Opioids Induce While Nicotine Suppresses Apoptosis in Human Lung Cancer Cells

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Abstract
Previously, we have shown that opioids acting via specific receptors inhibit the growth of human lung cancer cells while nicotine, acting through nicotinic acetylcholine receptors, reverses this inhibition. Therefore, we studied the role of apoptosis in these processes. Treatment of human lung cancer cells with 0.1–1 μm morphine or methadone resulted in morphological changes and cleavage of DNA into nucleosome-sized fragments characteristic of apoptosis. Quantitation of DNA fragmentation showed that a dose-dependent increase occurred within 2 h of opioid treatment and was blocked by the antagonist naloxone.

The apoptotic effect of opioids was suppressed by nicotine, while the nicotinic acetylcholine receptor antagonists, hexamethonium and decamethonium, reversed this suppression. In contrast, sphingosine, a protein kinase C inhibitor, caused significant DNA fragmentation which was not suppressed by nicotine. Unexpectedly, the combination of hexamethonium and opioids or hexamethonium and nicotine stimulated apoptosis. We found that nicotine, like phorbol 12-myristate 13-acetate, increased total protein kinase C (PKC) activity, while morphine and sphingosine decreased PKC activity, and nicotine reversed morphine inhibition of PKC activity. In contrast, methadone unexpectedly increased PKC activity. These results indicate that engagement of opioid receptors in human lung cancer cells induces apoptosis, while engagement of nicotine receptors suppresses apoptosis, which in some cases appear to be working through a PKC pathway. They also suggest complexities in the system where blockade of C6 or C10 nicotinic receptors can lead to facilitation of apoptosis. These findings suggest new strategies for treatment and prevention of cancer using opioids or nicotine receptors antagonists and are consistent with the idea that nicotine functions as a tumor promoter.

Introduction
Recently, we identified a growth regulatory system in human lung cancer involving opioids and nicotine (1, 2).

Lung cancer cells of diverse histological types express multiple, biologically active, high affinity membrane receptors for opioids and nicotine (1). Opioid agonists with IC_{50}s of 10–50 nM were found to inhibit the growth of lung cancer cells in vitro. While nicotine (at 100–1000 nM) had no effect on lung cancer cell growth, it blocked the opioid-induced growth inhibition in most of the lung cancer cell lines tested (1). Lung cancer cells also expressed endogenous opioid peptides, suggesting the presence of a negative autocrine/paracrine growth loop that could also be abrogated by nicotine (1). In addition to morphine and other opioids, we also found that the long-acting, synthetic narcotic, methadone, inhibited lung cancer cell growth (IC_{50} 50–100 nM) acting through a novel opioid-related receptor (2). However, the cellular mechanisms involved in morphine- and methadone-induced growth inhibition and opioid-nicotinic interactions in lung cancer cells have not been elucidated.

Apoptosis (programmed cell death) functions in many physiological processes such as embryogenesis, immune responses, and cell growth control (3–8). Several reports suggest that apoptosis may be an important mechanism in tumor cell death following treatment with chemotherapy and that alterations in apoptosis, such as those caused by the bcl-2 oncogene, can lead to tumorigenesis (8, 9). Apoptosis is characterized by cytoplasmic membrane blebbing and cell shrinking, DNA fragmentation into nucleosome-sized fragments, requires ATP, and involves new RNA and protein synthesis (8). We previously found that the growth-inhibitory effects of opioids could be reversed in the first 6 h of treatment by actinomycin D and cycloheximide, suggesting a requirement for de novo mRNA and protein synthesis (2). Thus, we were interested in whether opioids induced growth inhibition and whether its reversal by nicotine in lung cancer cells were associated, respectively, with initiation and suppression of apoptosis. In our previous studies, we demonstrated the growth-inhibitory effects of opioids, such as morphine and methadone, on a large panel of SCLC and non-SCLC (adenocarcinoma, squamous cell, and large cell carcinoma) cell lines (1, 2). In the present studies, we present data on the non-SCLC line NCI-H157 (squamous cell carcinoma expressing μ, δ, and methadone opioid receptors and C6 and C10 nAChRs) and the SCLC lines NCI-N417 (expressing μ, δ, κ, and methadone opioid receptors and C6 and C10 nAChRs) and NCI-H146 (expressing μ, κ, and methadone opioid receptors and C6, C10, and mecamylamine nAChRs) (1, 2). These lung cancer lines showed growth inhibition by morphine and methadone and have been widely distributed to investigators around the world.

