirm a positive association between NO production and tumor progression. Many authors have reported the presence of NOS protein and/or its activity in the tissue of tumors of different organs and also the correlation of the NOS protein expression with the degree of malignancy.

Nitric oxide synthases in human cancers

Human **breast tumors** were shown to express constitutive and inducible NOS forms where their presence correlated with the tumor grade [20]. Vakkala et al. [21] who investigated the immunohistochemical expression of eNOS and nNOS in 80 breast carcinomas revealed positive results in 65% and 11% of the cases, respectively. Neither eNOS nor nNOS expression was associated with vascular density, tumor grade or the TNM status of the tumor. A very strong correlation between expression of iNOS in malignant tissue and axillary lymph node metastasis was found by Duenas-Gonzalez et al. [22] in 22 patients with primary breast tumors.

The existence of a diverse pattern of nitric oxide synthase gene expression in four human **colon cancer** cell lines was reported by Jenkins et al. [23]. All lines (SW480, SW620, DLD-1 and WiDr) expressed mRNA for eNOS, while SW480 also expressed nNOS. The mRNA for iNOS was expressed by cytokine-stimulated and unstimulated SW480, SW620 and DLD-1 cell lines. The WiDr cells did not express iNOS at all. Enhanced expression of iNOS and eNOS in human colorectal cancers was confirmed immunohistochemically by Yagihashi et al. [24]. The iNOS immunoreactivity was detected in the 22 of 25 cases of colorectal cancer, yet this expression did not correlate with pathological grading, tumor size, lymph node metas-

tasis, p53 expression or tumor vessel density. Ambs et al. [25], after analysis of 118 colon tumors, found increased expression of iNOS in various tumors throughout the ascending, descending and sigmoid colon. The iNOS activity declined with advancing tumor stage and was at the lowest level in metastatic tumors and at the highest in adenomas. Immunohistochemical analysis localized iNOS mainly in tumor-infiltrating mononuclear cells and less frequently in endothelial cells within the tumors and in the tumor cells. A protective role of iNOS in colorectal carcinogenesis was also suggested by Ropponen et al. [26] based on the results of the study on 157 colorectal carcinoma patients. The authors found that iNOS immunostaining intensity was higher in Dukes A and B tumors than in Dukes C and D tumors and that low iNOS intensity was associated with higher histological grade. Moreover, cancer related survival was significantly lower among patients with a lower iNOS intensity and lower number of iNOS positive cancer cells.

Nitric oxide synthase activity was shown in human **gynecological cancers** [27]. The activity was highest in poorly differentiated tumors and was undetectable in normal tissue. Investigation of immunoreactivity revealed NOS activity only in malignant cells. NO biosynthesis was also detected in cells isolated from fresh ovarian tumor tissues [28] and ovarian carcinoma cell lines: SKOV-6, OVCAR-3 and HOC-7 following incubation with interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [29].

Accumulating data indicate overproduction of NO in patients with **lung cancer**. Arias-Diaz et al. [30] reported increased levels of nitrite/nitrate in the bronchoalveolar lavage (BAL) fluid of lung cancer patients (squamous cell carcinoma). Elevated levels of exhaled NO and enhanced nitrite generation in BAL fluid of primary lung cancer patients was further confirmed by Liu et al. [31]. The authors suggested that the increased NO production was most probably attributable to up-regulation of iNOS activity in alveolar macrophages as a consequence of the tumor-associated non-specific immunological and inflammatory processes of the host. An important role for NO in the metabolism and behavior of lung cancers (especially adenocarcinoma) was suggested by Fujimoto et al. [32] who investigated the activity and distribution of NOS in 72 primary lung cancer samples and in normal lung tissues. The total NOS activities in lung adenocarcinoma samples were significantly higher than those in other types of lung cancers or normal lung samples. Although, there was no correlation between tumor grade of the adenocarcinoma samples and NOS activity, cancer tissues from patients with N2 disease tended to have lower activity than those from patients with N0 or N1 disease. Marrogi et al. [33] studied immunohistochemically iNOS protein expression levels in 106 surgically resected human non-small cell lung cancer (NSCLC) specimens and found the immunoreactivity in 48% of the study subjects. The levels correlated with microvessel density at the tumor stromal interphase. Overexpression of iNOS was more frequent in adenocarcinomas and large cell carcinomas than in squamous cell carcinomas. Some results show prominent expression of iNOS in malignant mesothelioma and metastatic adenocarcinoma of the pleura when compared to healthy pleural mesothelium [34,35].

