Nitrosamine-induced Lung Carcinogenesis and Ca\(^{2+}\)/Calmodulin Antagonists

Hildegard M. Schüller

Experimental Oncology Laboratory, Department of Pathobiology, University of Tennessee, Knoxville, Tennessee 37901

Abstract

This review summarizes recent data which implicate cell membrane receptors and their associated signal transduction pathways as molecular targets of tobacco-related lung carcinogenesis as well as therapy of such cancers. It is shown that the two nitrosamines \(N\)-nitrosoditiamine and \(4\)-(methylnitrosamino)-1-(3-pyridyl)-1-butanone bind to nicotinic cholinergic receptors in hamster lung. Binding of the nitrosamines as well as nicotine to this receptor stimulates proliferation of human lung carcinoid cells \(in vitro\). These data suggest chronic stimulation of nicotinic receptors by nicotine and nitrosamines in smokers as one of the molecular events responsible for stimulation of neuroendocrine cell proliferation and ultimately the development of lung tumors with neuroendocrine differentiation.

On the other hand, a selective antiproliferative effect of the dihydropyridine derivative B859–35 on neuroendocrine lung tumor cells \(in vivo\) and \(in vitro\) suggests the potential use of such agents as cancer therapeutics. The demonstrated inhibition of Ca\(^{2+}\)/calmodulin and protein kinase C by B859–35 as reported in other \(in vitro\) systems suggests interference with such elements of signal transduction pathways as the molecular mechanism of the observed antiproliferative effects.

Introduction

\(N\)-Nitrosamines and their precursors are common environmental contaminants, and they are formed in the saliva, stomach, and gut from nitrate and amines (1, 2). Tobacco products contain significant levels of nitrosamines and are believed to contribute substantially to the high lung cancer risk of smokers (3). It is generally believed that nitrosamines are metabolically activated in the host organism to generate intermediates which react with DNA (4). The formation of the DNA-adduct \(O\)-methylguanine from the tobacco-specific NNK\(^3\) has recently been shown to correlate with the occurrence of mutations in codons 12 and 61 of the \(K\)-ras oncogene (5). Mutations of this particular type have been identified in many chemically induced and spontaneous tumors and are believed to be a key event in the molecular mechanisms leading to the development of many cancer types. In view of the many different cell types in the mammalian body capable of metabolizing nitrosamines, it is, however, difficult to understand why some members of this class of chemical carcinogens selectively cause cancers in the liver while others selectively affect other organs, such as the respiratory tract or pancreas (6).

DEN and the nicotine-derived nitrosamine NNK are both potent lung carcinogens in Syrian golden hamsters when s.c. administered (6, 7). However, there is a pronounced difference in the acute and chronic cellular responses when these two nitrosamines are administered under ambient air conditions as opposed to a simultaneous continuous exposure to hyperoxia. Under ambient air conditions, DEN and NNK both stimulate secretion and hyperplasia of pulmonary Clara cells (8, 9) and neuroendocrine cells (9, 10) during the first few weeks of exposure. When the treatment is continued, only Clara cells give rise to tumors (peripheral adenomas and adenocarcinomas) (8, 9), while the neuroendocrine cells undergo terminal differentiation into squamous cells without progressing into tumors (9, 11). On the other hand, treatment of hamsters exposed to continuous hyperoxia with an identical dosing regimen of DEN or NNK selectively stimulates peptide secretion by PNE cells during the acute phase (12), while chronic exposure results in the development of neuroendocrine lung tumors (9, 13).

The fact that the nitrosamines stimulated secretion by Clara cells and PNE cells during the initial phases of these experiments led us to investigate the role of signal transduction pathways which regulate secretion for cancer initiation by these chemical carcinogens. Subsequently, we have explored if signal transduction pathways involved in the regulation of cell proliferation can be exploited for the development of a target-oriented therapy of neuroendocrine lung tumors.