The abbreviations used are: IC_{50}, 50% inhibitory concentration; SCLC, small cell lung cancer; nAChR, nicotinic acetylcholine receptor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline.

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Results

Morphine and Methadone Induce Morphological Changes Consistent with Apoptosis. The effect of opioids and nicotine on the morphology of lung cancer cells was tested in the non-SCLC cell line NCI-H157 using the fluorescent nuclear DNA stain Hoechst-33258. Treatment of NCI-H157 cells with 100 nM morphine or methadone resulted, within 4 h, in morphological changes characteristic of apoptosis, including condensation of nuclear masses at the nuclear membrane and nuclear fragmentation resulting in groups of isolated pieces of condensed chromatin (Fig. 1). Similar to our previously reported growth studies (1), the opioid antagonist naloxone (1 μM) and nicotine (1 μM) blocked these morphological effects of opioids in the NCI-H157 cells (data not shown), indicating that morphine and methadone were acting through the opioid receptor in these cells and that this effect was also nicotine sensitive (see below for quantitative studies). Treatment with nicotine alone did not induce any morphological changes. In addition, in methadone-treated cultures after 24 h, the cells detached from the culture surface, whereas this effect was not observed if naloxone or nicotine was present with the opioids (data not shown).

Effect of Opioids and Nicotine on DNA Fragmentation. The apoptotic effect of opioids and nicotine on human lung cancer cells was also analyzed by electrophoresing DNA extracted from treated cells on agarose gels, followed by ethidium bromide staining (Fig. 2). Treatment of SCLC NCI-N417 cells for 2 h with 1 μM morphine or methadone resulted in cleavage of DNA into multiples of nucleosome-sized fragments (~180 base pairs), a “DNA ladder” pattern characteristic of apoptosis (7, 10). Of interest, the opioid antagonist naloxone, which competes with morphine and methadone for opioid receptor binding, also gave some DNA fragmentation in these experiments (see below for quantitative assays). Nicotine (1 μM) alone did not induce DNA fragmentation, and it blocked the opioid-induced DNA fragmentation changes in these cells (Fig. 2). We also found that sphingosine (50 μM), which is metabolized to ceramide, an inducer of apoptosis (16), also caused marked DNA fragmentation in these cells (Fig. 2).

Quantitative Analysis of Apoptosis using a DNA Release Assay. Quantitation of DNA fragmentation was determined in the SCLC cell line NCI-N417 by measuring the release of [3H]thymidine-labeled DNA as described previously (11). Time course studies showed that maximal DNA release was seen within 2 h of incubation with the opioids (Fig. 3). Similar to our previous growth inhibition studies (1, 2), maximum DNA release was seen at concentrations between 0.1 and 1 μM of morphine and methadone (data not shown). Thus, we tested 1 μM concentrations of morphine, methadone, nicotine, naloxone, various opioid agonists, and combinations of drugs after 2 h incubation in this assay (Table 1). We found morphine and methadone to reproducibly cause significant DNA release compared to control treatment. As shown in Fig. 2 above, naloxone by itself gave
some DNA release. However, naloxone (1 μM) was able to lower the effects of morphine and methadone on DNA release, indicating that these opioids were acting through naloxone-blocked opioid receptors. Similar to the morphological experiments reported above, nicotine by itself gave no DNA release, whereas the addition of nicotine to morphine or methadone treatment inhibited opioid induced DNA release (Table 1).

