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SHORT COMMUNICATION

Regulation of cell proliferation by β-adrenergic receptors in a human lung adenocarcinoma cell line

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We have recently reported that the tobacco-related nitrosamines *N*-nitrosodiethylamine (DEN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) stimulate cell proliferation in cell lines derived from human neuroendocrine carcinoma and adenocarcinoma (comprised of Clara cells) of the lung. In the neuroendocrine cell line, this effect was inhibited by antagonists of nicotinic cholinergic receptors which regulate the secretion of peptide hormones and cell proliferation of pulmonary neuroendocrine cells. No such inhibition was observed in the adenocarcinoma line. Clara cells reportedly do not have acetylcholine receptors and secretion of this cell type is regulated by β-adrenergic receptors instead. In this experiment, we test the hypothesis that the latter types of receptors are involved in the regulation of cell growth of normal and nitrosamine-stimulated adenocarcinoma cells with features of Clara cells. Our data demonstrate a pronounced stimulation of cell growth by the β-adrenergic agonist isoproterenol, DEN and NNK, as well as a dose-dependent inhibition of such growth-stimulating effects by the β-adrenergic antagonist propranolol. These findings suggest an important role of β-adrenergic receptors in the regulation of cell proliferation in lung tumors comprised of this cell type.

The incidence of peripheral pulmonary adenocarcinoma has increased dramatically in recent years (1). Like most lung tumors, this histological tumor type demonstrates a strong epidemiological link with cigarette smoking (1). Carcinogenic *N*-nitrosamines are among the most powerful chemical carcinogens contained in tobacco and tobacco smoke (2,3). The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) as well as the environmentally less important *N*-nitrosodiethylamine (DEN) are both very potent lung carcinogens in laboratory rodents (2–5,19). The tumors induced experimentally by either one of these nitrosamines in rodents are compatible with human peripheral lung adenocarcinomas by histopathology and electron microscopy (4,19) and are derived from Clara cells (4,19).

We have recently demonstrated that the agonist of nicotinic cholinergic receptors, nicotine, causes a pronounced and selective stimulation of cell proliferation in human neuroendocrine lung cancer cells (6,7). This effect was inhibited by the antagonist of nicotinic cholinergic receptors, hexamethonium (6). Similarly, DEN and NNK caused strong cell proliferation in this system which was antagonized by hexamethonium (6,7). Studies in smokers (8) and in hamsters (8,9) have provided evidence that nicotinic cholinergic receptors are involved in the regulation of peptide hormone secretion and cell proliferation of pulmonary neuroendocrine cells. It hence appears that the same receptor type which under physiological conditions regulates secretion and cell growth via uptake of the neurotransmitter acetylcholine in pulmonary neuroendocrine cells can also mediate the uptake of carcinogenic nitrosamines thus allowing the cells to respond by proliferation.

Secretion by Clara cells is not regulated by cholinergic receptors (10). Instead, it has been shown that β-adrenergic agonists stimulate Clara cell secretion which is inhibited by antagonists of β-adrenergic receptors (10). In this study, we have tested the hypothesis that adrenergic receptors are involved in the regulation of spontaneous and nitrosamine-induced cell proliferation in lung tumors with features of Clara cells. We have used the human lung adenocarcinoma-derived cell line NCI-H322 as test system. This cell line has been extensively characterized and demonstrates morphological and biochemical (cytochrome P-450-mediated xenobiotic metabolism) features of normal Clara cells (11,12).

The stock material of cell line NCI-H322 was supplied by Dr A.F.Gazdar (NCI-Navy, Clinical Oncology Branch). The cells, which grow as monolayers, were maintained in RPMI-1640 medium supplemented with L-glutamine (2 mM), fetal bovine serum (10% v/v) and gentamycin (50 μg/ml) at 37°C in an atmosphere of 5% CO_2/95% air. The cells were dissociated with trypsin (1 ml/25 cm^2, tissue culture flask for 5 min at 37°C) and the tissue culture flasks were inoculated with 5×10^4 cells at a density of 2×10^3 cells/cm^2. They were allowed to attach for 24 h. Nicotine (Sigma, St Louis, MO), DEN (Sigma), isoproterenol (Sigma) and NNK (Chemsyn Science Laboratories, Lenexa, KY) were added to the tissue culture media at equimolar concentrations of 1 μM. Pre-incubation with the competitive antagonist of nicotinic cholinergic receptors, hexamethonium (Sigma), the competitive antagonist of β-adrenergic receptors, propranolol (Sigma), and the competitive antagonist of α-adrenergic receptors, tolazoline (Sigma) was for 10 min at 0.1, 0.5, 1 and 10 μM concentrations. All treatment groups were comprised of quintuplicate culture vessels. After 72 h of incubation, the medium was removed, the cells washed with PBS, and the medium was then replaced. The medium was replaced again after 144 h. Cells were counted with a hemocytometer (Neubaur) in quintuplicate after dissociation with trypsin and staining with trypan blue at 72, 144 and 216 h after inoculation. Mean values of cell counts were analyzed for statistically significant differences by Student's *t*-test.

