Biochemical Pharmacology, Vol. 38, No. 20, pp. 3439–3442, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

CELL TYPE SPECIFIC, RECEPTOR-MEDIATED MODULATION OF GROWTH KINETICS IN HUMAN LUNG CANCER CELL LINES BY NICOTINE AND TOBACCO-RELATED NITROSAMINES*

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(*Received* 29 *July* 1988; *accepted* 27 *February* 1989)

Abstract—The objective of this study was to investigate a potential involvement of nicotinic cholinergic receptors in the mediation of cell type specific biological effects of nicotine and the two tobacco-related nitrosamines *N*-nitrosodiethylamine (DEN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on human lung cells. Three well differentiated human lung cancer cell lines that have been reported previously to possess ultrastructural and biochemical features of normal pulmonary neuroendocrine cells, Clara cells and alveolar type II cells, respectively, were used for these experiments. The effects of nicotine, DEN, and NNK on cell proliferation and its modulation by established antagonists of nicotinic and muscarinic cholinergic receptors were studied. In the neuroendocrine cell line, nicotine and the two nitrosamines caused a strong stimulation of cell proliferation that was inhibited by antagonists of nicotinic cholinergic receptors. In the cell lines with features of Clara cells and alveolar type II cells, nicotine did not stimulate cell proliferation. Both nitrosamines stimulated cell proliferation in the cell line with Clara cell features. This effect was not changed by pre-exposure to cholinergic antagonists. The data suggest a selective uptake of nicotine and the two nitrosamines via nicotinic cholinergic receptors in pulmonary neuroendocrine cells.

The autonomous nervous system which regulates a wide variety of nonvoluntary functions in the mammalian organism is comprised of the sympathetic and parasympathetic system [1]. Acetylcholine, the neurotransmitter of the parasympathetic system, is taken up via cholinergic receptors [1]. The highly selective action of nicotine and muscarine via different subtypes of such receptors has lead to the classic subdivision of cholinergic receptors into nicotinic and muscarinic receptors [1]. Such receptors are abundant in the central nervous system and at neuro-muscular junctions (synapses), while they are also involved in the regulation of endocrine and exocrine secretion in a variety of organs [1].

Recent experiments in Syrian golden hamsters have provided evidence that the secretion of calcitonin by pulmonary neuroendocrine cells is regulated via nicotinic cholinergic receptors [2, 3], and that nicotine stimulates such secretion while also causing a hyperplasia of pulmonary neuroendocrine cells [4–6]. A hyperplasia of pulmonary neuroendocrine cells and elevated levels of calcitonin [7] have also been reported as one of the early events during lung carcinogenesis induced by *N*-nitrosodiethylamine (DEN). With respect to their similarity in biological effects on pulmonary neuroendocrine cells, it is intriguing to speculate that DEN and possibly other tobacco-related nitrosamines may exert their carcinogenic effects in the lung via the same receptors that regulate the selective uptake of nicotine by certain cell types. A mechanism such as this could possibly mediate the hitherto unexplained organ and cell type specificity of nitrosamine carcinogenesis, and could also be involved in the selective propagation of certain lung tumor types by nicotine and tobacco-related nitrosamines in smokers.

Ideally, this theory should be tested in the normal cells of origin of the lung tumor types most commonly linked with cigarette smoking. Among these are neuroendocrine carcinoma [8] which is believed to be derived from pulmonary neuroendocrine cells [9] and peripheral adenocarcinoma [10] which is believed to arise from Clara cells [11, 12] or alveolar type II cells [13]. However, a system to study the reactions of such normal human lung cells under controlled conditions is not currently available. Therefore, as a compromise, this series of experiments was conducted in cell lines derived from well differentiated human lung tumors. The three cell lines used in this study have been shown previously to possess ultrastructural [14] and biochemical [15–18] features of normal pulmonary neuroendocrine cells, Clara cells, and alveolar type II cells respectively.

