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REVIEW

Nitric Oxide and Cancer

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ABSTRACT

Nitric oxide (NO) is a pleiotropic biological mediator which plays a key role in various physiological and pathological processes. It is synthesized with the help of the enzyme nitric oxide synthase (NOS), which has three isoforms. All these isoforms of NOS have been reported to be involved in promoting or inhibiting the aetiology of cancer. High levels of NOS expression in tumour cells may be cytostatic or cytotoxic, while low levels can have the opposite effect and may promote tumour growth. NO also has diverse effects in cancer treatment. It can enhance the cytotoxic efficacy of some chemotherapeutic agents as well as radiation. The modification of NOS activity in tumours can be considered to be a promising mean for selective tumour blood flow modification, thus providing a novel approach for reducing tumour oxygenation which is aimed at enhancing the efficiency of hypoxia- mediated, bioreductively activated modalities for cancer treatment.

Key Words: Nitric Oxide, Nitric Oxide Synthase, Cancer, Chemotherapy, Radiotherapy

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Nitric Oxide

Nitric oxide (NO), which was thought to be just one of the polluting gases produced in car exhausts earlier, has been recognised only recently as a reactive molecule with broad and diverse effects in human biology. NO is a hydrophobic diatomic gas that transmits signals in the organism. Signal transmission by a gas that is produced by one cell, penetrates through membranes and regulates the function of another cell, thus representing an entirely new principle for signalling in biological systems [1]. It appeared that there was a chemical similarity between endothelium derived relaxation factor (EDRF) and NO. Besides, both compounds exerted vasodilatation by means of cyclic guanosine monophospahte (cGMP) synthesis. The groups of Furchgott and Ignarro independently proposed in 1986 that EDRF was really NO [2]. Soon, the group of Moncada (1987) obtained the first results which supported that proposal. Moreover, they demonstrated that endothelial cells produced NO in sufficient amounts to explain the relaxation observed. So, the previous proposal was confirmed [3].

NO is also known as a free radical as it possesses an unpaired electron. A free radical prefers to steal electrons from the lipid membrane of the cell, thus initiating a free radical attack on the cell, which is known as lipid peroxidation [1].

Synthesis of Nitric Oxide

NO is synthesized during the conversion of arginine to citrulline. Nitric oxide synthase (NOS) catalyses the reaction. Three isoforms of NOS have been identified- endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible (iNOS). All have binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) near the carboxy terminus (the reductase domain) and the binding sites for tetrahydrobiopterin (BH_4) and heme near the amino terminus (the oxygenase domain). The reductase and oxygenase domains are linked by calmodulin binding sites. This enzyme exists in both a constitutive (calcium–dependent) and inducible (calcium- independent) form in endothelial cells, platelets and placental tissue [4],[5].

The calcium-calmodulin complex, in combination with BH_4, binds to nNOS and induces its translocation from the plasma membrane to the cytoplasm, as it is soluble in both aqueous and lipid media and also readily diffuses through the cytoplasm and the plasma membrane. NO activates various cGMP–regulating signalling pathways which in turn, enhance the release of neurotransmitters, thus resulting in smooth muscle relaxation and vasodilation [5].

Two families of NOS with molecular weights ranging from 125 to 160 KDa have been isolated. One family is iNOS and its expression is tightly controlled by several transcription factors, the specific inducers being variable with the cell type. Cytokines such as the tumour necrosis factor, interleukin–1 and gamma-interferon have been shown to promote the synthesis of the enzyme. The iNOS is mainly found in macrophages and is often called as macrophage NOS (mNOS) but monocytes, megakaryocytes, fibroblasts, neutrophils, hepatocytes and smooth muscle cells also possess it. The gene of this inducible enzyme is present on chromosome number 17 [1].

The other family is constitutive NOS (cNOS) which is synthesized at a constant rate, regardless of the physiological demand. The constitutive enzymes are regulated by calcium-calmodulin and phosphorylation. According to the sites of productions, the constitutive family of enzymes may be of two subtypes -(a) eNOS is found in the endothelial cells, platelets, the endocardium and the myocardium. The gene for eNOS is present on chromosome number 7. (b) nNOS is seen in the central and peripheral neurons. This enzyme is also termed as brain NOS (bNOS) as it is chiefly found in different areas of the brain. Its gene is present on chromosome number 12. The nNOS and mNOS are found in the cytosol, but the eNOS is predominantly localised in the plasma membrane [1].