**Analysis of Opioid Receptor Types Inducing Apoptosis.**

Opioids are known to exert their effects by interacting with the μ, δ, and κ opioid receptor types (12). In previous studies using highly specific ligands such as [α-Ala², N MePhe, Gly⁵-ol] enkephalin (μ agonist), [D-Pen², D-Pen⁵]-enkephalin (δ agonist), and U-50,488H (κ agonist), we found that the lung cancer cell lines exhibited multiple opioid receptor types (1, 2). In addition, these cell lines can exhibit high affinity binding sites for the non-opioid σ (SK&F 10,047) and phenycyclidine (MK-801) agonists (13). Our prior studies have shown that these specific opioid and nonopioid agonists caused a concentration-dependent growth inhibition of lung cancer cells (1, 2, 13). In order to determine the specific opioid receptor types that may be involved in opioid-induced apoptosis of lung cancer cells, we tested the effects of the specific opioid and nonopioid agonists (Table 1). The effects of the specific ligands were less than those observed for morphine or methadone at the same concentrations. In the SCLC line NCI-N417, DNA fragmentation was induced only by the κ- and σ-specific ligands, U50,488H and SK&F 10,047. Combinations of the ligands did not induce more DNA release than the specific ligands alone, except in the case of methadone and the κ-specific ligand U50,488H. In contrast, morphine plus U50,488H gave no increase compared to either agent alone (Table 1). Previously, we found other differences between methadone and morphine receptors in human lung cancer cells (2).

**Nicotine Suppresses Opioid-induced Apoptosis through a nAChR Pathway.** Our previous studies demonstrated that nicotine reversed the growth-inhibitory effects of opioids and that nicotine binding to lung cancer cells was displaced by antagonists for nicotine, indicating that nicotine was binding to classic nAChRs in these cells (1). Since nicotine abrogated the DNA release by both morphine and methadone (Table 1), we determined whether the nicotine antagonist, hexamethonium (a ganglionic antagonist marking C6 nicotine acetylcholine receptors seen in the peripheral nervous system) could reverse the inhibitory effect of nicotine. Hexamethonium blocked the inhibitory effect of nicotine on opioid-induced DNA release in SCLC cell line NCI-N417, indicating that nicotine was acting through classic nAChRs (Table 1). Similar results were obtained with decamethonium, a C10 nAChR antagonist active at the neuromuscular junction (data not shown).

<table>
<thead>
<tr>
<th>Treatment (1 μM)</th>
<th>No. of experiments</th>
<th>% DNA release Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Morphine</td>
<td>7</td>
<td>47 ± 7 P&lt;0.05</td>
</tr>
<tr>
<td>Methadone</td>
<td>15</td>
<td>47 ± 4 P&lt;0.05</td>
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<tr>
<td>Naloxone</td>
<td>6</td>
<td>21 ± 5 P&lt;0.05</td>
</tr>
<tr>
<td>Naloxone + Morphine</td>
<td>4</td>
<td>13 ± 4 b</td>
</tr>
<tr>
<td>Naloxone + Methadone</td>
<td>4</td>
<td>19 ± 5 b</td>
</tr>
<tr>
<td>Nicotine</td>
<td>12</td>
<td>9 ± 2 c</td>
</tr>
<tr>
<td>Nicotine + Morphine</td>
<td>8</td>
<td>9 ± 2 c</td>
</tr>
<tr>
<td>Nicotine + Methadone</td>
<td>8</td>
<td>15 ± 3 c</td>
</tr>
<tr>
<td>Hexamethonium</td>
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<td>12 ± 1</td>
</tr>
<tr>
<td>Hexamethonium + Morphine</td>
<td>6</td>
<td>63 ± 15 P&lt;0.05</td>
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<tr>
<td>Hexamethonium + Methadone</td>
<td>5</td>
<td>68 ± 13 P&lt;0.05</td>
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<tr>
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<td>48 ± 18 P&lt;0.05</td>
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<td>Hexamethonium + Nicotine + Morphine</td>
<td>2</td>
<td>69 ± 21 P&lt;0.05</td>
</tr>
<tr>
<td>Hexamethonium + Nicotine + Methadone</td>
<td>2</td>
<td>50 ± 30 P&lt;0.05</td>
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<tr>
<td>DAGO (μ)</td>
<td>4</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>DPDPE (δ)</td>
<td>4</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>U-50,488H (κ)</td>
<td>4</td>
<td>28 ± 5 P&lt;0.05</td>
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<tr>
<td>MK-801 (PCP)</td>
<td>2</td>
<td>6 ± 0.1</td>
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<tr>
<td>SK&amp;F 10,047 (σ)</td>
<td>4</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>DAGO + DPDPE</td>
<td>2</td>
<td>9 ± 2</td>
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<tr>
<td>DAGO + U-50,488H</td>
<td>2</td>
<td>30 ± 8 P&lt;0.05</td>
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<tr>
<td>DPDPE + U-50,488H</td>
<td>2</td>
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<td>32 ± 2 P&lt;0.05</td>
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<tr>
<td>Morphine + U-50,488H</td>
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<td>35 ± 2 P&lt;0.05</td>
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<tr>
<td>Methadone + U-50,488H</td>
<td>2</td>
<td>69 ± 4 P&lt;0.05</td>
</tr>
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</table>

*Significant *P*-tests values (Fisher PLSD) were obtained in comparison to control treatment. If no *P* were given, it indicates values are not significantly different from control.

b Naloxone + morphine or methadone not significantly different from naloxone alone.

