Tumors Face NO Problems?

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Abstract

The effect of nitric oxide (NO) synthase (NOS) II expression on cancer biology is unclear and difficult to define, with multiple reports of pro- and anti-cancer actions. Here we address the major factors that seem likely to account for these paradoxical behaviors, which include variability in NO production, heterogeneity in NO chemistry (and thus its cellular actions), and differential cellular responses. In addition, we suggest that a major determinant of the outcome of NO actions in the tumor environment is cellular adaptation-selection to the cytotoxic actions of NO. (Cancer Res 2006; 66(13): 6459-62)

Introduction

Inducible nitric oxide (NO) synthase (NOS II) is an enzyme with high NO output and has been identified in many types of cells in different animal species with high homology (1-5). NOS II also is expressed in tumors in various species, including human tumors of diverse tissue origins. However, NOS II expression in tumor and/or stromal cells, as well as the subsequent effect of NO production on tumor biology, is complex and remains unclear (3, 5). Some studies have found that NOS II is tumor suppressive whereas others have provided evidence that NOS II promotes tumor growth (3-6). Here we provide a brief examination of the major factors that, based on recent experimentation, seem to determine this heterogeneity of NO actions in the in vivo tumor environment.

NO Heterogeneity

The central concept required for accurate appreciation of the multiple actions of NO in the tumor environment is that of heterogeneity. This overall principle can be divided into chemical heterogeneity and biological heterogeneity.

Chemical heterogeneity. In the biological milieu, four chemical processes occur on exposure to NO: nitrosylation, oxidation, nitrination, and nitrosation. The relative abundance of these four processes at any spatial location is determined by the presence of key reactants (oxygen, superoxide, and CO₂) and targets (metals, thiols, and tyrosine) as well as the NO concentration. Even for an identical free NO concentration, therefore, homogeneity in NO biological actions should be the exception rather than the rule. Importantly, there are huge gaps in our understanding of the relative contributions of these four different chemical processes, which impart very different effects on targets, on the tumor biology of NO.

Biological heterogeneity. Heterogeneity in cellular production, consumption, and responses to NO compose the biological heterogeneity of NO actions. Without a doubt, one of the most important determinants of NO biological actions in the tumor environment is the location and abundance of NO synthase(s). NOS II protein expression, in particular the "high-output" NOS, varies widely depending on the availability of both endogenous and exogenous regulatory signals (3). Diverse intertumor and intra-tumor heterogeneity and complex genetic background and functional status of both tumor and stromal cells are crucial variables in NOS expression. An additional complication is that the effect of NOS II expression on human tumor development and progression has been inferred primarily from descriptive observations with staining of NOS II protein and measurement of NOS II activity in human specimens (7-9). Such measurements either provide no indication of the magnitude of NO formation (staining of NOS II protein) or yield only an average distribution throughout the tissue (NOS II activity). It also must be appreciated that regardless of NO sources, such as NO donors, NOS II activation, or NOS II gene transfer, the presumed effects of NO in cell cultures may have only limited in vivo physiologic and pathologic relevance (3, 7). Perhaps even more fundamentally troublesome, the definitions of NO output have been quite descriptive and inconsistent among different laboratories and there is consequently a dearth of ability to compare the relative magnitudes of NO output for different experimental studies, which may have directly opposite results (see below). Finally, in in vivo studies, various NOS activators and inhibitors have many known and unknown pharmacologic effects and have different influences on the expression and activity of different NOS isoforms and the actions of the NO produced at different locations. Therefore, NO donors or NOS II activators and/or inhibitors are quite likely to have global effects in animal models that may not mimic the physiologic/pathophysiologic environment (3, 10).

Quite possibly, the single most important component of biological heterogeneity to NO is the vastly differing responses of individual cells to NO, and in the tumor environment the canonical dichotomy of NO is its apparent antitumor role as an inducer of cytotoxicity and its apparent protumor role as an inducer of angiogenesis. In terms of cytotoxicity, numerous studies have shown that certain concentrations of NO induce cell arrest, apoptosis, and even necrosis in susceptible cells, whereas other concentrations of it can be antiapoptotic. In addition, cellular sensitivity to NO is not necessarily time invariant. Specifically, it
has been known for more than 10 years that small amounts of NO exposure can effect a preconditioning response, in which cells develop resistance to an otherwise lethal dose of NO (11). It was also shown that NO-induced preconditioning also imparts cross-resistance to other reactive species (i.e., H₂O₂). Of special interest to NO in the tumor environment is the relationship between NO cytotoxicity and p53 expression/function. Both endogenous and exogenous NO activate the wild-type p53 gene, which is closely associated with apoptosis (3, 8, 12–14). Therefore, loss of functional p53 may lead to reduced NO sensitivity. This led Amb et al. (14) to hypothesize that the cellular p53 status influences the fate of tumor cells exposed to either exogenous or endogenous NO, which may provide selection pressure for clonal expansion of cells with mutant p53 expression. However, several studies have shown that NO produces both p53-dependent and p53-independent apoptosis in tumor cells (3, 15, 16).

In terms of the protumor action of NO as an inducer of angiogenesis, several lines of evidence have suggested that a very low level of NO production, such as that produced via NOS III, can promote tumor migration and endothelial cell proliferation and differentiation. However, whether or not the level of NO produced via NOS II actually promotes tumor growth is uncertain (3–5, 7, 8). A number of studies have suggested that NO can alter the expression of genes important to tumor angiogenesis and metastasis (5, 6, 17). Notably, NO has been shown to increase the expression of several protumor factors (18, 19).

