The Inhibitory Effects of Nicotine on Azaserine Initiated Rat Pancreatic Carcinogenesis

Haydar Oztas and Deniz Yildiz
Department of Biology, Faculty of Science and Art, Mustafa Kemal Universiy, Antakya-Hatay, Turkey

Abstract: The objective of the present study was to investigate the tumor promoting effects of nicotine on exocrine pancreas in azaserine-rat model. Two weeks old male Lebed strain rats were given a weekly i.p. injection of azaserine (30 mg kg⁻¹ body weight) for five weeks. The other group was untreated with azaserine and kept as control groups. At the end of five weeks of treatment, rats were divided into four groups each group including 15 rats and exposed to nicotine for six months. At the end of the experiments, the animals were killed, the bodies were weighted and the pancreas were excised and weighted. The pancreas were kept in 10% formaldehyde for histological processes. The results of this study showed that exposure of azaserine for five weeks initiated the pancreatic cancer in rats. The standard diet plus nicotine mixture had an inhibitory effect on acinar cells of rat exocrine pancreas. The observed reduction in stereological parameters such as volume of Atypical Acinar Cell Foci (AACF) and mean focal diameter of foci (AACF) was significant (p<0.05) compared to azaserine initiated control rats. The results of the present study do not provide any evidence for that nicotine promotes exocrine pancreatic carcinogenesis in rat pancreas. Nicotine rather seems to be associated with the reduction of AACF that is produced by azaserine injection.

Keywords: Nicotine, rat, pancreas, carcinogenesis

INTRODUCTION

Several epidemiological studies have suggest that cigarette smoking is associated with a high risk of pancreatic cancer occurrence⁹. It is well known that pancreatic cancer continues to be a leading cause of death in men and women worldwide⁹. Peto et al.⁹ have estimated that in developing countries, there are about two millions of death attributed to cigarette smoking. Previously, nicotine has not been shown to have carcinogenic effects, but it is suspected that nicotine may promote tumor growth in the lung⁶. Shizouka et al.⁹ were the first to compare the efficiency of tobacco smoking on pancreas carcinogenesis. In a study, researchers detected a close association between tobacco smoking and AACF development in exocrine pancreas. It is well known that this kind of AACF may lead to exocrine pancreas in rats⁷. Hecht et al.⁹ claimed that smokers are repeatedly exposed to carcinogenic and genotoxic substances such as polynuclear aromatic hydrocarbons (PAHs), benz [a] pyrene and the nicotine-derived tobacco specific nitrosamine, 4-methyl1-nitrosamine-1-(3-pyridyl-1-butane) (NNK) which are powerful pulmonary carcinogens in rodent at doses similar to those encountered in a lifetime of smoking. Although the carcinogenic effects and metabolic pathways for PAH and NNK are well known, the carcinogenic effects and metabolic pathways for nicotine are not well described. In respect of pancreatic carcinogenesis, a tobacco smoke component, nicotine appears to be particularly important because of its relatively high level in cigarette smoke. Previously, it has been shown that a typical cigarette contains 6-11 mg of nicotine, of which the smokers can absorb 1-3 mg that is sufficient to establish and sustain nicotine dependence⁶,⁹. Performed researches have shown that nicotine acts on the neuronal nicotinic acetylcholine receptor (nAChR) which is a ligand-gated ion channels mediating the cholinergic neurotransmission⁶,¹¹. Maneckjee and Minna⁹ observed that some lung cancer types express nAChRs. Low concentrations of nicotine blocked the induction of apoptosis in these cells. So far researchers have evaluated the biochemical effects of nicotine and NNK on cultures of normal human bronchial epithelial cells⁹, but there is no study about the carcinogenic properties of nicotine on pancreas. For this reason, the initial objective of this study was to determine whether nicotine could promote exocrine pancreatic carcinogenesis in a well-known model, azaserine-rat model. One of pharmacologically important component of tobacco smoke component, nicotine, could provide further insight into the understanding carcinogenic effects of tobacco smoke.

Corresponding Author: Dr. Deniz Yildiz, Department of Biology, Faculty of Science and Art, Mustafa Kemal Universiy, Antakya-Hatay, Turkey Fax: 032624535867 E-mail: dyildiz@mkau.edu.tr
MATERIALS AND METHODS

Chemicals: Azaserine with a purity of 98% (evaluated by thin layer chromatography) was purchased from Sigma Chemical Co. (USA) Formalin and acetone was obtained from BDH Chemicals Ltd. (UK).