Nicotine, nicotine + morphine, and nicotine + methadone are not significantly different from each other or control values but are significantly (P < 0.05) different from morphine or methadone treatment.

**The nACR Antagonist Hexamethonium Unexpectedly Potentiates Opioid and Nicotine-associated Apoptosis.** By themselves, hexamethonium or nicotine had no effect on DNA release in N417 cells. However, unexpectedly, when hexamethonium was added to the cell culture medium together with opioids or nicotine or both drugs, a significant increase in DNA release was seen which, in some cases, was even higher than that seen with opioids alone (Table 1). These results suggest complexities in the system, including the possibility of several types of nicotine receptors triggering antagonistic pathways (see "Discussion" for a working model).
Study of Lung Cancer Cell Lines Where Nicotine Does Not Reverse Opioid-induced Growth Inhibition. We found previously that morphine-induced growth inhibition was blocked by nicotine in some, but not all, of the lung cancer cell lines tested (1). We refer to the lung cancer cell lines showing nicotine reversal of opioid growth inhibition as nicotine “sensitive” and those that did not show nicotine reversal as “resistant.” All of the lines expressed specific, high affinity nicotine receptors typical of nAChRs (1). [Prior quantitative studies showed that H157 bound 407 fmol, while N417 and H146 bound 1053 and 1024 fmol, respectively, of [3H]nicotine specifically per mg of membrane protein (1).] We found that, in the nicotine-resistant SCLC line NCI-H146, morphine induced DNA release. While nicotine treatment alone did not induce DNA release, when nicotine was added with morphine, DNA release, instead of being inhibited, was actually stimulated (Table 2). Thus, NCI-H146 also demonstrated “resistance” to suppression of apoptosis by nicotine.

Nicotine Cannot Reverse Sphingosine-induced Apoptosis. Other investigators have found that ceramide can induce apoptosis and that sphingosine, a well-documented inhibitor of PKC activity in cultured cells (14) including our lung cancer cells, is rapidly converted to ceramide in cultured cells, suggesting that ceramide mediates sphingosine action (15, 16). This led us to test the ability of sphingosine to induce apoptosis in lung cancer cells. We found that sphingosine induced a concentration-dependent increase in DNA fragmentation in the SCLC cells N417 (Fig. 4). Sphingosine (50 μM) induced approximately 80% DNA release in these cells and caused a typical pattern of DNA fragmentation on agarose gel analysis (Fig. 2). We next determined if nicotine could suppress sphingosine-induced apoptosis. In contrast to its effect on opioid-induced DNA release, nicotine did not inhibit the apoptotic effect of sphingosine. In fact, the combination of sphingosine and nicotine actually resulted in increased DNA release compared to sphingosine treatment alone (Table 3).