MATERIALS AND METHODS

The stock material for all three cell lines was obtained from Dr A. F. Gazdar (NCI-Navy, Clinical Oncology Branch, Bethesda, MD). The cell lines which grew as monolayers were maintained in RPMI 1640 medium supplemented with L-glutamine (2 mM), fetal bovine serum (10%, v/v), gentamycin

* Supported in part by the University of Tennessee Center of Excellence Grant R18-0101-03 and Grant CA48014 with the National Cancer Institute.

Table 1. Modulation of growth kinetics by nicotine and two tobacco-related nitrosamines and the effects of cholinergic receptor antagonists in the adenocarcinoma-derived cell line NCI-H358 (features of alveolar type II cells)

Treatment	(seeding)	No. of cells × 10^4 72 hr	144 hr	216 hr
Control	5	5.5 ± 0.2	13.5 ± 0.5	50.8 ± 0.3
Nicotine	5	5.6 ± 0.8	14.5 ± 1.0	51.3 ± 1.0
DEN	5	5.0 ± 0.1	14.8 ± 1.2	52.4 ± 1.1
NNK	5	5.1 ± 0.3	14.5 ± 1.3	51.4 ± 0.9
Hexamethonium + nicotine	5	5.2 ± 0.9	13.2 ± 1.2	50.1 ± 0.7
Atropine + nicotine	5	5.0 ± 0.7	12.9 ± 1.5	51.0 ± 0.4
Hexamethonium + DEN	5	5.3 ± 0.1	14.0 ± 0.6	51.2 ± 0.3
Atropine + DEN	5	5.6 ± 0.7	14.7 ± 0.2	50.9 ± 0.8
Hexamethonium	5	5.1 ± 0.6	12.9 ± 1.0	51.3 ± 0.7
Atropine	5	5.4 ± 0.4	13.2 ± 0.5	49.6 ± 0.9

Values are means ± SD.

(50 μg/ml) at 37°. The two adenocarcinoma-derived cell lines, NCI-H322 and NCI-H358, were maintained in an atmosphere of 95% air–5% CO_2 whereas the neuroendocrine cell line NCI-H727 required 92% air–8% CO_2. The cells were trypsinized and seeded at a density of 5×10^4 cells/ml. They were allowed to attach for 24 hr in the incubator. Preincubation with the antagonists of nicotinic cholinergic receptors, hexamethonium (Sigma Chemical Co., St Louis, MO) and pentolinium (Sigma), and with the antagonist of muscarinic cholinergic receptors, atropine (Sigma), was for 10 min prior to the addition of nicotine (Sigma) DEN (Sigma), or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, Chemsyn Research Laboratories, Lenexa, KS). All chemicals were used at a final concentration of 1 μM. After 72 hr of incubation with the chemicals, the medium was removed, the cells were washed with phosphate-buffered saline (PBS), and the medium was then replaced. The medium was replaced again after 144 hr. Cells were counted with a hemocytometer (Neubaur) in triplicate after trypsinization and staining with trypan blue at 72, 144 and 216 hr after seeding. Mean values of cell counts were analyzed for statistically significant differences by Student's *t*-test.

RESULTS

The growth kinetics of the adenocarcinoma-derived cell line NCI-H358, which has morphological [14] and biochemical [18] characteristics of alveolar type II cells, were not altered significantly by nicotine or the two nitrosamines (Table 1).

In contrast, both of the other two cell lines demonstrated a marked stimulation of cell growth by the two tobacco-related nitrosamines DEN and NNK (Tables 2 and 3). The carcinoid-derived cell line NCI-H727, which has morphological and biochemical features of pulmonary neuroendocrine cells [16, 17], demonstrated a pronounced stimulation of cell growth by nicotine (Table 2). On the other hand, nicotine did not stimulate cell proliferation in the two adenocarcinoma-derived cell lines NCI-H358 and NCI-H322 (Tables 1 and 3). Preincubation with the antagonists of nicotinic cholinergic receptors, hexamethonium or pentolinium [19], completely inhibited the growth-stimulating effect of nicotine, DEN and NNK in the neuroendocrine cell line NCI-H727 (Table 2). On the other hand, hexamethonium and pentolinium did not inhibit the growth-stimulating effect of DEN or NNK in cell line NCI-H322 (Table 3) which demonstrates morphological [14] and biochemical [15] features of Clara cells. The antagonist of muscarinic cholinergic receptors, atropine [20], did not significantly inhibit the stimulation of cell proliferation caused by nicotine, DEN, or NNK in the neuroendocrine cell line NCI-H727 (Table 2). Likewise, this antagonist did not alter the stimulation of cell proliferation by DEN and NNK in the adenocarcinoma-derived cell line NCI-H322 (Table 3).