Our data (Tables I and II) demonstrate that both nitrosamines as well as the β-adrenergic agonist isoproterenol (13) caused a pronounced stimulation of cell proliferation in NCI-H322 cells. In contrast, the agonist of nicotinic cholinergic receptors, nicotine (14), did not stimulate cell proliferation in this cell line. The antagonist of β-adrenergic receptors, propranolol (13,15), inhibited the stimulating effects of isoproterenol, DEN and NNK on cell proliferation. This inhibition was highly significant ($P < 0.01$) and dose dependent with all three chemicals. The

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; DEN, *N*-nitrosodiethylamine.

Table I. Modulation of growth kinetics by the two carcinogenic nitrosamines DEN and NNK and the effects of adrenergic and cholinergic receptor agonists and antagonists in the human lung adenocarcinoma cell line NCI-H322 (Clara cell features)

Treatment	(Inoculation)	No. of cells × 10^4/ml		
		72 h	144 h	216 h
Control	5	6.6 ± 0.3	25.3 ± 0.5	44 ± 0.7
DEN	5	44 ± 0.7	52.8 ± 0.4	66 ± 0.8
NNK	5	19.8 ± 0.3	57.2 ± 0.6	60.5 ± 1.0
Isoproterenol	5	42.5 ± 0.5	55 ± 0.9	64.5 ± 1.0
Nicotine	5	5.5 ± 0.1	20.1 ± 0.5	38.2 ± 1.0
Propranolol + DEN	5	6.6 ± 0.2	24.2 ± 0.4*	45.5 ± 0.9*
Tolazoline + DEN	5	45.5 ± 1.1	53.9 ± 0.8	67.1 ± 1.2
Hexamethonium + DEN	5	40.0 ± 1.3	49.8 ± 1.1	66.5 ± 0.8
Propranolol + NNK	5	7.7 ± 0.4	23.1 ± 1.0*	49.9 ± 0.5*
Tolazoline + NNK	5	22 ± 0.6	55 ± 0.7	68.2 ± 0.4
Hexamethonium + NNK	5	11.8 ± 0.3	47 ± 0.6	58.1 ± 0.5
Propranolol + isoproterenol	5	7.0 ± 0.4	24.3 ± 0.3*	43.1 ± 0.8*
Tolazoline + isoproterenol	5	40.8 ± 0.5	54.6 ± 0.9	65.2 ± 1.2
Hexamethonium + isoproterenol	5	41.9 ± 1.0	55.7 ± 1.1	63.8 ± 0.6
Propranolol	5	7.7 ± 0.2	26.4 ± 0.6	45.1 ± 0.7
Tolazoline	5	5.5 ± 0.4	25.3 ± 0.3	46.2 ± 0.3
Hexamethonium	5	6.1 ± 0.3	24.7 ± 0.5	41.9 ± 0.7

The nitrosamines and receptor agonists were added to the tissue culture medium at equimolar concentrations of 1 μm 24 h after inoculation of cells. Pre-incubation with the receptor antagonists propranolol, tolazoline and hexamethonium was for 10 min. The media were removed 72 h after inoculation and replaced with fresh RPMI medium after thorough washes with PBS. Each treatment group was comprised of quintuplicate culture vessels. Inhibitory effects of receptor antagonists $P < 0.01$ are marked by an asterisk.

Table II. Dose–response relationship of the inhibition of cell proliferation by the β-adrenergic antagonist propranolol in the human lung adenocarcinoma cell line NCI-H322 (Clara cells)

Treatment	(Inoculation)	No. of cells × 10^4/ml		
		72 h	144 h	216 h
0.1 μM propranolol + 1 μM DEN	5	22.3 ± 1.2	42.4 ± 0.9	65.0 ± 1.3
0.5 μM propranolol + 1 μM DEN	5	26.1 ± 0.7	36.3 ± 0.6	53.0 ± 1.0
1 μM propranolol + 1 μM DEN	5	6.6 ± 0.2	24.2 ± 0.4	45.5 ± 0.9
10 μM propranolol + 1 μM DEN	5	7.2 ± 0.4	24.8 ± 0.7	46.7 ± 1.1
0.1 μM propranolol + 1 μM NNK	5	20.0 ± 0.9	52.7 ± 1.3	60.0 ± 0.5
0.5 μM propranolol + 1 μM NNK	5	6.6 ± 0.2	24.2 ± 0.4	45.5 ± 0.9
1 μM propranolol + 1 μM NNK	5	7.7 ± 0.4	23.1 ± 1.0	49.9 ± 0.5
10 μM propranolol + 1 μM NNK	5	8.0 ± 0.5	24.3 ± 0.7	50.0 ± 0.8
0.1 μM propranolol + 1 μM isoproterenol	5	28.7 ± 0.9	50.2 ± 1.5	62.7 ± 0.9
0.5 μM propranolol + 1 μM isoproterenol	5	22 ± 0.6	55 ± 0.7	68.2 ± 0.4
1 μM propranolol + 1 μM isoproterenol	5	7.0 ± 0.4	24.3 ± 0.3	43.1 ± 0.8
10 μM propranolol + 1 μM isoproterenol	5	7.7 ± 1.4	23.9 ± 0.7	42.7 ± 1.1