Changes in PKC Activity following Treatment with Opioids, Nicotine, PMA, and Sphingosine. The experiments with sphingosine indicated that treatment with this PKC inhibitor led to apoptosis in lung cancer cells, and since PKC inhibition has been associated with apoptosis in other systems, we decided to measure PKC activity in these cells following treatment with PMA, sphingosine, nicotine, and opioids. In this study, we used two “nicotine sensitive” (SCLC cell line N417 and non-SCLC cell line H157) and the “nicotine resistant” SCLC cell line H146 (1), which varied ~ five-fold in their basal total PKC activity (Table 4). We found that 30 min treatment with 10 ng/ml PMA stimulated total PKC activity in N417 and H157 cells (Table 4). Treatment with 1 μM nicotine stimulated PKC activity in the two sensitive cell lines while causing a significant decrease in the resistant H146 cell line (Table 4). Treatment with morphine decreased total PKC activity in all three cell lines, while morphine added to PMA treatment decreased the PMA-stimulated PKC value in N417 and H157 cells. Thus, morphine treatment resulted in decreases in both basal and PMA-stimulated PKC activity in cultured lung cancer cells consistent with a role of PKC in opioid-induced apoptosis.
Nicotine reversed the inhibitory effects of morphine on total PKC activity in the two nicotine-sensitive lung cancer cell lines. In the H146 nicotine-resistant line, the PKC values after morphine and nicotine treatment were similar to those of nicotine treatment alone but were still significantly less than control values. Thus, nicotine treatment resulted in changes in PKC activity, again consistent with a role of PKC in nicotine suppression of apoptosis. As shown above (Figs. 2 and 4; Table 3), treatment with sphingosine resulted in apoptosis that was not reversible by nicotine. Treatment of H157 cells with sphingosine resulted in a decrease of total PKC activity, and while the combination of sphingosine plus nicotine gave higher PKC levels than with sphingosine treatment, they were still significantly less than control values. Compared to morphine, treatment with methadone gave entirely opposite effects on total PKC activity. Unexpectedly, exposure to 1 μM methadone increased the PKC activity in N417 and H157 cells and decreased it in H146 cells. Also unexpectedly, the combination of methadone and nicotine decreased PKC in all of the cell lines. This occurred despite the fact that treatment of N417 and H157 cells with nicotine or methadone alone resulted in significant increases in total PKC activity.

Discussion

We have demonstrated that opioids at concentrations of 0.1 to 1 μM induce human lung cancer cells to undergo apoptosis (programmed cell death). We have also found that sphingosine is a potent inducer of apoptosis in lung cancer cells. The morphological changes, DNA fragmentation, and DNA release found in human lung cancer cells after opioid or sphingosine treatments correspond to those seen with programmed cell death in other cell types (8). In the presence of 1 μM nicotine, opioid-induced apoptosis in lung cancer cell lines, such as NCI-H157 and NCI-N417, is inhibited. The effects of opioids and nicotine on apoptosis in these cells appear to involve their receptors, since naloxone and hexamethonium are able to reverse these effects. Also in studies on SCLC line NCI-N417, the opioid effect appeared to be mediated through κ- and α2-specific receptors. The opioid antagonist naltroxone, which acts by competing with opioids for binding to their receptors, also induced a low level of DNA release, suggesting that engagement of the opioid receptor alone may be sufficient to trigger the apoptotic pathway.

In a recent study, Wright et al. (17) showed that nicotine, at much higher concentrations (60–250 μM) than we used, inhibited apoptosis in several human tumor lines. This suppression involved apoptosis induced by diverse stimuli, including tumor necrosis factor, UV light, chemotherapeutic drugs, and calcium ionophore, and they postulated that nicotine may act as a tumor promoter. They found that the nicotine metabolite cotinine also inhibited apoptosis with an IC50 of 50 to 100 μM. However, the effect they saw was not stereospecific, and the nAChR antagonists, pentolinium and hexamethonium, did not block nicotine suppression of apoptosis, suggesting a lack of involvement of nAChRs (17). In contrast, our work in lung cancer cells indicated nicotine exerted its effect to suppress apoptosis through nAChRs. In addition, we find that the nicotine antagonist hexamethonium increased opioid-induced apoptosis. We also found, unexpectedly, that the combination of hexamethonium and nicotine actually induced apoptosis in lung cancer cells. Furthermore, in some human lung cancers (such as NCI-H146), nicotine does not block opioid-induced apoptosis, and in fact, the combination of nicotine and opioids led to increased apoptosis.

