Cytokine-induced nitric oxide formation in normal but not in neoplastic murine lung epithelial cell lines

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Thompson, David C., Stephanie E. Porter, Alison K. Bauer, Kumuda C. Das, Brandon Ou, Lori Dwyer-Nields, Carl W. White, and Alvin M. Malkinson. Cytokine-induced nitric oxide formation in normal but not in neoplastic murine lung epithelial cell lines. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L922–L932, 1998.—Cytokim, a mixture of interferon-γ, tumor necrosis factor-α, and interleukin-1β, induces nitric oxide (NO) production in lung epithelial cell lines. It is not known whether neoplastic transformation alters a cell’s ability to form NO in response to cytokines. The present study investigated NO formation in two murine lines of immortalized “normal” (nontumorigenic) lung epithelial cells of alveolar type II origin, E10 and C10, and their sibling spontaneous transformants, E9 and A5. Nontumorigenic cells elaborated much more NO after cytokim exposure than did their tumorigenic counterparts. NO production was prevented by inhibiting protein synthesis and NO synthase and attenuated by dexamethasone. Northern and Western blot analyses of inducible NO synthase (iNOS) demonstrated cytokim-induced induction of iNOS only in nontumorigenic cells. The deficiency of NO production in tumorigenic cells was not associated with reduced iNOS mRNA stability or with differences in cytokim-induced nuclear factor-κB activation. Although cytokim caused a greater production of NO in E10 cells than in E9 cells, the same treatment induced equivalent proliferation in both cell lines. These results indicate a specific deficiency in cytokine-induced NO synthase in transformed murine lung epithelial cells relative to their normal progenitor cells and provide a model for investigating iNOS regulation.

NITRIC OXIDE (NO) exerts important physiological and pathophysiological actions ranging from vascular relaxation and neurotransmission to mediating macrophage cytotoxicity (44). Indeed, many of the toxic effects of macrophages directed against tumor cells involve NO, with inhibition of both mitochondrial respiration and DNA replication as important mechanisms (34, 36, 57). Investigation of the role of endogenous NO in pulmonary pathophysiology is of great interest because concentrations of NO are elevated in the air expired by asthmatics (30) and by patients suffering from upper respiratory tract infections (31) or bronchiectasis (32). Immunohistochemical localization of inducible NO synthase (iNOS) in the pulmonary epithelium (25) indicates local generation as a potential source of exhaled NO. NO can exert either beneficial or deleterious effects depending on the amount of NO and its biological context. A great variety of cell types, ranging from macrophages to vascular smooth muscle cells, can be induced to produce NO in response to cytokine exposure (44). A mixture of interferon-γ, interleukin-1β, and tumor necrosis factor-α (empirically termed cytomim) is commonly used to promote NO generation. Cytokim evokes NO production in immortalized and neoplastic lines of bronchial and pulmonary epithelial cells (3, 26, 52, 53) and in primary cultures of human bronchial epithelial cells (3, 52).

In airway and lung epithelial cells, interleukin-1β and tumor necrosis factor-α induce nuclear translocation of nuclear factor-κB (NF-κB; see Refs. 12, 28, 51). This transcription factor appears to be an important inducer of iNOS expression (62, 63, 67). The identification of several NF-κB binding motifs on the iNOS promoter (66) supports this proposal. In addition, iNOS expression may be regulated by other transcription factors, including interferon regulatory factors (IRFs; see Ref. 37).

The role of NO in neoplasia remains to be clearly identified. Suggested actions of endogenously produced NO in tumors include reduced metastasis due to vasodilatation and decreased platelet aggregation (15), changes in cell proliferation (4, 11), and tumor angiogenesis (29). Elevated levels of nitrite and nitrate, which are stable metabolites of NO, were detected in bronchoalveolar lavage fluids of lung cancer patients (2). Whether the NO is derived from tumor cells or activated inflammatory cells remains to be defined. No studies have compared the NO-producing capacity of related tumorigenic and nontumorigenic lung epithelial cells. The E10 and C10 epithelial cell lines derived from mouse lung expressed characteristics of type II epithelial cells at early passage (39). E9 and A5 cells are spontaneous transformants of E10 and C10 cells, respectively, that produce tumors when injected into mice (55). Other features that distinguish the tumorigenic siblings from their nontransformed progenitors include lack of contact inhibition of cell growth (54), expression of mutant Kras (46), and constitutive expression of p53 (unpublished data). These cell lines have been extensively characterized in order to understand the biochemistry of pulmonary neoplasia (39).