Animals: Male inbred Leeds strain rats were obtained from our breeding colony. The rats were maintained under standard conditions (room temperature 23°C; lighting 7 am - 7 pm) on sawdust bedding. Rats fed with standard diet (Paterson and Christopher Hill Group, Protein Diet, PRD) plus 500 mg kg⁻¹ (equivalent to 10 mg rat/day) nicotine (one of control and experimental group) or only standard diet (one untreated control, one azaserine initiated control group). During the feeding process nicotine group’s standard diet was mixed with nicotine with a V-shaped blender for one hour to obtain a homogeneous preparation. For this purpose, nicotine was first dissolved in a minimal quantity of acetone prior to mixing with standard diet to a final concentration of 500 mg kg⁻¹ weight and then added to the standard diet. The prepared diets were stored separately in dark in a sealed container. Animals had access to food and water ad libitum and the powder feeders were replenished with fresh diet twice a week.

Starting at two weeks of age, totally 30 male rats received a weekly i.p. injection of 30 mg kg⁻¹ of azaserine for 5 weeks. The same dose of 0.9% NaCl solution injected by the same method to other 30 control rats. At the end of seven weeks, animals were divided into four different groups as the following; The first group (UnCt) received no azaserine treatment. Rats in this group were fed with only standard diet. The second group (AzCt) included the azaserine injected rats and was kept as control rats. The third group (NiCt) received no azaserine but was treated with nicotine. Rats in this group were fed with a mixture of 500 mg kg⁻¹ nicotine diet. The fourth group (AzNi) included the azaserine injected and nicotine treated rats. Rats in this group were fed with a diet containing 500 mg kg⁻¹ of nicotine. Animals were healthy at the end of experimental period. Animals were then killed at the end of six months.

Stereological analysis of pancreases: The pancreases were excised by autopsy and all of the adherent fat, the mesentery and the lymph nodes were carefully trimmed off. The wet weight of each pancreas was recorded before fixation in 10% buffered neutral formalin for approximately 8-18 h. Before immersion in the fixative solution, each pancreas was spread out on a piece of porous paper to ensure maximal transactional area for subsequent sectioning. Thus, a single section of maximal area was obtained for stereology for each pancreas. Sections were then cut into 5 μm on a microtome and stained with haematoxylin-eosine. Acidophilic foci in sections were identified and classified according to established criteria [10]. The total area of exocrine pancreatic tissue was measured directly in a single histological section from each pancreas by means of a VIDS III video image analyzer (Analytical Measuring Systems, Cambridge). The same instrument was used to count acidophilic and basophilic AACF and measured their transactional areas. The observed data was processed numerically by a computer software package (Volugen), which uses an algorithm based upon the mathematical formula of Campbell et al. [14] as modified by Pugh et al. [15]. In this model, the foci with areas below reliably detectable values are subtracted from total number of intersections counted.

Statistical analysis: Numbers of pancreatic AACF, mean values and standard error of means were determined for all data. Non parametric statistical analysis were performed using the one-way analysis of variance, the Mann-Whitney U-Test.

RESULTS

Body and pancreatic weights: The mean weight of the rats in the UnCt, AzCt, NiCt and AzNi groups did not show a significant difference (Table 1). The mean pancreatic weights of all of the rat groups also did not show any statistical differences. During a gross examination of pancreas, no pathologic changes related to nicotine toxicity was observed.

Quantitative analysis of foci: Quantitative analysis of atypical basophilic and acidophilic cell foci, AACF, developed during experimental processes in rat pancreas is shown in Table 2 and 3. As expected, UnCt had a very low rate of atypical acinar cell foci (acidophilic and basophilic) that arose spontaneously on rat pancreas. NiCt pancreases had slightly higher atypical acinar cell foci compared to UnCt. However, the differences between these groups did not reach to a statistically significant level (p<0.05). AzNi represented a greater number of basophilic atypical acinar cell foci per mm² (0.528 Vs 0.463) compared to AzCt (Table 2). Some minor differences were also found between these groups with respect to the number of foci per pancreas (692.11 Vs 550.00), mean focal volume of foci (0.072 Vs 0.11) and volume of foci as a % of pancreas (0.383 Vs 0.456) but differences was not statistically significant (Table 2). Comparison of Atypical
### Table 1: Effects of nicotine feeding, following initiation with azaserine, body and pancreatic weights (g). (No statistical differences between groups)