Recently, an increased expression of NOS protein was shown in **cancers of the head and neck**. The results obtained by Gallo et al. [36] indicated that NOS activity in tumor tissue from patients with head and neck cancer was increased threefold to fivefold in comparison to specimens of normal mucosa. Interestingly, the NOS activity was higher at the invasive tumor edge than in the tumor core. The authors also demonstrated that increases in NOS activity, cGMP levels, and microvessel density at the tumor periphery highly correlated with the metastatic phenotype of the head and neck cancer. In the study of immunohistochemical expression of iNOS in 41 cases of oral squamous cell carcinoma Brennan et al. [37] found significant association between the expression and lymph node metastasis. Although lymph node metastasis correlated with the degree and intensity of iNOS staining, there was no correlation between the degree of tumor differentiation and iNOS staining. Based on immunohistochemical study in 36 cases of oral dysplasia of varying severity, Brennan et al. [38] reported a significant correlation between iNOS staining and grade of dysplasia and

between p53 expression and iNOS staining. It has also been shown, that the iNOS is expressed in pleomorphic adenomas of the parotid [39].

Some data suggest that iNOS may play an important role in the **malignant transformation of melanocytes** and in **melanoma** growth. Ekmekcioglu et al. [40] showed that melanoma cells in the 12 of 20 cases expressed iNOS and that iNOS and nitrotyrosine immunostaining strongly correlated with poor survival in patients with stage 3 of disease. Also, Massi et al. [41] reported significant correlation between iNOS immunoreactivity and progression of malignant melanocytic lesions. A very important role of NO on human melanoma cell survival was confirmed by Salvucci et al. [42]. Inhibition of endogenous production of NO by melanoma cells led to their apoptosis, a process preceded by up-regulation of mRNA levels of *bax, caspase-1, -3, -6, gadd45beta, mdm2* and TNF-related apoptosis inducing ligand (TRAIL) genes.

Epithelial cells of **prostate carcinoma** tissues stained highly positive for iNOS, whereas the benign hyperplasia specimens did not [43]. Similarly, Uotila et al. [44] reported iNOS immunostaining being significantly stronger in prostate cancer cells than in the nonmalignant glandular epithelium of the control prostate. Moreover, iNOS was also overexpressed in the cells of prostatic intraepithelial neoplasia (PIN). The comparison of the clinical and histological data from 82 prostate cancer patients with the results of the immunohistochemical analysis of iNOS in cancer tissue revealed that a high expression of iNOS was related to a high pT classification of the tumor and the preoperative prostate specific antigen (PSA) level [45].

Positive iNOS immunostaining, although of highly inhomogenous pattern, was detected in samples of **bladder cancer** tissues, but not in nonmalignant tissues adjacent to malignant areas [46].

Cobbs et al. [47] examining human **brain tumors** for NOS isoforms detected the increased expression of nNOS and eNOS in astrocytic tumors with the highest levels found in higher grade tumors. Considerably higher iNOS mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) was reported by Ellie et al. [48] in the human glioblastoma than in the meningioma specimens.

As can be seen from the short preview of the studies on NOS involvement in human cancer development, it is not possible to clearly define the role of NO. Different cellular sources of the increased NO synthesis (activated macrophages, tumor or endothelial cells) make the assessment more complicated. In most cases it is also hard to correlate the intensity of NOS immunostaining with the tumor grade and stage. Although there are some reports which indicate a strong correlation between particular NOS isoforms activity or expression and patients' survival, it seems that the measurements cannot serve as a reliable marker in routine histopathological or clinical practice.