The present study investigates the ability of cytomim and individual cytokines to promote NO production in these sibling pairs of lung epithelial cell lines. Our results indicate that cytokine-induced NO production is deficient in the transformants.

METHODS

Cell culture and cytokine application. E10 and C10 (“normal” or nontumorigenic cells) and E9 and A5 cells (tumorigenic sibling cells of E10 and C10, respectively) are of type II
lung epithelial cell origin, as originally described by Smith and colleagues (54, 55). Type II features included the presence of lamellar bodies and the biosynthesis of surfactant. E10 and C10 are contact inhibited and anchorage dependent, whereas E9 and A5 are not. Cells were grown in culture in CMRL-1066 medium containing 10% fetal bovine serum and penicillin G (100 U/ml) - streptomycin (100 µg/ml) - amphotericin B (0.25 µg/ml) in a humidified atmosphere of 5% CO2 in air on either 100-mm plastic plates containing 10 ml of medium or 96-well plates containing 200 µl of medium. After confluence was attained, cells were serum deprived for 24 h before cytokine administration and throughout the 48-h course of the experiment to prevent exposure to any agents present in the serum that could modify iNOS expression. We determined in preliminary studies that removal of serum for at least 8 h was necessary to permit production of NO. Interferon-γ (murine, 15 U/ng; Sigma Chemical), tumor necrosis factor-α (human, 10 U/ng; Sigma Chemical), and interleukin-1β (murine, 10 U/ng; Genzyme) were applied singly or in combination to the medium. Cytomix is the combination of all three cytokines at a final concentration of 10 ng/ml each. After cytokine application, 100-µl samples of medium were withdrawn at various times and stored frozen for later nitrite assay.

Estimation of cellular NO production. Increases in medium concentration of nitrite were used as a measure of NO production. This was validated by demonstrating the sensitivity of cytokin-induced nitrite production to inhibition by the NO synthase inhibitors aminoguanidine and monomethyl-l-arginine (Sigma Chemical). In other experiments, glucocorticoid regulation of NO production in E10 cells was examined by application of dexamethasone (American Reagent Laboratories). Each agent was administered concurrently with cytokinx to produce the desired medium concentration. Nitrite concentrations were determined in 20- to 100-µl aliquots using chemiluminescence detection of NO as described previously (27). Briefly, medium samples were introduced into a stream of helium (ultrapure)-degassed reducing solution (0.1 M sodium iodide in 0.1 M phosphoric acid). NO produced in the aqueous phase was released through gas-permeable tubing in the Dunham cell and swept by a stream of ultrapure helium (General Air) into an NO chemiluminescence detector (Sievers 270B). Peak areas were recorded on an integrator (Hewlett-Packard 3390A), and sample concentrations were determined by interpolation from a sodium nitrite standard curve. Nitrite levels for each treatment were pooled (4–8 replicates) and represented as means with associated SE. Comparisons between control and cytokine-treated cells were made by Student’s unpaired t-test, with P < 0.05 being significant.

Western blot analysis. Cellular levels of iNOS protein were estimated by immunoblotting. Twenty-four hours after cytokinix application, cells were harvested by scraping and sonicated in an ice-cold homogenization buffer consisting of 20 mM HEPES-10% glycerol buffer (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (TNS buffer, blocking buffer) for 60 min and subsequently incubated with a specific iNOS mouse monoclonal antibody (Transduction Laboratories) at a 1:2,000 dilution for 60 min at 25°C. Blots were rinsed five times for 5 min with wash buffer (0.1% Tween 20 + 1% milk in TNS) and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Transduction Laboratories) as a secondary antibody for 60 min at 25°C. After additional rinses with wash buffer, Immunoblots were visualized by application of enhanced chemiluminescence Western blotting reagents (Amersham) to the blot and exposure to autoradiographic film. A single band was identified as iNOS by comparison with a macrophage lysate standard (Transduction Laboratories) and molecular weight markers.

