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Long-Term Adaptation of Lung Tumor Cell Lines with Increasing Concentrations of Nitric Oxide Donor

James A. Radosevich[*,1,2], Kim M. Elseth[1,2], Benjamin J. Vesper[1,2], Gabor Tarjan[3] and G. Kenneth Haines III[4]

[1]*Center for Molecular Biology of Oral Diseases, College of Dentistry, University of Illinois at Chicago, Chicago, IL 60612, USA*

[2]*Jesse Brown VAMC, Chicago, IL 60612, USA*

[3]*Department of Pathology, John H. Stroger, Jr. Hospital of Cook County, Chicago, IL 60612, USA*

[4]*Department of Pathology, Yale University School of Medicine; New Haven, CT 06510, USA*

Abstract: The free radical nitric oxide (NO) is known to play an important role in the biology of human cancers, including lung cancer. However, it is still not clear how elevated amounts of nitric oxide affect tumor development and propagation. Herein we develop an *in vitro* model system to study these effects in lung tumor cells. Two cell lines—one human lung adenocarcinoma (A549) and one mouse adenocarcinoma (LP07) cell line—were adaptively grown in increasing concentrations of the NO donor DETA-NONOate over several months. Both cell lines were successfully adapted to high levels of NO (HNO). Experiments validated the adaptation occurred as a result of the exogenous NO produced by the DETA-NONOate, and was not merely a response to the chemical composition of DETA-NONOate. No morphological differences were observed between cells that were adapted to the HNO and cells which did not undergo the adaptation process (i.e., "parent cells"). Parent cells were unable to survive when placed directly in media containing high levels of DETA-NONOate, suggesting that the adapted cells underwent a biological change enabling them to survive and grow in a HNO environment. The adapted cells were found to grow faster than the parent cells under both normal growth conditions and stressful growth conditions (serum-less media, growth on soft agar) even when the DETA-NONOate was removed from the HNO culture media. These adapted cell lines can serve as a novel tool for use in future experiments designed to better understand the role nitric oxide plays in lung cancer.

Keywords: Adenocarcinoma, nitric oxide, nitric oxide synthase (NOS), lung cancer, nitrogen and oxygen reactive species, cellular adaptation.

INTRODUCTION

Despite extensive research, lung cancer remains the leading cause of cancer death in the world. It is estimated that over 215,000 new cases of lung cancer were diagnosed in the United States in 2008, and 29% of U.S. cancer deaths in 2008 resulted from lung cancer [1]. Oxidative/reductive (redox) DNA damage is thought to be a significant contributor in the progression of cancer [2,3], and one redox species in particular, nitric oxide (NO), has been implicated in a number of physiological and pathophysiological processes in a variety of human tissues [4]. NO and a number of its metabolic byproducts are involved in two completely opposite functions: they can serve as either protective agents against malignancies, or as antagonists which can promote carcinoma formation and development [5]. In the case of the former, NO is produced as both an antimicrobial and an anti-tumor immune response, while in the case of the latter, unregulated and prolonged NO expression can induce mutational events that may lead to cancer [6,7].

Nitric oxide is produced from the amino acid L-arginine by a family of isoenzymes called nitric oxide synthase (NOS) [8,9]. There are three isoforms of NOS, defined according to their activity or the tissue type in which they were first described: endothelial constitutive NOS (ecNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) [10]. Our laboratory has investigated NOS and nitrotyrosine (a marker of NOS activity) expression levels in a variety of carcinomas, including head and neck [4,11], salivary [12], and esophageal tumors [13]. Over-expression of at least one NOS isoform was observed for each tumor type tested, relative to normal tissue, and nitrotyrosine over-expression was observed in the head and neck tumors, the only tumor type we tested for nitrotyrosine expression.

To date, however, there is still uncertainty regarding the role of NO and NOS in lung adenocarcinomas. While a small number of studies have failed to detect a difference in NOS expression between lung tumor cells and normal cells [14,15], the majority of work in this area has found that levels of NOS (particularly iNOS) and/or nitrotyrosine are higher in lung carcinomas than in normal tissue [15-19]. Furthermore, a recent study found a correlation between smoking—a known source of high levels of reactive nitrogen and oxygen species—and increased expression of iNOS:

*Address correspondence to this author at the Center for Molecular Biology of Oral Diseases, University of Illinois at Chicago, College of Dentistry, 801 S. Paulina St., Chicago, IL 60612, USA; Tel: (312) 996-9538; Fax: (312) 996-9592; E-mail: jrados@uic.edu