Pro- and antitumorigenic effects of nitric oxide

The phenomenon of tumor growth and spreading depends on several processes, including tumor cell proliferation and capacity for angiogenesis, invasiveness and tendency for metastasis. Although, the mechanisms of protumorigenic effects of NO are not as far fully understood, the role of NO in each of the afore mentioned processes has been well documented.

Tumor growth

Inhibition of *in vivo* NO production by the treatment with the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), reduced growth of colon adenocarcinoma in rat [49]. Although EMT-6 murine mammary carcinoma cells, stimulated to NO production by lipopolysaccharide (LPS) and interferon-gamma (IFN-gamma), grew much more slowly in *in vitro* culture, after injection into BALB/c mice, they demonstrated a twofold increase in subcutaneous tumor growth and experimental pulmonary metastases over control cells [50]. Simultaneous treatment with L-NAME *in vitro* blocked LPS/IFN stimulation of NO production and restored cell growth to near control levels. In *in vivo* tests L-NAME reduced tumor size and number of lung metastases to control levels. Similarly, Jenkins et al. [51] showed that human colon adenocarcinoma cell line DLD-1 engineered to overexpress iNOS proliferated at a slower rate *in vitro* than the control cells. However, the line subcutaneously injected into nude mice grew faster and had greater capacity for angiogensis than the wild-

type and/or transfection controls. The results suggest a dual role for NO in cancer. When it is produced at high concentrations it has antitumor activity (apoptosis, cytotoxicity), but at lower concentrations it promotes cancer growth.

Angiogenesis

Growth of solid tumors cannot proceed beyond a microscopic size without the development of an extensive vascular system. Endothelial cells interact with tumor cells during tumor vascularization and during the metastatic process. Tumor cells produce factors like vascular endothelial growth factor (VEGF) that influence endothelial functions, whereas endothelial cells produce adhesion molecules and soluble molecules that interact with tumor cells. An important role of NO in angiogenesis was shown by Lee et al. [52] who found impaired angiogenesis and wound healing in eNOS-deficient mice. Similarly, Murohara et al. [53] observed suppressed angiogenesis in response to tissue ischemia in eNOS-deficient mice.

Several studies confirm the hypothesis that eNOS-derived NO is a downstream signal for growth factor-induced angiogenesis. Vascular endothelial cells during cancer development can express three high affinity VEGF receptors: e-f-ms-like tyrosine kinase-1 (lft-1), kinase insert domain-containing receptor/fetal liverkinase-1 (KDR/flk-1) and flt-4. Among the receptors, KDR/flk-1 is believed to play the predominant role in angiogenesis [54]. It has been shown that KDR/flk-1 mediates NO production induced by VEGF through formation of the KDR/flk-1-*c-src* complex, which triggers Ca^{2+} release through the inositol triphosphate (IP_3) a second messenger pathway. As a result, eNOS Ca^{2+}-dependent on and constitutively expressed in vascular endothelial cells, is almost immediately activated after tissue exposure to VEGF. One possible mechanism of angiogenesis modulation by NO is the suppression of protein kinase C-δ (PKC-δ) [55]. Another mechanism is the modulation of adhesion molecule expression on the surface of endothelial cells. NO has been shown to maintain the functional expression of $\alpha_v\beta_3$-integrin, a mediator for endothelial cell migration, survival, and angiogenesis [56].

An important role of NO produced after VEGF receptor activation in cancer development can be illustrated using breast cancer as an example. In this type of cancer the expression of VEGF correlates with high microvessel density, and both features are associated with poor prognosis [57]. Transfection of VEGF121 isoform into human MCF-7 breast carcinoma cells (the obtained cell line was designated as V12 cells) has been shown to enhance tumor growth and vascular density *in vivo* and promote a strong angiogenic response [58]. Similarly, Ziche et al. [59] reported that V12 cells implanted into rabbit cornea induced a strong angiogenic response, which was efficiently blocked by systemic NO synthase inhibition.