Northern blot analysis. Cellular concentrations of iNOS mRNA were estimated by Northern blotting. Murine iNOS cDNA doped into pUC19 was generously provided by R. A. Robbins (University of Nebraska). Plasmids were amplified in Escherichia coli and purified with a plasmid preparation kit (QIAGEN, Chatsworth, CA). cDNAs were isolated from the vectors by treatment with Nco I, gel purified, and labeled with a random-prime DNA labeling kit (BRL). Total RNA was isolated from cells using an RNeasy kit (QIAGEN) and quantitated spectrophotometrically. Standard molecular biology protocols (7) were followed. Fifteen micrograms of RNA were subjected to electrophoresis on a 1% agarose-2.5 formaldehyde gel using a buffer containing 20 mM 3-(N-morpholino)propanesulfonic acid and 1 mM EDTA (pH 7.4). RNA was transferred to a nylon membrane (MSI) in 10× SSC buffer (1.5 M NaCl and 0.15 M sodium citrate) and prehybridized for 30 min at 65°C in buffer containing 5× SSC, 5× Denhardt’s, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. Blots were hybridized overnight with iNOS cDNA labeled to a specific activity of 10⁶ counts-min⁻¹ µg⁻¹ using [α-³²P]cytidine 5'-triphosphate in prehybridization buffer at 65°C. The blots were washed three times in a solution containing 2× SSC and 0.1% SDS and finally rinsed in a solution of 0.2× SSC and 0.1% SDS. All washes were conducted at 65°C. Blots were processed on a phosphorimager (Molecular Dynamics).

In these studies, cells were prepared for Northern blot analysis 24 h after treatment with cytokinmix or medium as a control. To evaluate RNA stability, cells exposed to cytokinmix for 24 h were treated with 100 µM 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB), a transcriptional inhibitor, and prepared for Northern blot analysis 3, 6, 9, or 12 h thereafter. Northern blot analysis

NF-κB nuclear translocation. Nuclear extracts were obtained from cells exposed to cytokinmix or a medium control for 2 h. Nuclear extracts were prepared according to methods described previously (56) with the following modifications. Approximately 2 × 10⁶ cells were rinsed in 10 ml of phosphate-buffered saline (PBS) and subjected to centrifugation (1,500 g for 5 min). The pellet was resuspended in 1 ml of PBS and centrifuged (16,000 g for 15 s). The supernatant was removed, and the pellet was resuspended in 400 µl of 10 mM HEPES, pH 7.8, 10 mM KCI, 0.1 mM EDTA, 2 mM dithiothreitol (DTT), 1 mM PMSF, 0.5 mg/ml leupeptin, and 0.3 mg/ml antipain. After a 15-min period during which cells were allowed to swell on ice, 25 µl of 10% Nonidet P-40 were added, and the tube was vortexed for 10 s. The lysate was centrifuged for 30 s in a microcentrifuge, and the nuclear pellet was resuspended in 20 mM HEPES, pH 7.8, 0.42 M NaCl, 5 mM EDTA, 1 mM PMSF, and 10% (vol/vol) glycerol. The tube was then rocked gently on a shaking platform for 30 min at 4°C, and this nuclear extract was centrifuged in a microcentrifuge for 10 min at 4°C. The supernatant was collected and stored at −70°C for later electrophoretic mobility shift assay (EMSA).
results

Application of cytomix to the cell lines generated NO, with the amount of NO being cell line dependent. The nontransformed cells E10 and C10 produced more NO (34- to 78-fold) than did their respective neoplastic siblings E9 and A5 (1- to 13-fold; Fig. 1). NO production was estimated by measurement of elevations in medium concentration of nitrite. The validity of this procedure was verified by demonstrating that increases in nitrite induced by cytomix administration to cells were inhibited by the NO synthase antagonists aminoguanidine and monomethylarginine (Fig. 2). We characterized this induction further. Concurrent treatment of cells with the protein synthesis inhibitor cycloheximide prevented production of NO (Fig. 3). We infer that this reflects a requirement for iNOS synthesis. Concentrations of dexamethasone as low as 1 µM attenuated both basal and cytomix-induced NO generation (Fig. 4).