<table>
<thead>
<tr>
<th>Groups (n=15)</th>
<th>UnCt</th>
<th>AzCt</th>
<th>NicCt</th>
<th>AzNi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>326±19.8</td>
<td>342.7±17.6</td>
<td>302.5±33.6</td>
<td>266.3±16.7</td>
</tr>
<tr>
<td>Pancreatic weight (g)</td>
<td>1.25±0.31</td>
<td>1.35±0.166</td>
<td>1.26±0.179</td>
<td>1.31±0.152</td>
</tr>
</tbody>
</table>

### Table 2: Effects of nicotine feeding, following initiation with azaserine, on induction of Atypical Basophilic Acinar Cell Foci (AACC)

<table>
<thead>
<tr>
<th>Groups (n=15)</th>
<th>UnCt</th>
<th>AzCt</th>
<th>NicCt</th>
<th>AzNi</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of AACC per mm²</td>
<td>0.009±0.012</td>
<td>0.022±0.013</td>
<td>0.0012±0.025</td>
<td>0.098±0.027</td>
</tr>
<tr>
<td>No. of AACC per mm²</td>
<td>0.007±0.023</td>
<td>0.463±0.025</td>
<td>0.0023±0.0125</td>
<td>0.528±0.163</td>
</tr>
<tr>
<td>No. of Aacc per pancreas</td>
<td>4.12±0.518</td>
<td>55.00±24.85</td>
<td>6.23±0.124</td>
<td>69.11±0.232</td>
</tr>
<tr>
<td>Volume of AACC as % of pancreas</td>
<td>0.020±0.005</td>
<td>0.456±0.243</td>
<td>0.004±0.002</td>
<td>0.383±0.188</td>
</tr>
<tr>
<td>Mean focal diameter (mm)</td>
<td>0.71±0.416</td>
<td>0.260±0.02</td>
<td>0.210±0.02</td>
<td>0.240±0.085</td>
</tr>
<tr>
<td>Mean focal volume (mm³)</td>
<td>0.003±0.0026</td>
<td>0.110±0.008</td>
<td>0.002±0.001</td>
<td>0.072±0.007</td>
</tr>
</tbody>
</table>

### Table 3: Effects of nicotine feeding, following initiation with azaserine, on induction of Atypical Acidophilic Acinar Cell Foci (AACC)

<table>
<thead>
<tr>
<th>Groups (n=15)</th>
<th>UnCt</th>
<th>AzCt</th>
<th>NicCt</th>
<th>AzNi</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of AACC per mm²</td>
<td>0.006±0.012</td>
<td>0.036±0.013</td>
<td>0.0015±0.018</td>
<td>0.023±0.0156</td>
</tr>
<tr>
<td>No. of AACC per mm²</td>
<td>0.0015±0.0037</td>
<td>0.0628±0.0259</td>
<td>0.0022±0.0081</td>
<td>0.063±0.044</td>
</tr>
<tr>
<td>No. of Aacc per pancreas</td>
<td>0.55±0.174</td>
<td>90.18±43.38</td>
<td>3.51±0.1035</td>
<td>69.24±0.2315</td>
</tr>
<tr>
<td>Volume of AACC as % of pancreas</td>
<td>0.001±0.001</td>
<td>0.973±0.539</td>
<td>0.0050±0.010</td>
<td>0.032±0.216 * Vs AzCt</td>
</tr>
<tr>
<td>Mean focal diameter (mm)</td>
<td>0.320±0.09</td>
<td>0.600±0.16</td>
<td>0.21±0.015</td>
<td>0.470±0.192 *Vs AzCt</td>
</tr>
<tr>
<td>Mean focal volume (mm³)</td>
<td>0.005±0.001</td>
<td>0.176±0.125</td>
<td>0.030±0.004</td>
<td>0.066±0.056</td>
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</table>

Acinar Cell Foci (AACC) parameters of UnCt with NicCt indicates that NicCt rats are higher than UnCt rats (3.51 Vs 0.55), but differences did not reach to significant level (p<0.05). The other parameters observed for both groups (NicCt Vs. UnCt) did not show any significant differences (p<0.05). When AzNi is compared with AzCt, AzNi represented a reduced quantitative parameter (Table 3). The differences on the volume of atypical acinar cell foci (AACC) (0.032 Vs 0.973) and mean focal diameter of cell (AACC) (0.47 Vs 0.68) were statistically significant (p<0.05). Also the volume of AACC as % of pancreases were reduced by 7.76%, but the differences did not reach to a significance level.