Invasiveness and metastasis

The sequence of biochemical events during tumor cell invasion of the extracellular matrix can be described by a three-step theory: 1. tumor cell attachment to the matrix mediated through specific matrix glycoproteins (eg. laminin and fibronectin) and through tumor cell receptors; 2. secretion of hydrolitic enzymes (eg. serine proteinases, neutral metalloproteinases and cysteine proteinases) by tumor cells which locally degrade the matrix; and 3. tumor cell locomotion into the area modified by proteinases.

Suggestions of metastases promoting role of eNOS were made by Lala and Orucevic [60] who found that tumor cells at primary sites were highly heterogenous in eNOS expression, whereas those at lung metastases were strongly and homogenously stained for eNOS. Moreover, L-NAME treatment reduced the migratory capacity of highly (C3L5) and weakly (C10) metastatic cell lines clonally derived from a spontaneously arising mammary tumor in mice [61]. The migratory capacities of the cell lines were restored to baseline levels after additional treatment with excess L-arginine (proof of NO-specific effects).

There are some possible explanations for NO-stimulated increase in tumor invasiveness:

- it was shown that endogenous NO was involved in matrix degradation by C3L5 cells as a result of altered bal-

ance between matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) [62]. While constitutive NO production by tumor cells evoked down-regulation of TIMP-2 and TIMP-3, additional inductive NO production led to up-regulation of MMP-2;

- NO is able to up-regulate urokinase-type plasminogen activator (uPA) in endothelial cells [63], uPA, which converts plasminogen to plasmine, can finally lead to activation of numerous MMPs;
- NO is known to degrade human, bovine, and rabbit articular cartilages by stimulating MMPs (e.g. collagenases or stromelysin) in chondrocytes [64,65];
- peroxynitrite ($ONOO^-$) - product of the reaction of NO with superoxide anion - can inactivate TIMP-1 protein *in vitro* [66].

Although the above data confirm protumorigenic effects of NO, there are reports documenting inhibitory role of NO in cancer development. Because the issue of the antitumorigenic effects of NO is beyond the scope of this paper, only some reports are quoted just to confirm the existence of such effects. Xie et al. [67] found that administration of multilamellar vesicle liposomes (MLV) with lipopeptide CGP31362 combined with murine IFN-gamma caused regressions of liver metastases after the i.v. injections of M5076 murine reticulum sarcoma cells into C57Bl/6 mice. This effect was most probably mediated by the activation of iNOS in sarcoma cells and increased production of NO, which eventually led to tumor cells apoptosis. Similar results were obtained using murine K-1735 melanoma cells and human SN12PM6 renal carcinoma cells in which engineered overexpression of iNOS diminished capacity for metastasis [68,69].

CONCLUSIONS

The roles of nitric oxide in carcinogenesis are very complex and hard to be clearly defined. There are actually two conflicting views on NO involvement in cancer development, considering the molecule as pro- or antitumorigenic agent. The data presented in this paper confirm genotoxic properties of nitric oxide, as well as the existence of a diverse pattern of NOS protein expression and/or its activity in tissue of tumors of different organs in humans. Although increased expression of NOS protein was shown in many tumor types it is difficult to draw uniform conclusions as to the correlations between the intensity of particular NOS isoform immunostaining and the grade and stage of the tumor and also patient's survival. Many results of studies conducted using different experimental models confirm active involvement on nitric oxide in tumor cells proliferation, their capacity for angiogenesis, invasiveness and tendency for metastasis. In these processes dual effects of NO can be noticed. They are particularly distinct in the case of tumor growth. Nitric oxide produced in excess by tumor cells shows antitumorigenic activity (cytotoxicity, apoptosis), but at lower concentrations it promotes cancer growth. The short preview presented in this paper unequivocally shows that there is still much to be done to finally characterize the complex protumorigenic effects of nitric oxide.

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