Previous studies have shown that the PKC activator, PMA, inhibits apoptosis in a variety of model systems (18–23). Thus, it is of interest that nicotine can stimulate PKC activity in adrenal chromaffin cells (24). In this study, we also found that nicotine stimulated total PKC activity to levels equivalent to or higher than those stimulated by PMA. In association with this elevation of PKC activity, nicotine treatment was able to suppress apoptosis in nicotine-sensitive but not in nicotine-resistant lung cancer lines. Recently, ceramide, an inhibitor of PKC activity, was found to induce apoptosis in U937 monoblastic leukemia cells (16), and the effect of ceramide on DNA fragmentation was prevented by the PKC activator PMA. PKC has also been shown to be involved in the suppression of apoptosis in hematopoietic cells by deprivation of granulocyte-macrophage colony-stimulating factor and interleukin 3 (25). In this study, we showed that sphingosine, which is converted to ceramide, besides inducing apoptosis, also significantly lowered total PKC activity, and this change in PKC was only partially altered by nicotine. Although G-protein-mediated inhibition of adenylate cyclase remains the best characterized biochemical effect of opioid receptor activation, recent studies suggest that alternate mechanisms, including the PKC (26–28) or phosphoinositide signal transduction (29, 30) pathways, may account for opioid actions. Thus, we tested for PKC activity after drug treatment in lung cancer cells. In this study, we found that morphine lowered both basal and PMA-stimulated PKC activity in lung cancer cells, whereas nicotine significantly reversed this change in PKC activity in the nicotine-sensitive but not the nicotine-resistant lung cancer cells. Thus, the results with morphine and nicotine were consistent with involvement of the PKC pathway in apoptosis, with morphine lowering and nicotine raising PKC activity. However, the results with methadone were unexpected in that methadone increased PKC activity, whereas the combination of methadone and nicotine lowered total PKC activity. Our previous studies showed other differences between morphine and methadone receptor activation in lung cancer cells, including the observation that growth inhibition by morphine was reversed by nicotine, while that induced by methadone was not (1, 2). Why in this study we saw reversal of methadone-induced apoptosis, whereas previously we failed to see reversal of growth inhibition, is currently a mystery. It is possible that study of the changes in different PKC isoforms could explain these differences.

Fig. 5 provides a working model to deal with these findings. We suggest that pathways exist (labeled O1 for morphine and O2 for methadone), triggered by ligands binding to opioid receptors, that induce apoptosis in the majority of lung cancer cell lines tested (such as in NCI-N417, H157, and H146 cells). The number of steps in these pathways is unknown. Why morphine results in a decrease in total PKC activity (Fig. 5, PKC ↓), while methadone results in an increase in the PKC (Fig. 5, PKC ↑) activity, despite the fact that both induced apoptosis, is also unknown. Nicotine triggers a second pathway (Fig. 5, N1), acting through C6 and C10 nAChRs, that can increase total PKC activity (Fig. 5, PKC ↑) and suppress apoptosis induced via the opioid pathways. Wright et al. (17) have shown that nicotine is also able to inhibit apoptosis through a non-nAChR-mediated
Fig. 5. Working model of induction and suppression of apoptosis in human lung cancer cells by opioids, nicotine, and sphingosine (ceramide). The opioid pathways are labeled for morphine as O1 and for methadone as O2 (opioid-induced pathways 1 and 2). The morphine and methadone receptors are known to be distinct (2). The nicotine pathways are labeled as N1 (suppressing apoptosis) and N2 (facilitating apoptosis when the N1 receptors are blocked). The N1 pathway can originate through C6 and C10 nAChRs, but the origin of the N2 pathway is unknown, and the diagram for its putative receptor is labeled with a "?". In addition, there must be another nicotine-triggered pathway (N3) that suppress apoptosis induced by a variety of cellular insults, which, as shown by the work of Wright et al. (17), does not act through nAChRs (17). The ceramide pathway is labeled as "C." PKC with up or down arrows indicate the change in total PKC activity after treatment with the various drugs, while the PKC ? indicates that the status of PKC activity after such treatment is unknown. The sequential arrows indicate the possibility of several steps but are not meant to imply the number of such steps.