**DISCUSSION**

Tobacco smoke contains at least 43 compounds known to induce tumors in laboratory animals and tobacco smoking is a well-established risk factor for lung cancer. It has been shown that nicotine derived compounds such as 4-methyl (nitrosamine)-1-(3-pyridyl)-1-butane (NNK) may cause neoplastic disease especially in lungs of laboratory animals. But so far, carcinogenic effects of nicotine and related compounds in exocrine pancreatic carcinogenesis are not shown. Previous investigations of epidemiology suggest that tobacco smoking is associated with increased pancreatic carcinogenesis. The first objective of this study was to determine the possible promoting effects of nicotine by evaluating the AACC formation that occurs following long-term exposure of smokers to the carcinogens. But the results of this investigation have shown that a six months exposure of rats to a nicotine mixed diet did not promote AACC development in exocrine pancreas. In contrast, our results claim that nicotine may reduce the development of preneoplastic AACC development which potentially leads to pancreatic carcinogenesis. Quantitative stereological parameters of basophilic AACC in AzNi group was increased when compared with other groups, but differences were not significant. Basophilic AACC seems to have only a low potential for growth and progression to neoplasm, whereas a higher fraction of lesions that classified as acidophilic seem to have the potential for such a progression. Rao et al. have reported that the size of basophilic AACC tends to remain constant, that they have a consistently low or absent proliferative capacity. Acidophilic AACC have been previously observed in low numbers by several investigators in normal aging rats not exposed to any chemical carcinogen. It has been reported that incidence of these acidophilic AACC increase with age. The initiating effects of azaserine is well known on rat exocrine pancreas. In the early stage it mostly causes the development of precursor AACC and tumors. In our study, we observed a decreased AACC development in AzNi rats. The decreases in focal parameters in AzNi rat pancreases indicates that at least in the light of this findings nicotine cannot be a promoter of atypical acidophilic cell foci. The observed decrease in quantitative parameters of acidophilic AACC showed that in exocrine pancreas, nicotine is somehow effective in reduction of atypical acinar cell foci development in the early stage of the experiment. To interpret this reducing effect of nicotine on AzNi on AACC is difficult. Brognard et al. found that the expression of nicotinic acetylcholine receptor subunits types, timing and duration of the Akt response and effects of Akt phosphorylation of downstream substrates varied.
between normal human bronchial epithelial cell and small airway epithelial cells following stimulation with nicotine or NNK. Nicotine also caused a loss of contact inhibition at high cell densities in culture and when lung epithelial cells were challenged with a variety of apoptotic stimuli, nicotine and NNK inhibited the induction of apoptosis\(^{[9]}\). It was speculated that based on the early studies phosphorylation of Akt on the physiologically relevant serine and threonine residues was active in lung cancer cells derived from smokers and hypothesized that compounds such as nicotine might affect the Akt pathway in normal lung cells. It has been suggested that activation of this signal transduction pathway may change the behavior of normal lung epithelial cells, making them more similar to cancer cells\(^{[10]}\). But there is not any finding about activation of Akt pathway on normal pancreatic cells. It is well known that serine/threonine kinase Akt pathways regulated various cellular activities, such as cell growth and apoptosis. Akt is a multifunctional serine-threonine protein kinase which is in a low-activity conformation in quiescent cells cytosol. In this study, it is possible to speculate that possible lack of metabolic activation of nicotine by pancreatic acinar cells for Akt could be partially responsible for reduction of atypical acinar cell foci parameters. But further research is needed about the biochemical activity of Akt in exocrine pancreatic carcinogenesis. It could be interesting to find out how nicotine or carcinogenic derivatives of nicotine acting through the nicotinic acetylcholine receptors in acinar cells cause a loss of contact inhibition and development of resistance to apoptosis. Previous studies have demonstrated that the initiating/promoting effects of polycyclic aromatic hydrocarbons, heterocyclic amines and N-nitrosamines in lung carcinogenesis, but carcinogenic effects of this substances on pancreatic carcinogenesis remain to be elucidated. Hemingfield et al.\(^{[11]}\) demonstrated that a typical cigarettes contain 6-11 mg of nicotine of which the smokers can absorb 1-3 mg. The researchers found that nicotine and NNK, at concentrations equivalent to those reached in bloodstream of smokers, activated the Akt pathway, but mechanism by which the two compounds acted was different. This study provides that the development of pancreatic carcinogenesis depending on tobacco smoke seems to be more complex than previously thought\(^{[12]}\). A decrease in the focal parameters of azaserine initiated–nicotine fed rats may indicate that at least nicotine cannot be a promoter of atypical acinar cells developed after azaserine treatment. It is not always possible to ascribe these variations to any specific experimental conditions, although the total dosage of azaserine can be expected to increase the number of foci. Additional research would be required using various doses of nicotine to elucidate whether it is a promoter or non-promoter in exocrine pancreas.

REFERENCES