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lung tumor tissues taken from smokers exhibited higher levels of iNOS than lung tumor tissues taken from nonsmokers [20]. The patient population in this study was controlled to study only smokers versus nonsmokers; all patient properties and tumor characteristics (including age and sex of patient, tumor type and stage, etc.) were effectively controlled. The spectrum of NOS expression could be linked to the biological behavior of the tumors, therefore suggesting NOS expression could be used to predict patient outcome. The results of this work suggest that differences in NOS- and NO-related gene products are fundamental properties of these tumors. There are a number of clinical issues that need to be addressed: 1) It is currently unclear how such differences in NO biology affect lung tumor carcinogenesis, 2) It is unclear if the treatment of tumors influences NO expression, and 3) It is also unknown if tumors with varying expression levels of NO necessitates different therapeutic approaches. Currently, there is no reasonable way to test in humans all of the possible parameters involved with this spectrum of NO expression and its relationship to the biology of the tumors. Therefore, a model system that represents the spectrum of NO expression in tumors would be of considerable value to test and determine a wide spectrum of biological properties related to NO tumor biology.

In an effort to better comprehend the underlying biological effects of NO exposure in lung cancer, we herein develop a model cell line system to study human and mouse lung tumors “adapted” to increasing levels of nitric oxide over a long period of time. Previous *in vitro* studies of NO exposure to lung tumor cells, including those performed by our laboratory [21,22], have focused exclusively on the NO donor being delivered in a short time frame (typically no longer than 72 hours). In contrast, the system developed herein sought to expose cells to a slow, gradually, increasing concentration of NO donor over an extended period of time, with the belief that these cells would eventually sustain long-term viability in a high-free radical environment that would otherwise be toxic to cells that had not undergone adaptation. Successfully creating these adapted cell lines would result in a practical model system to study the mechanisms by which cells differentially respond to varying degrees of nitrosive stress, as seen *in vivo*.

MATERIALS AND METHODOLOGY

Cell Culture and Cell Lines

All media and supplements were purchased from Invitrogen (California, USA), except where noted. A549, a human lung adenocarcinoma cell line [23,24], and LP07, a mouse lung adenocarcinoma cell line [25-27], were used in this study. The A549 cell line was purchased from American Type Culture Collection (Virginia, USA); the LP07 cell line was previously obtained as a generous gift from Dr. Slobodanka Klein at the University of Buenos Aires, Argentina. A549 was grown in RPMI-1640 media, and LP07 was grown in MEM media. All media was supplemented with 10% fetal calf serum inactivated at 56ºC for 30 minutes, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 2.5 μg/mL Amphotericin B solution. The MEM media was additionally supplemented with 100 mM MEM nonessential amino acids and 1 mM sodium pyruvate (CellGro, Inc., Virginia, USA). Cell lines were grown in a humidified incubator at 37 ºC and 5% CO_2. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Cell Adaptation Process

Both A549 and LP07 were “adapted” to the nitric oxide donor (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate (DETA-NONOate). DETA-NONOate was purchased from Sigma Life Sciences (St. Louis, Missouri, USA) and used without further purification. Stock solutions of DETA-NON Oate were prepared in sterilized water and sterile filtered using 0.22 micron filter units. To begin the adaptation process, cells were passaged with trypsin-EDTA and transferred to a new flask containing media supplemented with 50 μM DETA-NONOate. The flask was placed in the incubator and grown at 37 ºC and 5% CO_2. After reaching ~90% confluency, the cells were again passaged with trypsin-EDTA; the resulting cultures were grown in media treated with 75 μM DETA-NONOate. This process was repeated, with the NO donor concentration being increased in 25 or 50 μM increments, until the concentration of DETA-NONOate reached the highest desired level (600 μM for the A549 cells and 300 μM for the LP07 cells). These end point concentrations were chosen on the basis that they were a known dose able to kill 100% of the cells in 24 hours, when the parent cell line was “shocked” (rather than adapted to) into media containing that level of DETA-NONOate. The ability to grow in a lethal dose of DETA-NONOate would imply that biological changes had taken place in such cells. As the adaptation process progressed, aliquots of the adapted cells were periodically removed for cryostorage.

Separate “parent” cells were maintained as controls. Parent cells were grown under standard conditions (i.e., no nitric oxide donor was added).

During both the adaptation process and the subsequent maintenance of the fully adapted cells, the cells were replenished with media containing DETA-NONOate every 2-3 days. Parent cells were also replenished with fresh media every 2-3 days.