pathway (Fig. 5, N3). These opioid and nicotine pathways would explain opioid- and naloxone-induced apoptosis and the ability of nicotine to inhibit opioid-induced apoptosis (such as in NCI-N417 and H157 cells). Sphingosine induces apoptosis, either because of inhibition of PKC or because of conversion to ceramide which inhibits PKC. In Fig. 5, we indicate that sphingosine triggers a separate pathway (labeled C) that decreases PKC activity (Fig. 5, PKC ↓) and appears to act downstream of the opioid and nicotine pathways, since nicotine cannot reverse sphingosine-induced apoptosis. However, nicotine also appears to activate another pathway (Fig. 5, N2) that by itself does not generate but can facilitate apoptosis. When both the N1 and N2 pathways are activated, N1 is dominant. This is the case with nicotine treatment alone, where apoptosis is not seen (such as in NCI-N417 and H157 cells). However, if the N1 pathway is inhibited while the N2 pathway is activated (by concurrent treatment with hexamethonium and nicotine), apoptosis is induced (as in N417 cells). Thus, we envision N417 and H157 cells to possess the O1, O2, N1, N2, and C pathways. In contrast, H146 cells appear deficient in the N1 pathway. In H146 cells, opioids induce apoptosis, and nicotine, instead of reversing actually potentiates apoptosis, presumably working through the N2 or a similar pathway. The fact that hexamethonium treatment alone did not lead to apoptosis but the combination of opioids and hexamethonium resulted in greater apoptosis than seen with opioids alone suggests that the N1 pathway, in the absence of nicotine, may also play some tonic role in suppressing apoptosis. We know from prior studies (2) that methadone interacts with an opioid-like receptor distinct from the morphine receptor. As shown here, methadone in some lung cancers can stimulate while morphine inhibits PKC activity. Finally, the finding that the combination of sphingosine and nicotine enhanced apoptosis indicates that the ceramide pathway (C) and some nicotine-activated pathway (potentially N2) can act together to potentiate apoptosis.

In conclusion, we have shown that opioids, such as morphine and methadone, induce apoptosis in human lung cancer cells. These opioids are commonly used in the treatment of cancer pain, and methadone is being tested in a clinical trial to see if it has anticancer activity in advanced lung cancer patients. Cell death plays an important role in controlling cell growth, and certain tumors grow because programmed cell death does not occur (8). Hence, the regulation of apoptosis in cancer cells could lead to new approaches for the treatment of cancer. In fact, there is substantial evidence that conventional anticancer chemotherapy invokes an apoptotic pathway and that, for efficient execution, this pathway often requires function of the wild-type p53 tumor suppressor gene (9). p53 is frequently mutated in human cancer, and one possibility is that p53 mutations could lead to acquired cross-resistance to anticancer agents. However, p53 mutations are common in lung cancer, and the majority of lung cancer cells (including N417 and H157) which show opioid-induced apoptosis and growth inhibition have mutations in their p53 genes (31–35). Thus, opioids can induce apoptosis in human lung cancer cells, despite the presence of p53 mutations. The present study is also consistent with the hypothesis that nicotine could function as a tumor-promoting agent by blocking apoptosis (17). We suggest it would act to inhibit apoptosis through stimulation of PKC activity. Although the normal source of nicotine is from cigarette smoking, the use of nicotine patches and gum as antismoking aids provides another source of nicotine that could potentially play a role in tumor promotion. Therefore opioids, nicotine receptor antagonists such as hexamethonium, and inhibitors of PKC activity could have potential value as chemopreventive agents for lung cancer in cigarette smokers. In fact, in some instances, by blocking the N1 pathway, the induction of apoptosis may actually be increased by the presence of nicotine found in persons continuing to smoke or using nicotine patches (such as by activating the N2 or a similar pathway). Thus, it will be important to see if these agents can induce apoptosis in vivo in tumor or preneoplastic cells harboring mutations.

Materials and Methods

Cell Lines. Previously characterized SCLC and non-SCLC cell lines were grown in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum as described (36). These cells have been deposited by our group in the American Type Culture Collection (Bethesda, MD). The cells used in the DNA fragmentation assays were changed to 2.5% fetal calf serum just prior to the addition of [3H]thymidine. The cells were free of Mycoplasma contamination as indicated by a molecular hybridization assay used according to the vendor's instructions (Gen-Probe, San Diego, CA).

Reagents. Commercial sources for chemicals were: (+)-methadone hydrochloride, (-)-nicotine di tartarate, hexamethonium bromide, D-sphingosine, naloxone hydrochlo-
ride, and Hoechst 33258 stain (Sigma Chemical Co.); morphine sulfate (Malinckrodt); [d-Ala², N MePhe, Gly⁷-ol] enkephalin and [D-Pen², D-Pen⁵]-enkephalin (Peninsula Laboratories); U-50,488H, MK-801, and SK&F 10,047 (Research Biochemicals); ³H-thymidine (specific activity, 82 Ci/mmol), and protein kinase C assay kit (Amersham).