DISCUSSION

Our data demonstrate that nicotine caused a highly selective stimulation of cell proliferation in the tumor cell line comprised of neuroendocrine cells (NCI-H727). The reason for this selective effect of nicotine appears to be the presence of nicotinic cholinergic receptors in pulmonary neuroendocrine cells as evidenced by the inhibition of nicotine-induced cell proliferation by the antagonists of nicotinic cholinergic receptors, hexamethonium and pentolinium, and the lack of effect of atropine. With respect to the consistently high incidence of neuroendocrine lung cancer in smokers [8], these findings suggest that nicotine itself may act as a "growth factor" for pulmonary neuroendocrine cells and may, therefore, selectively propagate the development of neuroendocrine lung tumors. In the normal (non-neoplastic) pulmonary neuroendocrine cell, nicotinic cholinergic receptors are involved in the regulation of calcitonin secretion [2, 3]. It is disturbing to realize that neoplastic neuroendocrine cells such as our tumor cell line obviously retain their nicotinic recep-

Table 2. Modulation of growth kinetics by nicotine and two tobacco-related nitrosamines and the effects of cholinergic receptor antagonists in the neuroendocrine human lung cancer cell line (NCI-H727)

Treatment	(seeding)	No. of cells × 10^4		
		72 hr	144 hr	216 hr
Control	5	7.5 ± 1.2	14.3 ± 0.8	29.0 ± 1.1
Nicotine	5	12.2 ± 0.9	33.0 ± 1.5	97.8 ± 2.0
DEN	5	9.9 ± 0.6	31.0 ± 0.9	89.4 ± 1.8
NNK	5	20.0 ± 1.8	22.6 ± 0.4	62.5 ± 1.0
Hexamethonium + nicotine	5	7.2 ± 0.7	15.0 ± 1.3	30.3 ± 1.0
Pentolinium + nicotine	5	7.0 ± 0.5	14.3 ± 0.6	29.6 ± 0.8
Atropine + nicotine	5	7.6 ± 0.9	33.8 ± 0.4	98.9 ± 1.0
Hexamethonium ± DEN	5	5.6 ± 0.2	10.0 ± 0.4	26.7 ± 0.7
Pentolinium + DEN	5	5.8 ± 0.1	9.3 ± 0.6	28.7 ± 1.0
Atropine + DEN	5	5.9 ± 0.5	29.1 ± 0.5	86.5 ± 0.1
Hexamethonium + NNK	5	7.0 ± 0.6	13.1 ± 0.5	32.0 ± 1.0
Pentolinium + NNK	5	6.8 ± 0.3	14.0 ± 1.1	30.1 ± 1.2
Atropine + NNK	5	7.3 ± 0.4	24.6 ± 1.1	60.3 ± 0.7
Hexamethonium	5	6.9 ± 0.2	12.8 ± 0.7	29.5 ± 1.2
Pentolinium	5	7.6 ± 0.9	14.5 ± 1.2	28.7 ± 0.8
Atropine	5	7.7 ± 1.0	13.5 + 0.6	30.3 ± 1.4

Values are means ± SD.