The production of NO induced by cytomix was time dependent, with elevations occurring 8 h after cytomix application and continuing for up to 48 h (Fig. 5). The amount of NO elicited by the combination of cytokines (as cytomix) exceeded that produced by any single cytokine alone. Interferon-γ induced significant NO production by E10, A5, and C10 cells (Figs. 5 and 6). In E10 cells, the amount of NO generated by interferon-γ was comparable to that elicited by cytomix (Fig. 4). The effect of interferon-γ in promoting NO formation was concentration dependent in E10 cells, and significant increases were observed at concentrations exceeding 0.1 ng/ml (Fig. 7). NO production was unaffected by tumor necrosis factor-α or interleukin-1β except in E10 cells in which tumor necrosis factor-α caused a modest induction of NO production (Figs. 5-7). Although transformed A5 cells produce about three times as much NO as do E9 cells, this remains approximately 10-fold less than their nontransformed, growth-regulated sibling counterpart C10 (Fig. 6).

To investigate the mechanism causing the decreased ability of the transformants to synthesize NO, iNOS content was examined in cells exposed to cytomix for 24 h. Under these conditions, iNOS expression was observed in the cytosol of cytomix-exposed E10 and C10 cells. In the tumorigenic cell lines, cytomix elicited a small induction of iNOS in A5 cells, but none was observable in E9 cells (Fig. 8). These cell line differences in the extent of iNOS protein induction correlated well with their respective amounts of NO synthesis.

Northern blot analysis revealed that 24 h of cytomix exposure caused a much larger increase in iNOS mRNA in E10 cells than in E9 cells (Fig. 9). However, no differences were apparent between these cell lines with respect to iNOS mRNA stability (Fig. 9). This was assessed by inhibiting RNA synthesis at a peak in cytomix induction of iNOS and analyzing the amount of iNOS mRNA at different times thereafter. Thus greater
The instability of the iNOS message does not account for the decreased iNOS mRNA content in E9 cells. Further mechanistic studies were conducted in E10 and E9 cells to address the lack of iNOS mRNA in cytomix-treated E9 cells. NF-\(\kappa\)B appears to be involved in the actions of various cytokines (1, 12, 28, 51). Upon activation, cytosolic NF-\(\kappa\)B is released from binding to its inhibitory component I\(\kappa\)B and translocates into the nucleus to transcribe genes (35). In both E10 and E9 cells, cytomix induced the nuclear translocation of the transcription factor NF-\(\kappa\)B to an apparently equal extent (Fig. 10). Thus a failure in NF-\(\kappa\)B activation does not underlie the E9 cell resistance to cytokine induction of NO.

To consider whether the lack of effect of cytomix on NO production in the neoplastic cells was due to a general defect in cytokine responsiveness or was specific for cytokine induction of iNOS, the proliferative
actions of cytomix on the cell lines was evaluated. In these studies, cytomix caused a 40–50% increase in cell number in both lines (Fig. 11). This proliferative effect of cytomix was prevented by the iNOS inhibitor aminoguanidine and by dexamethasone (Fig. 12).