NO is inhibited by asymmetrical dimethylarginine (ADMA). ADMA is metabolised by dimethylaminohydrolase (DDAH) and excreted by the kidneys. In 1992, Vallance et al first described the presence of ADMA as an endogenous inhibitor of eNOS in human plasma and urine. Since then, the role of this molecule in the regulation of eNOS has attracted increasing attention. ADMA inhibits vascular NO production within the concentration range which is found in patients with vascular disease. ADMA also causes local vasoconstriction when it is infused intra-arterially and increases systemic vascular resistance and impairs renal function when it is infused systemically. Thus, elevated ADMA levels may explain the 'L- arginine paradox'; i.e. the observation that supplementation with exogenous L-arginine improves NO-mediated vascular functions in vivo [7].

Biological Actions of Nitric Oxide

The classical pathway by which NO exerts many of its actions is via the activation of the enzyme soluble guanaylate cyclase (sGC) and the resultant conversion of

guanosine 5´–triphosphate (GTP) to the second messenger cGMP. However, recent studies have established that NO can also act via cGMP-independent pathways in various systems, particularly during the inhibition of platelet aggregation and the regulation of inflammatory cell apoptosis. NO may have an additional beneficial effect on blood coagulation by enhancing fibrinolysis via an effect on plasminogen [1],[6].

As NO is a free radical, it is a highly reactive molecule within biological systems, reacting with other free radicals, molecular oxygen and heavy metals. It has been suggested that the biological effects of NO can be mediated by the products of different NO metabolites e.g. nitrite, nitrate, S-nitroso-thiols or peroxynitrite. NO has also been reported to bind rapidly and with high affinity to ferrous iron (Fe^{2+}). NO can bind easily to free iron, iron within iron-sulphur centres, and iron within haemoproteins. NO is unusual because it reacts with both the ferric and ferrous forms of heme iron. The binding of NO to ferrous ions is irreversible and occurs with very high affinity i.e. 10,000 times more than for oxygen.[3] NO may also be involved in the regulation of protein activity through S-nitrosylation. In the extracellular milieu, NO reacts with oxygen and water to form nitrates and nitrites. The toxicity of NO is linked to its ability to combine with superoxide anions to form peroxynitrite, an oxidising free radical that can cause deoxyribonucleic acid (DNA) fragmentation and lipid peroxidation. In the mitochondria, peroxynitrate acts on the respiratory chain complex and manganese superoxide dismutase to generate superoxide anions and hydrogen peroxide respectively [3],[4].

NO is a vasoactive substance which is produced by endothelial cells. NO is a vasodilator and has been believed to be decreased in pre-eclampsia [7].

Recent studies have revealed that NO can also modulate apoptosis or programmed cell death in a variety of cell types, including human inflammatory cells. Apoptosis of inflammatory cells is a highly regulated process whereby cellular death occurs without disruption of the cell membrane and by the subsequent release of pro-inflammatory and histotoxic contents of the dying cell [8]. NO can be both pro and anti-apoptotic, depending on local concentration and the specific cell type in question. Current evidence suggests that lower concentrations of NO produced by eNOS and nNOS are cytoprotective, while supraphysiological concentrations produced by the iNOS trigger cell death. This paradox may be explained, at least in part, by the free radical nature of NO and hence, the ease with which it reacts with other radicals, particularly reactive oxygen species (ROS), present in the milieu to form various NO-related species in vivo [4].

Endothelial cell dysfunction can be linked to vascular diseases such as atherosclerosis and hypertension. Endothelial cells produce biologically active molecules such as prostacyclins, NO and endothelins. These molecules are believed to play a major role in the control of vascular diameter and tone. It is now believed that the dysfunction of endothelial cells can contribute to inappropriate vasoconstriction and platelet aggregation, which are early signs of atherosclerosis, hypertension and coronary and cerebral vasospasm or thrombosis [7].

Moncada et al showed that other situations in which high amounts of NO may be synthesized include inflammation, re-endothelialisation and angiogenesis. In these situations, the inducible isoforms of iNOS, the enzyme that catalyzes the formation of NO from the terminal guanidino nitrogen of L-Arginine, are expressed after induction by cytokines or endotoxins. Regarding angiogenesis, a process that is highly relevant to proliferative vitreoretinal disorders, NO has been shown to be a potent inhibitor of the cytokine-induced proliferation of endothelial cells [3].

Various authors suggested that tumour cells utilize certain NO-mediated mechanisms for the promotion of growth, invasion and metastasis and proposed that NO-blocking drugs may be useful in treating certain human cancers. There is also evidence that tumour-derived NO promotes tumour angiogenesis as well as the invasiveness of certain tumours in animals, including humans [9].