Nicotinic Cholinergic Receptor of PNE Cells and Nitrosamines

Under physiological conditions, the secretion of calcitonin and mammalian bombesin by PNE cells is stimulated by the neurotransmitter acetylcholine via high-affinity ligand binding to cholinergic receptors in the cell membrane (14). Exposure to tobacco smoke or nicotine has been shown to stimulate calcitonin secretion in humans and hamsters (14–16). This effect was inhibited by specific antagonists of nicotinic cholinergic receptors (14–16), an expression of pharmacological properties of the nicotinic subtype of cholinergic receptors. Although the signal transduction pathway and molecular biology of nicotinic cholinergic receptors have been extensively investigated, all such studies have been conducted on muscle or neuron receptors (17, 18). Since signal transduction pathways and molecular biology of nicotinic cholinergic receptors in muscles and neurons are slightly different (18), it is conceivable that this receptor expresses yet another variation of functional aspects when located in PNE cells. Discussions and interpretations on the mechanisms of action of this receptor in PNE cells and tumor cells with neuroendocrine differentiation may therefore change in the future as more data become available. Nevertheless there are a number of functional similarities among the reported subtypes of nicotinic receptors which are likely to apply to this receptor in PNE cells as well. Binding of an agonist to the receptor results in the opening of an ion channel with high affinity for Ca\(^{2+}\) via membrane depolarization. Agonist/receptor binding results in a momentary desensitization of the receptor which is mediated by phosphorylation of the receptor via cyclic AMP-dependent protein kinase (19), protein kinase C (20), and tyrosine-specific-protein kinase (21). Prolonged exposure to agonists results in permanent desensitization of the receptor accompanied by channel closing and an increase of receptor affinity by two orders of magnitude (17). It has been shown in neuroendocrine cells of the adrenal medulla and in pheochromocytoma cells (which are derived from these cells) that stimulation of the nicotinic receptor by agonist binding causes the nonspecific secretion of the entire contents of neu-

---

\(^1\) Presented at the NCI Workshop "Investigational Strategies for Detection and Intervention in Early Lung Cancer," April 21–24, 1991, Annapolis, MD.

\(^2\) To whom requests for reprints should be addressed.

\(^3\) The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; DEN, \(N\)-nitrosoditiimine; SCLC, small cell lung cancer; PNE, pulmonary neuroendocrine.
neuroendocrine storage granules by exocytosis (22). Thus opioids, catecholamines, and neuropeptides were simultaneously secreted in this system (22).

Structural similarities of DEN with acetylcholine and of NNK with nicotine along with the observed stimulation of PNE cell secretion by these nitrosamines in the hamster led us to investigate their potential interaction with the nicotinic cholinergic receptor which regulates the secretory activity of this cell type under physiological conditions (14–16). Radioreceptor assays with $^3$H-labeled (S)-(−)-nicotine in cell membrane fractions (23) from normal hamster lung homogenates revealed measurable and saturable binding under steady-state conditions (24). However, when an identical assay was conducted on cell membrane fractions from hamsters which had been preexposed for 4 wk to hyperoxia (resulting in PNE cell hyperplasia), the levels of specific nicotine binding were substantially increased (24). Saturation was reached in both cases at a concentration of 20 nM $^3$H-labeled (S)-(−)-nicotine, suggesting that the observed increase in specific binding was due to an increase in receptor number rather than changes in the sensitivity of receptors. Scatchard analysis confirmed that binding was to a single class of binding sites (24). Competition experiments with non-radioactive DEN and NNK resulted in complete displacement of $^3$H-labeled (S)-(−)-nicotine from the receptor at equimolar dose levels (24). Even 20 times lower concentrations of the nitrosamines caused significant displacement of nicotine. Accordingly, the affinity of DEN and NNK to the nicotinic receptor is significantly higher than that of nicotine itself.

Having thus established that DEN and NNK do in fact bind to nicotinic cholinergic receptors in the lungs, we then asked the question: Does such binding result in a stimulation of PNE cell proliferation? In the absence of an in vitro system of normal PNE cells, we studied the effects of DEN and NNK on the growth kinetics of the human cell line NCI-H727 which is derived from a lung carcinoid. This cell line is very well differentiated, grows as a monolayer, and expresses several neuroendocrine markers as well as functional bombesin and epidermal growth factor receptors (25–28). Contrary to human lung cancer lines of other histological types, NCI-H727 only grows in tissue culture when maintained at relatively high CO2 levels (8 to 10%), thus mimicking the well-established dependence of normal PNE cells in vivo on abnormal pulmonary oxygen levels for proliferation (reviewed in Ref. 27). Exposure of the cells for 48 h to various concentrations of acetylcholine, nicotine, DEN, or NNK resulted in a dose-dependent stimulation of cell proliferation in all cases (26, 29). This effect was inhibited by preexposure to hexamethonium or pentolineum, which are specific antagonists of nicotinic cholinergic receptors. Antagonists of other receptor types, such as muscarinic cholinergic or adrenergic receptors, did not inhibit the mitogenic effects of DEN, NNK, nicotine, or acetylcholine. Our data thus confirm the hypothesis that the two nitrosamines stimulate proliferation of cells with PNE cell differentiation via direct interaction with the nicotinic cholinergic receptor. A mechanism such as this is likely to contribute significantly to the well-documented prevalence of lung cancer with neuroendocrine differentiation in smokers (30). Moreover, the mitogenic effects of nicotine revealed in line NCI-H727 strongly suggest that nicotine itself aids in the selective propagation of a neuroendocrine lung tumor type.