Verification of Adaptation End-Point

Following adaptation of the cells lines to 300 and 600 μM DETA-NONOate for LP07 and A549, respectively, we sought to confirm that these adapted cells had been developed to withstand a HNO environment that would not support the growth of the corresponding parent cells. For each cell line, parent (A549 & LP07) and adapted cells (A549-HNO & LP07-HNO) were seeded (100 μL) into 96-well plates and grown for 24 hours, to ~70% confluency. (LP07-HNO cells were grown in media containing 300 μM DETA-NONOate; A549-HNO cells were grown in media containing 600 μM DETA-NONOate.) The media was then removed, and 100 μL of media without DETA-NONOate was added to the HNO cells, while 100 μL of media containing either 300 μM (LP07 cells) or 600 μM (A549 cells) DETA-NONOate was added to the parent cells. Plates were then incubated for an additional 72 hours, at which time 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT) cell proliferation/viability assays were

performed. The assay was performed as follows: the media was removed, and 100 µL of 2 mg/mL MTT (Sigma, St. Louis, Missouri, USA) in phosphate buffer saline (PBS) was added. Plates were incubated at 37ºC for 5 hours, after which time the MTT was aspirated. The remaining purple formazan crystals were dissolved in 100 µL of Dimethyl Sulfoxide (DMSO), and the absorbance of each well was read at 540 nm using a SpectraMax® Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, California, USA). A minimum of three independent trials were carried out for each experiment, and each data point represents the average of at least four microtiter wells for each plate. Thus, a minimum of 12 independent values were measured for each cell line at each time point. Individual trials were normalized and averaged.

Validation of Nitric Oxide Donor Source

To confirm that the adapted cells were being affected by the NO production from the DETA-NONOate, and not the chemical makeup of the donor itself, adapted cells were grown in the presence of different NO donors. A549-HNO cells were seeded (100 µL) into 96-well microtiter plates and grown in RPMI-1640 media containing 600 µM DETA-NONOate for 24 hours, to ~70% confluency. The media was then removed, and fresh media containing 600 µM of one of the following donors was added: DETA-NONOate; (N)-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitroso-hydrazino]butyl]-1,3-propanediamine (spermine-NONOate; Oxis International, Beverly Hills, CA, USA); (±)-S-nitroso-N-acetylpenicill-amine (SNAP; Sigma Life Sciences, St. Louis, Missouri, USA); or N-(β-D-glucopyranosyl)-N^2-acetyl-S-nitroso-D,L-penicillaminamide (glyco-SNAP; Oxis International, Beverly Hills, CA, USA). Stock solutions of spermine-NONOate, SNAP, and glyco-SNAP were prepared in sterilized distilled water and sterile filtered, analogous to the solution preparation of DETA-NONOate described above. The microtiter plates were then returned to the incubator, and MTT cell proliferation/viability assays were performed after 24, 48, and 72 hour exposure to the desired NO donor.

H_2O_2 Exposure Growth Assays

Parent and HNO adapted cells were seeded (100 µL) into 96-well plates in the appropriate media and incubated overnight. In order to find the concentration range of hydrogen peroxide that the cells could tolerate, 0.1 mL of 30% hydrogen peroxide solution was added to first column of wells in a 96-well microtiter plate and diluted by 1:1 serial dilution. Then, the cells were incubated at 37 ºC overnight and MTT assays were used to measure the cell viability of both parent and HNO adapted cell lines, as described above. The assays were repeated three times to verify the concentration ranges for each of the cell line pairs.

Normal Media Growth Assays

The MTT assay was used to measure cell proliferation/viability of the parent and HNO adapted cells under the growth conditions established in this study. Parent and HNO adapted cells were seeded into 96-well microtiter plates at 100 µL. Parent cells were seeded and grown in the presence of the appropriate media formulation (RPMI-1640 for A549; MEM for LP07). The HNO adapted cells were seeded and grown in media containing either 300 µM (LP07-HNO) or 600 µM (A549-HNO) DETA-NONOate. MTT assays were carried out as described above, at the desired time points (24, 48, or 72 hours after seeding).

Serum-Less Growth Assays

Parent and A549-HNO cells were seeded (100 µL) into 96-well plates in the appropriate media (i.e., standard media for parent cell lines; media treated with 600 µM DETA-NONOate for the adapted cells). The plates were incubated for 24 hours, after which time the media was replaced with analogous serum-less media (i.e., lacking the 10% FCS). With the exception of the FCS, all other components of the media, including the additional DETA-NONOate for the HNO adapted cells, were identical to the original formulation used. Plates were then incubated for an additional 24, 48, and 72 hours. MTT assays were performed at each time point, as described above. LP07 and LP07-HNO cells were not studied in this assay format.