The nitrosamines and isoproterenol were added to the tissue culture medium 24 h after inoculation of cells. Pre-incubation with propranolol was for 10 min. The media with chemicals were removed 72 h after seeding and replaced with RPMI medium after thorough washes with PBS. All treatment groups were comprised of quintuplicate culture vessels.

antagonist of α-adrenergic receptors, tolazoline (13,15), did not inhibit the growth-stimulating effects of isoproterenol, DEN or NNK at any of the dose levels tested. Similarly, the antagonist of nicotinic cholinergic receptors, hexamethonium (14), did not alter the stimulation of cell proliferation by isoproterenol, DEN or NNK.

Our data support our earlier reported findings that DEN and NNK stimulate cell proliferation in a variety of human lung cancer cell lines including peripheral adenocarcinoma derived from Clara cells (6,7). Although we have shown that this effect is dose dependent (6,7) it is important to note that stimulation of cell proliferation was only observed at relatively low dose levels (1 μM and below) while higher dose levels were toxic (6,7). Such low dose levels are typically employed in experiments aimed at investigating the role of cholinergic and adrenergic receptors (15,16), because these receptors have a very high affinity for their ligands thus enabling them to take up and bind specific ligands against a high gradient and at minute dose levels (15,16).

The observed stimulation of cell proliferation by the β-adrenergic agonist isoproterenol suggests that β-adrenergic ligands can act as growth factors in lung tumors comprised of this cell type, although less well differentiated tumors of the same category may react differently. Although such findings from neoplastic cells cannot be generally extrapolated to normal (non-neoplastic) cells of the same histological type, the reported effects of such agonists on normal (non-neoplastic) Clara cell secretion (10) suggests that proliferation of such normal Clara cells is also regulated by β-adrenergic receptors and their ligands. In keeping with this assumption, it has been shown that propranolol inhibits the mitogenic action and stimulation of

ornithine decarboxylase by L-epinephrine in normal human bronchial epithelial cells (17).

It has been amply demonstrated that among the many effects of nicotine on the mammalian organism, there is a pronounced stimulation of the sympathetic nerve system that results in increased levels of catecholamines such as norepinephrine and epinephrine (14), which are physiological ligands for α- and β-adrenergic receptors. In light of our findings, it seems quite possible that the nicotine-induced increase in the levels of adrenergic ligands in smokers is critically involved in the initiation of smoking-related lung cancer derived from Clara cells.

It has recently been speculated that the sympathetic part of the autonomous nerve system may play a role in the initiation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced gastric tumors in hypertensive rats (17). However, the authors interpreted their findings as indicating that central parts (nerve cells, ganglia) of the sympathetic system play the critical role in such mechanism (16). On the other hand, our data are derived from an *in vitro* system devoid of such central components (there are no nerves, ganglia or nerve cells in this tumor cell line). They clearly show that the regulation of cell proliferation in Clara cells may proceed without involvement of central nerve components and is mediated by the most peripheral portion of the sympathetic system, the adrenergic receptor, via interaction with its ligands.

In this context, it is very important that the two carcinogenic nitrosamines DEN and NNK, which both induce Clara-cell-derived lung tumors in laboratory rodents (4,19), apparently stimulate cell proliferation in our Clara-cell-derived human lung tumor cell line via the same receptor-mediated mechanism as the β-adrenergic agonist isoproterenol. As is clearly evidenced by the nitrosamine-induced cell proliferation—which parallels that of isoproterenol, in conjunction with the selective and dose-dependent inhibition of such effects by the β-adrenergic antagonist, propranolol—β-adrenergic receptors are critical mediators of nitrosamine-dependent cell proliferation in our system.

This is not the first report suggesting a potentially important role of cell-type-specific receptors for nitrosamine carcinogenesis. We, as well as others, have recently shown that pulmonary neuroendocrine cells, which are the origin of smoking-related neuroendocrine lung cancer (18) selectively take up nicotine as well as DEN and NNK *in vitro* and *in vivo* via nicotinic cholinergic receptors (6–9) which regulate cell proliferation and peptide hormone secretion in this cell type (8,9). The data provide evidence that the proliferative response to nicotine and the two nitrosamines of pulmonary neuroendocrine cells and tumors derived from them is regulated by nicotinic cholinergic receptors.

In light of our present findings it appears that cell-type-specific receptors which are physiologically involved in the regulation of product synthesis (e.g. secretion) and cell proliferation may be important mediators of carcinogenesis induced by *N*-nitroso compounds in many organs and cell types, a hypothesis that would explain the well-known target cell specificity of this class of chemical carcinogens and certainly deserves further study.

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