DISCUSSION

The function of NO generated by lung epithelial cells remains to be defined. Insofar as NO exerts cytotoxic and cytostatic actions and is induced by cytokines, a role in inflammation and/or cellular defense against pathogens has been proposed (40, 53). A striking feature of induced NO generation is the seeming cellular ubiquity with which cytokines can promote this process. NO production may be induced in most of the cell types along the pulmonary tree. Primary cultures and immortalized lines of bronchial (3, 53) and pulmonary epithelial cells (3, 26, 48, 52, 53) generate NO in response to the application of cytomix with or without the bacterial endotoxin lipopolysaccharide. The results of the present study are largely consistent with these previous investigations in that cytomix can induce NO formation in immortalized but nontumorigenic murine lung epithelial cell lines. However, sibling cells displaying neoplastic characteristics were resistant to this effect. The generation of NO was time dependent; the initial lag time required between cytokine application and accumulation of significant nitrite in the medium (~8 h) is consistent with the requirement for synthesis of iNOS. The sensitivity of NO production to inhibition by the protein synthesis inhibitor cycloheximide confirms the necessity for iNOS synthesis in NO production.

The primary focus of the present study was to determine whether NO generation was altered in transformed lung epithelial cells. Cytokine-induced NO production has been examined in cultured lung type II epithelial cells derived by clonal selection (L2; see Refs. 16, 26), by primary isolation from normal lung (48), and in type II cell lines derived from lung tumors (A549, LA-4; see Refs. 3, 21, 52, 53). Although all of these cell lines produce NO, it is difficult to compare production in nontumorigenic (or normal) cells with that in tumorigenic cells because the normal cells were isolated from a different species (rat) than were the cells from tumors (A549, human; LA-4, mouse). The cell lines used in the present study are derived from murine lung type II epithelial cells. The advantage of these particular cells is that the tumorigenic cells (E9 and A5) are spontaneous...
ous transformants of the normal cells (E10 and C10, respectively) and are therefore close genetic relatives. The neoplastic cells generated considerably less NO in response to cytomix than did their respective nontransformed progenitors. This indicates that the ability to produce NO was diminished after neoplastic transformation. Western blot analyses in the cell lines confirmed the cytomix enhancement of iNOS expression in nontumorigenic cells with little, if any, induction in the tumorigenic cells. This indicates that the ability to produce NO was diminished after neoplastic transformation. Western blot analyses in the cell lines confirmed the cytomix enhancement of iNOS expression in nontumorigenic cells with little, if any, induction in the tumorigenic cells. The deficiency of iNOS protein in tumorigenic cells after cytomix treatment suggests that an insufficiency of NO synthase cofactors such as tetrahydrobiopterin is unlikely to be a factor underlying the difference in NO production by these cells. Northern blot analyses in E10 (nontumorigenic) and E9 (tumorigenic) cells were consistent with the Western blot data in that cytomix-induced iNOS mRNA was reduced in E9 cells relative to E10 cells. This may be a consequence of reduced iNOS gene transcription in E9 cells or diminished iNOS mRNA stability. With respect to the latter, previous investigations have demonstrated iNOS mRNA stability as a possible site of modulation of NO synthesis (33, 47). To examine this possibility, iNOS mRNA levels were examined in cells after inhibition of transcription using DRB. These studies revealed that, despite the different intracellular mRNA concentrations, iNOS mRNA stability was comparable in E10 and E9 cells. Hence, the possibility that message destabilization was responsible for diminished NO production by the tumorigenic cells can be excluded.

NF-κB is an important transcriptional inducer of iNOS expression in a variety of cells (62, 63, 67). Indeed, two NF-κB binding motifs are present in the murine iNOS promoter (66). The two cytokines used in the present study, interleukin-1β and tumor necrosis factor-α, have been shown to induce NF-κB translocation into the nucleus (12, 28, 51). In the present study, cytomix induced equivalent nuclear translocation of NF-κB in both E10 and E9 cells. As such, the likelihood that a defect in translocation of NF-κB was responsible for the diminished NO production by E9 cells seems remote. Putative transcription factor consensus sequences identified in the iNOS promoter region in murine macrophages include nuclear factor interleukin-6 binding sites, an NF-κB site, and a tumor necrosis factor response element (37). Interleukin-6 had no effect on NO production in E10 cells (unpublished observation). Interferon-γ, on the other hand, was the most effective cytokine in eliciting NO production in nontumorigenic E10 cells, suggesting that IRFs may play an

Fig. 7. Concentration dependence of cytokine-induced induction of NO production by E10 cells. E10 cells were exposed to medium (open bar) or different concentrations of IFN, TNF, or IL-1. Samples of medium were taken after 48 h incubation with the cytokines and measured for nitrite. Data represent means ± SE from 4 experiments. *P < 0.05, unpaired Student's t-test, compared with control (untreated) cells.