NOS Expression in Tumours

The first report of NOS expression in human tumour cell lines belongs to Radomski et al (1991), who studied human colorectal adenocarcinoma cell lines from a primary tumour (SW-480) and a lymph node metastasis (SW-620) derived from the same patient. Both cell lines were shown to express constitutively, a calcium-independent NOS activity. The constitutive expression of calcium dependent NOS has also been reported in the cervical epithelial cell line, ME-180 [10].

Fujimoto et al detected the increased expression of iNOS in 74% and 96% of malignant mesotheliomas and metastatic pleural adenocarcinomas, respectively and the expression of iNOS was found to be more in epithelial and mixed mesotheliomas as compared to the sarcomatoid subtype. They also observed that eNOS was found in 89% of the mesotheliomas and there was prominent iNOS and nNOS expression in metaplasia-dysplasia-lesions. So, they postulated that there was a divergent role of NOS in carcinogenesis and the destruction of the alveolar epithelium in the emphysematous lung. It was also observed by them, in samples obtained from patients of lung cancer, that iNOS was detected in 40% cases, while 89% and 81% cases were positive for eNOS and nNOS, respectively. It was also observed that increased levels of eNOS was seen more often in adenocarcinomas than in squamous cells carcinomas and raised levels of iNOS was seen more often in grade I-II tumours than in grade III tumors [11].

Pan JW et al estimated the eNOS and VEGF levels in blood samples from 37 patients of primary astrocytomas and four patients of astrocytic hyperplasia. They suggested that eNOS and VEGF may have a cooperative effect in tumour angiogenesis and may play an important role in the pathogenesis of primary astrocytoma [12].

Zhan et al conducted a study in 26 patients of choledochal cyst to assess the relationship between the expression of iNOS and the p53 gene, as well as the pathogenesis of choledochal cysts. Hyperplasia of the mucosa of the cysts and the amylase level in the bile were also investigated by them. It was observed by them that patients with a high level of amylase in the bile had higher expression of iNOS than those with a low level of amylase and a higher expression of iNOS was related to hyperplasia and carcinogenesis of the mucosa of choledochal cysts [13].

Jaiswal et al observed that there was increased expression of iNOS in gastrointestinal malignancies and NO contributed to carcinogenesis in gastrointestinal tissues by causing DNA lesions, thus inhibiting DNA repair enzymes such as human 8-oxodeoxyguanosine DNA glycosylase1, blocking apoptosis via nitrosylation of caspase and functioning as an angiogenesis factor [14].

Vaninetti et al studied patterns of p53 mutations and the expression of iNOS in oesophageal adenocarcinomas. They noticed a progressive increase in the iNOS messenger ribonucleic acid (mRNA) expression in tissues (63%) in oesophageal adenocarcinoma [15].

Begnami et al compared the expressions of apoptosis related proteins and NOS between Epstein Barr virus (EBV) positive and EBV negative gastric carcinoma. They observed raised expressions of NOS-1 and NOS-3 in

EBV associated gastric carcinoma and postulated that EBV positive gastric carcinoma showed a high expression of cNOS that could influence tumour progression and aggressiveness [16].

Gunel et al, in their study, measured serum interleukin-18 (IL-18) and nitrate and the nitrite levels in 56 patients with non-metastatic breast cancer and 14 control subjects. They observed that serum IL-18 and nitrate and nitrite levels were significantly higher in patients with breast cancer when compared to the control subjects. They demonstrated that increased NO activity positively correlated with oestrogen receptor (ER) expression in breast carcinoma. It was postulated by them that serum IL-18 and NO activity can serve as a prognostic predictor in patients with breast cancer [17].

MacLeod et al found that the rate of breast tumour growth and metastasis was significantly reduced when a dietary component of protein (arginine that is needed for the synthesis of NO) was removed from the diet of mice [18].

Marcelo et al, in their study, analysed the expression of iNOS in 15 untreated patients with acute myeloid leukaemia (AML) and in 7 normal controls. By using flow cytometry and immunocytochemistry, they demonstrated that patients with AML had a high expression of iNOS when compared to controls [19].

Zhao et al investigated the expression of the different isoforms of NOSs in B cell-Chronic lymphocytic leukemia (B-CLL) to delineate a possible role for NO in the control of the apoptosis of the tumoral cells. They observed that all B-CLL cells expressed iNOS mRNA, whereas eNOS mRNA was undetectable. It was also found by them that the NO released, exerted an anti-apoptotic effect on B-CLL cells [20].