Ca$^{2+}$/Calmodulin Antagonists and Neuroendocrine Lung Cancer

Ca$^{2+}$/calmodulin antagonists have been suggested as therapeutics for neuroendocrine lung cancer, because the signal transduction pathways of many growth factors for this tumor category are Ca$^{2+}$/calmodulin dependent (31). In particular, mitogenic action of the neuropeptides gastrin-releasing peptide (synonym, mammalian bombesin), bradykinin, cholecystokinin, galanin, neurotensin, and vasopressin involves Ca$^{2+}$/dependent signaling events (32). However, the use of conventional Ca$^{2+}$/antagonists such as verapamil as cancer therapeutics is prohibited by their pronounced cardiovascular effects.

Recently, a novel type of Ca$^{2+}$/calmodulin antagonists has been developed from the dihydropyridine, niguldipine, by separation of the racemic mixture into two stereoisomers (33). While the Ca$^{2+}$/ channel-mediated cardiovascular effects rest primarily with the (+)-enantiomer, the (−)-enantiomer (B859–35) has a 40-fold lower affinity to Ca$^{2+}$/ channels, therefore affecting the cardiovascular system only at relatively high dose levels (33). On the other hand, B859–35 is a potent inhibitor of calmodulin (34) and protein kinase C (35) and is thus likely to inhibit cell proliferation stimulated by pathways involving these regulatory components. We therefore tested the antiproliferative effects of B859–35 in the neuroendocrine, lung tumor cell line NCI-H727. Exposure of cells for 48 h to B859–35 caused a dose-dependent inhibition of cell proliferation, which was highly significant until the end of the assay (216 h after seeding of cells or 168 h after termination of exposure to B859–35) (Fig. 1). Concentrations as low as 1 μM were still highly effective in inhibiting cell growth in these assays. In contrast, verapamil inhibited cell proliferation under identical exposure conditions only at significantly higher concentrations (10 to 100 μM), and this effect was tapering off during the last part of the observation period (144 to 216 h after seeding) (Ref. 36; Fig. 1).

In order to explore if B859–35 also has an antiproliferative effect on neuroendocrine lung tumors in vivo we conducted a bioassay experiment in hamsters. The animals were simultaneously exposed to hyperoxia and DEN as previously described (13) to induce neuroendocrine lung tumors. Nitrosamine injec-
tions were then discontinued, and the animals were returned to ambient air conditions and given B859–35 by stomach tube every day excluding weekends for 20 wk, while a positive control group was maintained under identical conditions but without receiving B859–35. In analogy to the in vitro studies with the human carcinoid-derived cell line (see above), B859–35 demonstrated a pronounced antiproliferative effect on neuroendocrine lung tumors in this system (37). In contrast, Clara cell-derived peripheral adenomas induced in the same animal species under ambient air conditions by DEN progressed further when treated with an identical dosing regimen of B859–35 (37). In analogy to the in vitro studies with the neuroendocrine hamster lung tumors are atypical carcinoids, a well-differentiated and only moderately malignant tumor type. A potential effect when treated with an identical dosing regimen of B859–35 (37).

In conjunction with the data obtained with cell line NCI-H727 (see above), these data suggest that B859–35 may be effective in the treatment of neuroendocrine lung cancer. In order to arrive at a realistic assessment of these data, we have to consider that line NCI-H727 and the neuroendocrine hamster lung tumors are atypical carcinoids, a well-differentiated and comparatively poor differentiation, is difficult to predict at this time. Clinical trials in human cancer patients which are currently in progress at the Thompson Cancer Survival Center, Knoxville, TN (clinical investigator, Dr. J. J. Costanzi) and the University of Colorado Health Sciences Center (clinical investigator, Dr. P. A. Bunn, Jr.), as well as at the Clinical Center, Phillips University, Marburg, Germany (clinical investigator, Dr. K. Havemann), will have to show if the high expectations in this novel type of antitumor agent are justified.