Fig. 8. Western blot analysis of inducible NO synthase (iNOS). E10, E9, C10, and A5 cells were exposed to medium (−) or cytomix (+) for 24 h. Cytosolic fractions were subjected to Western blot analysis using mouse monoclonal anti-iNOS antibodies. Labeled proteins had the same retardation factor as the macrophage lysate (mac) standard, with a size of ~130 kDa.

Fig. 9. Cytokine induction of iNOS mRNA and its stability. E10 and E9 cells were exposed to medium (−) or cytomix (+) for 24 h. Cellular RNA was subjected to Northern blot analysis using mouse iNOS cDNA. Inset: relative levels of iNOS mRNA 24 h after treatment with medium (−) or cytomix (+). Graph demonstrates time course of iNOS mRNA after treatment of cells with 100 µM of the transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole applied 24 h after cytomix administration. In both cell lines, iNOS mRNA at each time point is expressed as a percentage of that measured at time (t) = 0.
important role in NO induction in these murine lung epithelial cells. Deletion constructs in iNOS reporter assays (37, 41) substantiate the importance of at least one of the IRF binding elements for the full expression of interferon-\(\gamma\)-induced NO production in RAW 264.7 cells. Consistent with this report was the demonstration that NO induction by interferon-\(\gamma\) in pancreatic cells was preceded by increases in nuclear IRF-1 protein (19). Should the transcriptional activation of iNOS by interferon-\(\gamma\) in murine lung epithelial cells be similarly mediated by IRF-1, the tumorigenic E9 and A5 epithelial cells may have a defect in the IRF transduction pathways.

Individual cytokines had varying effects on NO production in the cell lines. In all cell lines, interleukin-1\(\beta\) failed to elicit NO production. This contrasts with interleukin-1\(\beta\)-induced increases in NO from L2 cells (a rat nontumorigenic type II cell line; see Ref. 26) and from rat adult and fetal primary type II cells (24, 48). The amounts of induced NO in these rat and human cells were less than those caused by a mixture of cytokines, however. In A549 cells, a line derived from human lung carcinoma, exclusion of interleukin-1\(\beta\) from cytomix plus lipopolysaccharide reduced iNOS gene expression by 41\% (3), indicating a contributory role of this cytokine in NO induction. An absence of receptors for interleukin-1\(\beta\) on cells used in this study could be responsible for the failure of this cytokine to affect NO production in cells; this bears further study. Induction of NO synthesis by tumor necrosis factor-\(\alpha\) appeared to be cell line selective in that a modest induction of NO synthesis occurred in E10 cells but not in C10, E9, or A5 cells. In other lung epithelial cell lines, the effects of this cytokine are equivocal. In A549 cells, exclusion of tumor necrosis factor-\(\alpha\) from the cytomix cocktail decreased NO synthesis by an amount equivalent to that caused by deleting interleukin-1\(\beta\) (5). On the other hand, tumor necrosis factor-\(\alpha\) did not affect NO production by lung epithelial cells cultured from the fetal or adult rat (24, 48) or by L2 cells (26). Of
all the cytokines included in the cytomix cocktail, interferon-γ was the most effective by itself. Indeed, interferon-γ elicited NO induction in E10 and A5 cells approaching that induced by cytomix. These results are in accordance with other studies in lung epithelial cells wherein interferon-γ plays a pivotal role in NO induction either alone or in combination with other cytokines (3, 26, 48).