Soft Agar Growth Assays

The 96-well soft agar assay method was used to compare the growth properties of the A549 parent and A549-HNO, as well as LP07 and LP07-HNO; more malignant cells grow better on soft agar than do less malignant cells [28]. The soft agar mixture consisted of 50% low melting point agarose (Sigma A-9539, gelling temperature approximately 36ºC; Sigma, Missouri, USA) and 50% 2x RPMI supplemented with 20% fetal calf serum, 200 U/mL penicillin, 200 µg/mL streptomycin, 4 mM L-glutamine, and 5 mcg/mL Amphotericin B solution. After the agarose was autoclaved and incubated at 42 ºC for at least 30 minutes, the agarose and 2x RPMI-1640 were mixed and incubated at 42ºC until used. The agarose mixture (100 µL) was loaded into a 96-well microtiter plate and allowed to harden. Parent and HNO adapted cells (100 µL) were then added on top of the solidified agar. (Parent cells were added in standard 1X growth media; HNO adapted cells were added in standard 1X growth media supplemented with 300 or 600 µM DETA-NONOate; LP07-HNO and A549-HNO respectively.) The plates were then incubated for 12 hours at 37ºC, enabling the cells to attach to the agar. Following incubation, the media above the agar was removed, and the plates were returned to the incubator for an additional 24, 48, or 72 hours. At the desired time point, MTT assays were performed: 100 µL of 2 mg/mL MTT in PBS was added, and the plates were incubated at 37ºC for 5 hours. The MTT solution was then removed, and the remaining purple formazan crystals were dissolved in 100 µL of DMSO. The resulting (purple) solution in each well was then transferred to a new 96-well plate; absorbance readings were obtained as described above.

Data Analysis and Statistics

For each experiment type reported herein, at least three independent experiments were conducted. A minimum of four wells were run in each experiment for a total of twelve wells. All values that were more than two standard deviations from the mean were not included in the final analysis. The independent experiment values were normalized with respect to each other and then averaged. The error bars reported represent one standard deviation from the averaged normalized mean. Data was graphed using SigmaPlot version 9.0 (Systat Software, Inc., Chicago, IL, USA).

Fig. (6). MTT proliferation/viability assays of A549 and LP07 parent and HNO cells on soft agar. The media of the HNO cells was supplemented with 300 μM (LP07-HNO) or 600 μM (A549-HNO) DETA-NONOate.

cells, i.e. 300 μM is lethal to the parent LP07 cell line. Future studies will focus on determining the extent of the biological differences between the parent and adapted cell lines. It is interesting to note that LP07-HNO cells were not "cross" adapted to modest levels of H_2O_2 (Fig. **3**), as were the A549-HNO cells, implying that LP07-HNO cells lack some protective mechanism that was not induced by the NO exposure. Future molecular studies will be directed at elucidating this mechanism. In addition, future studies will be directed at using H_2O_2 to adapt cells to determine if the same molecular changes are induced. If so, this would be an important finding as it would broaden the versatility of this model system to reflect any free radical selective pressure encountered by tumors. It will also be of interest to see if the HNO cells are resistant to sulfur based free radicals, and if such exposure would make them NO resistant, as many fossil fuels produce reactive sulfur based components. Such a mechanism could explain how air pollution changes the nitric oxide biology of the upper aerodigestive tract and results in tumors arising in these tissues.

As mentioned previously, DETA-NONOate was selected for use in the adaptation process due to its favorable kinetic properties, most notably its longer half-life and higher delivery rate of NO relative to the other donors considered. The ability of the A549-HNO cells to survive and grow in the presence of other NO donors (Fig. **2**) suggests that the cells have adapted to the NO produced by the donor, and not to any inherent toxicity resulting from exposure to the DETA-NONOate donor itself. Given this observation, we opine that the adaptation process would have been successful regardless of the donor chosen. However, we note that because the four donors considered (DETA-NONOate, spermine-NONOate, SNAP, and glyco-SNAP) possess different kinetic properties [22], the adaptation time and concentration (as defined here, in terms of the NO donor, and not in terms of the NO produced by the donor) required to reach an *equivalent NO-environment* would have varied. Such differences in donor kinetics likely account for the different growth curves obtained in Fig. (**2**) between the NONOate and thiol-based donors. The NONOate donors (DETA-NONOate and Spermine-NONOate) contribute two moles of NO per mole of donor, while the thiol donors contribute only one. Thus, the decreased viability of the A549-HNO cells in the presence of the NONOate donors, relative to the thiol donors, was likely a result of the higher free-radical environment created by the additional NO generated. In reality, the kinetics of adaptation is not important. Patients that smoke, for example, may vary their exposure and amount to cigarette smoke over time. Some of these patients will present with clinical tumors earlier and with fewer pack-years, than other patients. The difference between these patients may not only be the differences in their exposure levels, but also in their biological constitution, as noted by the differences seen between the two cell lines used herein.