NO as an Inhibitor of Tumour Growth

In human tumours, the role of NO has not been established. NO produces multiple effects that can influence the outcome of tumour growth and metastasis. This molecule regulates vasodilatation and platelet aggregation [21],[22],[23] which affect tumour cell arrest in capillaries[24]. NO is also a major cytotoxic mediator which is secreted by activated macrophages[25] and endothelial cells[24]. It is shown to be responsible for the destruction of tumour cells passing through capillary beds. The production of endogenous NO is associated with the apoptosis of tumorigenic cells [26],[27]. Cytotoxicity as a result of a substantial NO-formation is established to initiate apoptosis which is characterized by the upregulation of the tumour suppressor p53, changes in the expression of pro- and anti-apoptotic Bcl-2 family members, cytochrome c relocation, activation of caspases, chromatin condensation and DNK fragmentation [28]. Taken together, these results suggest the possibility that the production of endogenous NO may be detrimental to tumour cell survival and the production of metastasis [27]. Numerous *in vivo* and *in vitro* studies support this hypothesis [26],[29],[30]. An inverse correlation has been found between the production of endogenous NO and the ability of circulating K-1735 tumour cells to survive and produce metastases [30]. The data demonstrates that the introduction of an enzymatically active iNOS gene into highly metastatic murine melanoma K-1735 C4 cells (which express low levels of iNOS) induces apoptosis, suppresses growth and abrogates metastasis [29]. Some other reports have also suggested that the expression of iNOS can influence tumour growth and metastasis by regulating vasodilatation and platelet aggregation [21],[31], inhibiting angiogenesis [32] and inducing programmed cell death [28],[33].

NO as a Promotor of Tumour Growth

While NO had been shown to have anti-tumour properties [34], Jenkins et al [35] first reported the surprising finding that human carcinoma cells transfected with a murine iNOS cDNA cassette (DLD-1 cells generating 20 pmol min-1 mg-1 NOS activity) showed increased tumour growth, rather than decreased growth. By using a nude mouse/xanograft model, it was shown that the growth of these NO-generating tumours was accompanied by increased neovascularization. These results were supported by Ambs et al, who used recombinant iNOS expressing Calu-6 and HT-29 human carcinoma cell lines containing mutant p53 [36] to look at tumour growth. The authors demonstrated that an NO-mediated up-regulation of VEGF corresponded with increased vascularisation in the xenograft tumours. Therefore, it is possible that NO generated by NOS (located either within the tumour or in the surrounding stroma) may promote new blood vessel formation by up-regulating VEGF. This neovasculaturization not only enhances the ability of the tumour to grow, but also increases its invasiveness and metastatic ability.

The role of NO in tumorigenesis is multifactorial. NO could participate in the complicated process of carcinogenesis by mediating DNA damage in the early phases of tumorigenesis and support tumour progression through the induction of angiogenesis and the suppression of the immune response [9]. It has been demonstrated that oxygen radicals and nitrogen oxide derivatives such as peroxynitrite and nitrogen dioxide can effectively damage DNA despite the presence of multiple antioxidant defense and repair systems. Such damage is thought to make a significant contribution to the age-related development of cancer [37]. It is also proposed that increased NO production may select mutant p53 cells and contribute to human carcinogenesis and tumour progression [38]. Furthermore, bradykinin is known to activate an eNOS. Because bradykinin is generated effectivelly in tumours, the bradykinin-NO interplay may become an important issue in tumour growth [37]. The invasion-stimulating effects of NO are also due to the upregulation of matrix metalloproteases and the down regulation of their natural inhibitors. Recent reports implicate NOS involvement in the degradation of articular cartilage and show that NO activates metalloproteinase enzymes in chondrocytes and cartilage tissue from human, bovine and rabbit sources. The subsequent loss of integrity of the extracellular matrix and the basement membranes would promote angiogenesis, invasion and the metastatic process [38],[40].

Possible Mechanisms for the Dual Effect of NO on Tumour:

It is proposed that low concentrations of NO can be pro-angiogenic and pro-tumour growth inducers, whereas higher NO concentrations can have the opposite effects [41]. The effect of NO production in tumour biology may change during tumour progression [38]. This hypothesis is supported by data investigating the role of NO in cancer metastasis. After *in vitro* incubation with cytokines or lipopolysaccharide (LPS), non-metastatic cells exhibited a high level of inducible NOS activity and NO production, whereas metastatic cells did not [42]. Cancer growth can be stimulated as well as inhibited by the immune system. The intratumoural macrophageal arginine metabolism is one molecular explanation for the dual ability of the immune system to inhibit or stimulate tumour growth. It has been suggested that arginine metabolism in the tumour bed, yielding citrulline and NO, favours tumour rejection, whereas the production of ornithine and urea could promote tumour growth [43].