In epithelial cell lines derived from human (A549), rat, or mouse lungs, dexamethasone inhibits cytomix-induced NO production (26, 52, 53). Similar results were obtained in the present study using murine E10 cells. The inability of dexamethasone treatment to completely prevent NO production may relate to this glucocorticoid being administered at the same time as cytokine application. Indeed, many of the effects of the glucocorticoids require alterations in protein synthesis (23), a process that usually takes several hours. In support of this notion is the observation that the concentration and duration of exposure to dexamethasone can influence NO generation and interleukin-1β release from rat alveolar macrophages (6). However, preincubation of E10 cells with dexamethasone for 24 h before cytokine application did not enhance the inhibitory effect of dexamethasone on NO induction (P. Kazakoff, personal communication). Accordingly, signal transduction pathways activated by cytomix in lung epithelial cells appear to be both glucocorticoid sensitive and insensitive. NO production in nonpulmonary cell types can also be modulated by concurrent glucocorticoid treatment (13, 49).

Cytomix enhanced cell proliferation in both E10 and E9 cells by 40–50% over a 24-h period. The influence of NO on cell proliferation in other systems is equivocal, with both increases (11) and decreases (4) on extrapulmonary tumor cell proliferation having been reported. The physiological consequences of NO production on lung epithelial cell growth are currently not known, although transfection of a human bronchial epithelial cell line (BEAS-2B) with iNOS had no effect on cell proliferation even though c-fos expression was stimulated (18). Cytomix-induced proliferation was prevented by aminoguanidine, an agent that shows relative selectivity as an inhibitor of iNOS (43), suggesting a role for NO in mediating the proliferative action of cytomix. However, other evidence suggests that the relationship between NO production and the proliferation may be coincidental. Cytomix administration induced proliferation in E9 cells, which produce little, if any, NO. Dexamethasone prevented the proliferation but did not abolish NO production. Accordingly, other actions of aminoguanidine aside from NOS inhibition may underlie the inhibition of cytomix-induced proliferation, such as inhibition of polyamine metabolism (42) and the glycosylation of cell matrix (8).

Spontaneously transformed murine lung epithelial cells produce less NO in response to cytokine activation than do their nontumorigenic counterparts. Neoplasia may promote resistance to cytokine-induced NO synthesis in certain cell types. The reduced iNOS expression and activity in human gastric (50) and colorectal tumor tissue (45) relative to normal tissue is consistent with such a proposal. Deficient NO could encourage tumor cell proliferation, inhibit differentiation (10), or prevent neoplastic cells from undergoing apoptosis (64). The complexity of the role of NO in cancer is illustrated by the observation that NO has both pro-apoptotic and anti-apoptotic effects (13), the cellular fate depending on the cell type and level of NO production. A role for...
endogenously produced NO in tumor development has been demonstrated in a mouse model of hepatic metastasis in which induction of iNOS in the tumor cells led to regression of the metastases (65). It is clear that the capacity of neoplastic cells to produce NO varies from cell type to cell type. Indeed, the ability of cytokinotransformants used herein, together with elevated NOS expression or activity in human breast cancer (61), central nervous system (11), and gynecological cancers (60), argues against deficient NO being common to all neoplastic cells. The amounts of NO generated by the tumor-derived cell lines relative to the cells from which they transformed is unknown. Conceivably, the non-tumorigenic cells may respond with significantly higher levels of NO than their neoplastic derivatives. This consideration emphasizes the value of the sibling cell pairs used in the present study. Some human tumors, iNOS expression was not increased as much as other NOS isoforms (11, 60), and in breast cancer, iNOS expression was localized to the tumor-infiltrating macrophages rather than the tumor parenchyma (61). The ability of neoplastic cells to produce NO and/or respond to NO may be dictated by factors such as malignancy, differentiation status (60), and metastatic potential (15, 64). The microenvironment affects the response of tumor cell lines (EMT-6 murine breast cancer, DLD-1 human colon adenocarcinoma) in that NO overexpression inhibits tumor cell growth in vitro but enhances tumor growth in vivo (17, 29). The role of NO in neoplasia is thus not a simple one and warrants further investigation. The sibling lung epithelial cell lines described herein represent unique systems for examining in molecular detail the components that regulate cytokine induction of NO.

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