Table 3. Modulation of growth kinetics by nicotine and two tobacco-related nitrosamines and the effects of cholinergic receptor antagonists in the adenocarcinoma-derived cell line NCI-H322 (Clara cell features)

Treatment	(seeding)	No. of cells × 10^4		
		72 hr	144 hr	216 hr
Control	5	6.6 ± 0.2	25.0 ± 0.6	42.0 ± 0.9
Nicotine	5	5.5 ± 0.1	20.1 ± 0.5	38.2 ± 1.0
DEN	5	42.5 ± 0.8	51.0 ± 0.7	67.2 ± 1.1
NNK	5	11.0 ± 0.3	46.0 ± 0.6	57.8 ± 0.9
Hexamethonium + nicotine	5	4.6 ± 0.1	18.7 ± 0.8	37.5 ± 1.2
Pentolinium + nicotine	5	5.0 ± 0.4	19.8 ± 0.6	38.4 ± 1.3
Atropine + nicotine	5	5.5 ± 0.7	20.4 ± 0.4	39.3 ± 0.7
Hexamethonium + DEN	5	40.0 ± 1.3	49.8 ± 1.1	66.5 ± 0.8
Pentolinium + DEN	5	41.8 ± 0.7	50.5 ± 0.6	67.7 ± 1.0
Atropine + DEN	5	42.7 ± 1.0	51.3 ± 1.3	66.9 ± 0.7
Hexamethonium + NNK	5	11.8 ± 0.3	47.0 ± 0.6	58.1 ± 0.5
Pentolinium + NNK	5	10.9 ± 0.5	46.5 ± 1.1	56.7 ± 0.3
Atropine + NNK	5	11.5 ± 1.2	45.4 ± 0.8	58.0 ± 1.0
Hexamethonium	5	6.1 ± 0.3	24.7 ± 0.5	41.9 ± 0.7
Pentolinium	5	6.7 ± 0.4	25.2 ± 0.7	42.6 ± 0.9
Atropine	5	7.0 ± 1.2	26.1 ± 0.9	43.0 ± 1.5

Values are means ± SD.

tors and thus provide additional portals of entry for nicotine. It does not require much imagination to recognize that such a phenomenon is likely to greatly amplify the growth factor-like effect of nicotine on pulmonary neuroendocrine cells and tumors deriving therefrom.

The lack of effect of nicotine on the two cell lines with features of Clara cells and alveolar type II cells, respectively, is in agreement with published data which reported regulation of Clara cell secretion by non-cholinergic receptors [21]. In conjunction with our data, these findings indicate that neither Clara cells nor alveolar type II cells have nicotinic cholinergic receptors and hence lack the ability for nicotine uptake. Accordingly, tumors derived from these cell types, such as our adenocarcinoma-derived cell lines, are unlikely to express this receptor type.

It is important that the growth-stimulating effect

of both tobacco-related nitrosamines investigated in this experiment is apparently mediated in neuroendocrine cells via the same subtype of cholinergic receptors as nicotine. It has been known for 12 years that several carcinogenic nitrosamines, including DEN, cause a pronounced hyperplasia of pulmonary neuroendocrine cells [4–6] accompanied by increased secretion of calcitonin [7]. Until today, there was no logical explanation for this phenomenon. Our data, in conjunction with published articles [2–7], suggest that, similar to nicotine, the nitrosamines are taken up by pulmonary neuroendocrine cells and tumors derived from them via nicotinic cholinergic receptors, thus mimicking the secretagogic and cell proliferative effects of the neurotransmitter acetylcholine on this cell type. This, in turn, raises the possibility that a mechanism unrelated to the DNA-alkylation theory of chemical carcinogenesis [22] may be instrumental in mediating the organ and cell type specific carcinogenicity of nitrosamines.

The stimulation of cell proliferation by DEN and NNK in the adenocarcinoma-derived cell line NCI-H322 (features of Clara cells) was not inhibited by the antagonists of nicotinic cholinergic receptors, hexamethonium and pentolinium, nor by the antagonist of muscarinic cholinergic receptors, atropine. In conjunction with the finding that nicotine did not alter the growth kinetics of this cell line and the published non-cholinergic regulation of Clara cell secretion [21], it has to be concluded that Clara cells and the adenocarcinoma cell line used in this experiment are devoid of nicotinic cholinergic receptors. Since there was, nevertheless, a pronounced stimulation of cell growth by the the two nitrosamines in this cell line, it is possible that non-cholinergic receptors of yet unknown nature are involved in this effect. This theory is currently under investigation in our laboratory.

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