**Toward Homogeneity**

In an attempt to decrease the number of confounding variables in NO tumor biology, several groups have studied genetically modified tumor cells and mouse models including NOS-expressing human tumor cells in nude mice (6, 20). In our recent study, we used adenoviral expression to induce human tumor cells to produce NO (20). By incorporating various NOS mutations, we were able to generate some cell lines that produced NO at higher levels than previously reported, whereas others produced NO at lower or similar levels (6, 9, 20). Using this unique system, we found that the extent of NO-mediated cytotoxicity and antitumor activity is directly proportional to *in vitro* NOS activity and NO production. Therefore, this is the first biological system to show concentration-dependent NO cytotoxicity *in vitro* and *in vivo*. Importantly, wild-type NOS expression showed maximal suppression of primary tumor growth and distant metastasis in spite of its concomitant up-regulation of protumor factors vascular endothelial growth factor and interleukin-8, suggesting that the antitumor cytotoxic actions of NO in this model outweigh potential protumor actions (Fig. 1).

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**Figure 1.** NOS II and tumor progression. In a heterogeneous tumor, uneven NOS II expression and NO production in both tumor and stromal cells lead directly to cytotoxicity in tumor cells that produce NO and the immediate surrounding tumor cells. However, induction of protumor factors by NO in surviving tumor cells and stromal cells may lead to two major consequences: development of NO resistance in tumor cells and promotion of angiogenesis. Therefore, NO cytotoxic effect favors the survival and expansion of more malignant cells.
Moreover, a predominant source of NO production in a growing tumor could be that from host stromal cells (21). The actual level of NO production in tumor bed is contributed by NO production in both stromal and tumor cells (3, 21). Therefore, a combined use of NOS II−/− animal and tumor cells engineered to produce defined levels of NO using those mutant NOS II genes would further limit the number of confounding variables and provide definitive evidence on concentration-dependent NO tumor biology. Indeed, our recent studies using both NOS II−/− animal and tumor cells have shown that NO produced by host stromal cells can be sufficient to execute antitumor activity (21).

In addition, NOS II transduction almost totally abrogated the growth of various human tumor cells, including the PC3 prostate cancer, AGS gastric cancer, DLD-1 colon cancer, HT-1080 fibrosarcoma, MDA-MB-453 breast cancer, 253J BV bladder cancer, SKOV3 ip1 ovarian cancer, and SN12PM6 renal cell carcinoma cell lines (20). These cell lines exhibited diverse p53 functional statuses, ranging from wild-type (e.g., A375SM) and mutant (e.g., Km12SM) forms of the gene to loss (e.g., COLO357-L3.3) of it. Our results suggest that the antitumor cytotoxic actions of NO in this model outweigh potential NO resistance and growth advantage rendered to tumor cells by a mutated p53 gene as Amb et al. previously showed (6). However, in both cases, NO production and action are compared among different human tumor cell lines which differ not only in p53 status but also in overall genetic background (different cell lines). In addition, the influence of NO production by host stromal cells has not been accounted for in these human tumor xenograft models (6, 20); in particular, the levels of NO production defined in vitro may not necessarily represent the levels in the tumor bed. Therefore, it seems premature to conclude the definitive role of p53 in tumor growth and progression. To address these two issues by limiting those confounding factors, NOS II−/− nude or severe combined immunodeficient mice should be used to clearly define the effect of different concentrations of NO on human tumor growth in vivo of isogenic cell lines differing only in p53 status, such as p53-wild-type and p53-knockout HCT116 cell lines. Likewise, to test the effect of a given variable on NO cancer biology, it is crucial to make other variables constant while changing the variable to be tested.

**NO Further Problems**

Tremendous efforts have been made to address the induction of NO expression in tumor and/or stromal cells as well as the subsequent NO production and its effect on tumor biology. Before logically designing an effective preventive and therapeutic strategy by targeting tumor-associated NOS II/NO, one must clearly understand the mechanisms of NOS II expression and NO production and their actions in the tumor bed, which have yet to be defined. NOS II gene transfer apparently can achieve NO-mediated tumor suppression regardless of the tumor type and increased expression of protumor factors, suggesting that in vivo NOS II–mediated tumor suppression differs mechanistically from in vitro NO-mediated cytotoxicity. These recent findings raise several new issues that must be addressed. First, for a specific cellular action, which of the four chemical processes resulting from NO (nitrosylation, oxidation, nitration, and nitrosation) is responsible? Second, is it possible to restore NOS II expression and NO production in an established growing tumor, and if so, what are the consequences? Third, do NO-mediated cytotoxicity and up-regulation of protumor factors occur through independent pathways, or are they causes/effects of each other? Fourth, given the fact that tumor cells grow in the tumor bed with consistent NO production, is it possible to reverse NO resistance and render tumor cells susceptible to NO produced as a result of the existing level of NOS II expression? Fifth, is it possible to establish a relevant model system to test the hypothesis that NO actually mediates clonal selection and expansion? Sixth, what is responsible for differences in sensitivity to NO cytotoxicity in vitro and to tumor suppression in vivo? Seventh, a more sophisticated model system is needed to define the role of p53 in sensitization to tumor suppression by NO. Finally, recent studies have uncovered several important regulators of NOS II expression (e.g., transforming growth factor-β and cyclooxygenase-2; ref. 22, 23) and signaling mechanisms for NO-mediated apoptosis (e.g., AKT pathway; refs. 24, 25). Then, how are those new and other information translated to effective NOS II/NO–based cancer therapy? We believe that tumor-associated NO production by NOS II is a major problem that has to be faced because of the cytotoxic nature of NO. However, for researchers, the problems of NO chemistry in the tumor environment, NO output, NO protumor activity, and the target cell type remain to be further investigated by moving from in vitro biology to more relevant animal models.

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