Conclusions

Our data suggest that the nicotinic cholinergic receptor in normal and neoplastic lung cells with neuroendocrine differentiation plays an important role in the mechanisms of tumor initiation and progression of smoking-related lung cancer. The pronounced mitogenic effect of nicotine and tobacco-related nitrosamines on normal and carcinoid-derived neuroendocrine lung cells is mediated by binding of these amines to this receptor type. Multiple exposures to these three nicotinic receptor agonists over 20 to 30 yr thus provide neuroendocrine tumor cells with a growth advantage over other cell types, a fact clearly reflected in the prevalence of SCLC in smokers. On the other hand, such chronic exposure to receptor agonists will ultimately lead to receptor desensitization. In the case of a Ca\(^{2+}\) channel-operated cell membrane receptor, such as the nicotinic cholinergic receptor, this results in a higher receptor affinity to its ligands which is reflected by receptor saturation at substantially lower agonist concentrations (17). At the same time, the closing of Ca\(^{2+}\) channels characteristic for desensitized receptors of this category (17) blocks the intracellular transport chain normally initiated by binding of an agonist to the receptor. This, in turn, results in an inhibition of cellular responses such as secretion of peptides or cell proliferation regulated by the receptor. The data presented by J. D. Minna in this conference and elsewhere (38) on functional aspects of nicotinic cholinergic receptors in SCLC lines are a classic example for desensitized receptors; saturation of the receptor in such highly malignant tumor cells, which are the end stage of decades of exposures to nicotine and nitrosamines by smoking, is reached at significantly lower concentrations of nicotine than in the normal PNE cells of our hamster experiments. Moreover, because signal transduction via these receptors is blocked by closed Ca\(^{2+}\) channels, nicotine fails to stimulate cell proliferation in these cells.

The mitogenic effect of nitrosamines via binding to nicotinic receptors does not exclude additional molecular events commonly associated with carcinogenesis, such as metabolic activation and DNA interaction. In light of the fact that agonists bound to a cell membrane receptor are usually internalized and enzymatically degraded, such high-affinity uptake will result in substantially higher nitrosamine concentrations in normal and neoplastic PNE cells than in other cell types which are lacking this mechanism. As we have previously shown with the carcinoid-derived cell line NCI-H727 and DEN, neuroendocrine lung cells are well able to metabolize nitrosamines (28). Whether the frequently reported changes in oncogene expression are the result of such DNA/metabolite interactions or an adaptive response to continuous receptor stimulation and subsequent receptor desensitization remains yet to be established.

The antiproliferative effect of the dihydropyridine derivative B859–35 and the phenylalkylamine verapamil on lung tumor cells with neuroendocrine differentiation in the hamster model and in the human carcinoid cell line NCI-H727 is unrelated to the discussed functional aspects of the nicotinic cholinergic receptors. Although we have shown that B859–35 inhibits the mitogenic effects of nicotinic agonists in line NCI-H727 by blocking this receptor-mediated pathway, the experiments discussed in this review are dealing with an antiproliferative effect on cells not stimulated by nicotinic agonists. As is evident from the relatively high concentrations of verapamil required to yield a significant antiproliferative effect in line NCI-H727, blocking of Ca\(^{2+}\) channels alone results in significantly less inhibition of proliferation than the simultaneous inhibition of calmodulin and protein kinase C by the dihydropyridine, B859–35. Calmodulin and protein kinase C interface with a large number of different signal transduction pathways and Ca\(^{2+}\)-dependent intracellular reactions involved in the regulation of cell proliferation (39, 40). Their inhibition is thus likely to inhibit proliferation in many different cell types. The selective effect of B859–35 on tumor cells with neuroendocrine differentiation observed in our in vitro and in vivo studies may in part be explained by the dependence of many neuropeptides which are autocrine growth factors for these cells on Ca\(^{2+}\)/calmodulin. Moreover, selective uptake by dihydropyridine receptors may further potentiate the effects of B859–35 in neuroendocrine tumor cells by yielding a relatively high intracellular concentration of the drug.

Although our findings to date support the development of inhibitors of second messengers such as Ca\(^{2+}\) channels, protein kinase C, or calmodulin as cancer therapeutics, it is possible that cancer cells such as SCLC are less susceptible to their actions than the relatively well-differentiated carcinoid cells used in our studies. Moreover, it is conceivable that, during progression to a more malignant state under continuous exposure to multiple carcinogetic signals from the environment, multiple adaptive changes occur in these cells, thus providing them with their well-documented superiority over normal cells. Signal transduction pathways which regulate the proliferation of normal cells in response to external stimuli may thus become replaced by new ones. Further studies are needed to elucidate the molecular changes in such signal transduction pathways under continuous exposure to environmental agents, such as the nitrosamines and nicotine, and during progression into a highly malignant state.

H. M. Schüller, unpublished observations.
References


Nitrosamine-induced Lung Carcinogenesis and Ca\textsuperscript{2+} /Calmodulin Antagonists

Hildegard M. Schüller

Cancer Res 1992;52:2723s-2726s.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/52/9_Supplement/2723s

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.