Our previous studies found that the mode and kinetics of NO delivery (i.e., the identity of the NO donor) strongly impacted the potential for genotoxicity and apoptosis at the enzymatic and gene expression levels of lung tumor cell lines following short-term exposure to NO donors [21,22]. At this time it is impossible to make similar conclusions about the biological properties of the adapted cells prepared in this study for a number of reasons: 1) Only one donor, DETA-NONOate, was used in the adaptation process, 2) In contrast to previous studies, the cells in the current experiment were exposed to donor for long periods of time, thus likely affecting additional and different biological pathways than are affected by short-term exposure, and 3) We have not yet identified the biological alterations arising within the HNO cells. Regardless of the donor chosen, however, we believe the cyclical nature of the NO concentration exposed to the cells during adaptation—repeated treatment of the cells with fresh media containing donor, followed by periods of donor decay—likely serves as

a good *in vitro* method to mimic the cyclical exposure to nitrogen and oxygen reactive species that many lung cancer patients receive (for example, from long-term, periodic tobacco use). Furthermore, the successful adaptation of the LP07 mouse adenocarcinoma cell line suggests that we will be able to use these HNO cell lines in future *in vivo* animal models. It remains to be determined if the application of low dose, long-term exposure of any NO donor could result in *in vivo* tumor formation.

The results of the MTT assays (Figs. **4-6**) indicate the adapted cells exhibit more aggressive growth than the parent cells under both normal growth and harsh conditions. These results suggest that we have successfully developed a model system consisting of two extreme cell types: 1) the parent cells, which are able to survive and grow in low-NO environments with nutrient supplements, and 2) the HNO cells, which represent a stronger tumor capable of growing robustly in a high-free radical environment, and other harsh conditions. The adaptation process carried out in this study further illustrates how such a prolonged, gradual increase in exposure to reactive nitrogen and oxygen species might transform a tumor originally only able to survive in a low-NO/nutrient environment into one that is able to survive and grow in a harsh, high free-radical environment. This observation offers tremendous support to the current belief that nitric oxide plays a key role in tumor progression.

This is the first time that cell lines have been "adapted" to such high levels of nitric oxide. Moreover, studies carried out to date (including those from our own laboratory [21,22]) have typically only investigated short-term exposure to high levels of NO donor, rather than the slow adaptation approach used here. Given the simplicity in which the adaptation process was carried out in the current study, we believe that our adaptation method can be easily extended to the study of other cell lines, as well as to other chemical stimuli affecting cellular function. It will be important to undertake these studies to prove that this is a universal biological property not only between different cell lines of origin, but also between various pathological subtypes arising within various tissues. Defining the molecular changes within this matrix of cell types and tissue origins will provide new insights into NO tumor biology.

CONCLUSION

We have successfully adapted human and mouse lung tumor cells in increasing concentrations of the NO donor DETA-NONOate, resulting in a set of new, biologically different cell lines. The development of these HNO cell lines represents a significant finding unto itself, while also introducing a number of questions which will need to be addressed with future experiments. First, how do the HNO cell lines differ biologically from the parent cell lines at the molecular level? For example, do the HNO cell lines exhibit greater expression of oxidative stress proteins and/or other key cellular enzymes? Second, what happens when the NO source is removed indefinitely from the adapted cell line? Do the cells lose their ability to survive in the HNO environment, thereby reverting back to their original properties (i.e., the parental cell line), or is the ability to survive the harsh environment a permanent genetic change? Third, and most importantly, how do the properties of these HNO cell lines correspond to tumors observed clinically, and how can the cell lines be used to better understand the clinical behavior of tumors? Do tumors growing in higher levels of nitric oxide need to be treated differently than tumors growing in lower levels on NO? Future experiments will be needed to answer these and many other questions.

In conclusion, while we do not yet know the extent of the biological differences between the HNO cell lines and their corresponding parent cell lines, we believe that the adaptation process outlined here represents a truly novel approach to studying the role of NO in human and mouse tumors. The HNO cell lines developed herein will serve as an important tool in future work aimed at understanding the role nitric oxide plays in the development and progression of lung cancer.

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