Role of NO in Cancer Treatment

Several studies have demonstarted that NO releasing agents can kill tumour cells and as a consequence, there have been attempts to deliver NO to cells. While NO-releasing drugs are under developement, an attractive alternative mechanism for delivery would be to transfer NOS- encoding cDNA sequences into cancer cells for gene therapy purposes [44]. Several studies have shown that this approach may work. For example, by using a mouse model, it was demonstarted that the transfection of K-1735 melanoma cells with an iNOS cDNA expression cassette suppressed tumourogenicity and abrogated metastasis [45]. Transfection of human renal carcinoma cells with a retroviral iNOS cassette showed similar results [46]. A problem with current approaches however, is that the constitutive expression of NOS can quickly result in the death of the transfectant, thus shortening the time in which NO can be generated and potentially limiting the utility of the approach. NOS transfectants often have to be cultured under conditions that reduce toxicity (for example, in the presence of a NOS inhibitor) and transfection attempts may result in cells that are capable of relatively low levels of NO-generation [36]. As discussed above, this may result in concentrations of NO that promote tumour growth rather than cell killing. Another significant point is that NOS enzyme activity requires a panel of substrates and co-factors for full activity and these may be missing from the target cell type. For example, the synthesis of the important co-factor, tetrahydrobiopterin (BH_4), requires transcriptional regulation of the rate- limiting enzyme GTP cyclohydrolase,which may not be induced in all target cells [47].

Tumour cytokine therapy using Interleulin-2 (IL-2), IL-1β, or cytokine-inducing agents such as flavone acetic acid (FAA) or analogues, has been shown to induce NOS inman. Several of these studies associate increased NO production with the toxic effects of cytokine therapy, such as circulatory changes and hypotension. But there are studies indicating that patients with the highest plasma nitrate levels show the highest response to the therapy [39], [48]. It has also been proposed that selected NO blocking drugs may be useful in the treatment of certain human cancers – either as single agents or as a part of combined therapies. In accordance with that, the treatment of tumour-bearing mice with NO-blocking agents reduce the growth and vascularity of primary tumours and their spontaneous metastases [9]. The NO-inhibitory ϖ3 polyunsaturated fatty acids are known to reduce the risk of colon cancer in both man and rat [49]. Arginine analogues block the promotional phase of the neoplastic transformation of mouse fibroblasts. On the other hand, they could potentiate the pulmonary metastasis of Lewis lung carcinoma and B16 melanoma cells [50].

Radiotherapy and NO:

One of the major factors that limits the effectiveness of radiation therapy is the presence of radioresistant hypoxic tumour cell populations. Over the past 50 years, there has been a concerted effort to identify the agents which are effective hypoxic cell radiosensitizers. To date, there have been few substances which are able to effectively overcome the hypoxic effect. However, several papers have reported that NO can radiosensitize hypoxic cells extensively. Howard-Flanders first showed in 1957 that NO radiosensitized hypoxic bacteria [51]. Nearly four decades later, it was shown that NO and NO donor compounds effectively radiosensitize hypoxic mammalian cells too, with a substance enhancement ratio (SER) of 2.4 [52]. These findings demonstrate that NO is equally as effective as oxygen at enhancing the sensitivity of hypoxic cells to radiation.

NO-mediated radiosensitization of hypoxic cells is proposed to occur via a mechanism which is similar to that of oxygen molecules (O_2) [51],[52]. Carbon-centered radicals are initially generated by ionizing radiation on DNA. In the absence of NO or O_2, these reactive radicals scavenge the nearby protein

hydrogen atoms, thus facilitating DNA repair. This process limits the number of DNA lesions per photon. However, it has been postulated that NO reacts with these high energy carbon-centered radicals to form complexes which are not capable of abstracting protein hydrogen atoms. The fixing of the radiation induced damage increases the number of lesions per photon, thus increasing radiation-mediated cell death. An advantage of using NO as a hypoxic cell radiosensitizer is that it penetrates into the tissue farther than oxygen due to its higher diffusion coefficient [53],[54]. The lower concentrations of NO and the higher diffusion rates suggest that NO may be an ideal candidate for new strategies in radiotherapy.

Conclusion

Thus, NO can be considered to be a 'double edged sword' in cancer. High concentrations may mediate cancer call apoptosis and the supression of cancer growth, while low concentrations may promote tumour growth and proliferation. It is also important in understanding the treatment protocol and the various modalities being used in the treatment of various cancers. Thus, NO performs a multifactorial role and appears to be a promising molecule for further research in the diagnosis, management and monitoring of cancer patients.

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