**Quantitative Assay of DNA Fragmentation.** DNA fragmentation was quantitated using a previously described assay (11). Lung cancer cells were labeled with 20 μCi [³H]thymidine/5 × 10⁶ cells and incubated for 20 h at 37°C. The cells were washed three times and resuspended in the same medium; 100 μl was then aliquoted into Eppendorf tubes for use in the assay. Appropriate concentrations of the drugs were added to the aliquoted cells, and the tubes were incubated at 37°C for the times indicated in "Results." At the end of the incubation period, 0.8 ml of cold PBS was added to each tube and centrifuged at 6000 × g for 1 min in a microfuge. Supernatants were removed, and the pelleted cells were lysed by the addition of 0.4 ml of 0.2% Triton X-100 and 2 mM EDTA in PBS without Ca and Mg. Then the fragmented DNA was separated by centrifugation at 13,000 × g for 15 min. Supernatants, lysates, and pellets were counted in a scintillation counter, and the % DNA release calculated as:

\[
\text{% DNA release} = \left( \frac{\text{Supernatant cpm} + \text{lysat cpm}}{\text{Supernatant cpm} + \text{lysat cpm} + \text{pellet cpm}} \right) \times 100
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Statistical analysis was performed using ANOVA tests in the computer program Statview SE (Abacus Concepts, Berkley, CA).

**Morphological Determination of Apoptosis.** Morphological changes occurring during apoptosis were evaluated using the fluorescent Hoechst 33258 stain. Lung cancer cells (NCI-H157) were plated on glass slides placed in Petri dishes and allowed to attach and grow as a monolayer in RPMI 1640 containing 10% fetal calf serum at 37°C. When the cells appeared confluent, they were treated with the appropriate drugs and incubated for 2-, 4-, 6-, and 24-h periods. At the end of the incubation periods, the cells were fixed with methanol/acetic acid, air dried, and stained directly with the Hoechst stain for 20 min. The slides were washed with distilled water and mounted in Vector Shield. Fluorescence was observed using a Nikon microphot FXA fluorescence microscope equipped with a 60X oil NA 1.4 lens.

**Analysis of DNA Fragmentation in Agarose Gels.** DNA fragmentation in human lung cancer cells was visualized by gel electrophoresis as described previously (10). Cells (2 × 10⁶) were incubated at 37°C for 2 h with 1 μM concentrations of the various drugs. Following this incubation, the cells were washed in PBS and centrifuged at 1000 rpm. The cells were then incubated at 37°C for 4 h in digestion buffer [200 mM Tris (pH 8.5), 100 mM EDTA, 50 μg/ml proteinase K, and 1% sodium dodecyl sulfate]. The DNA was extracted with equal volume of phenol, and the aqueous phase was dialyzed overnight in 10 mM Tris (pH 7.5)-1 mM EDTA. After the dialysis, the DNA solution was incubated at 37°C with 50 μg/ml of RNase A for 4 h and with 120 μg of proteinase K for an additional 4 h. The DNA was then extracted with an equal volume of phenol/chloroform. NaCl was then added to the aqueous phase (final concentration, 150 mM) and precipitated with 2 volumes of ethanol at -20°C overnight. Pellets were air-dried and re-suspended in 50 μl of distilled water. The DNA concentration was calculated by determining the absorbance at 260 nm. Horizontal electrophoresis of the DNA was performed in 1% agarose gel in TBE buffer [90 mM Tris, 90 mM boric acid, and 2 mM EDTA (pH 8.1)]. DNA was visualized by ethidium bromide staining.

**PKC Measurements.** Cell extracts were prepared from confluent cells as described previously (24, 37). Prior to the addition of drugs, the cells were placed in serum-free medium. The drugs were added at appropriate concentrations, and the cells were incubated for 30 min and 24-h time periods at 37°C. At the end of the incubation, the medium was discarded, the cells were washed with PBS, and then disrupted by sonicating in ice-cold lysin buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 mg/ml leupeptin, and 0.3% w/v β-mercaptoethanol. Homogenates were centrifuged for 60 min at 100,000 × g at 4°C. The resulting supernatant was the source of soluble PKC activity. The pellets were suspended in the same buffer containing 0.1% Triton X-100 and centrifuged at 120,000 × g for 60 min at 4°C. Enzyme activity recovered from the resulting supernatant was considered as particulate PKC activity. PKC levels in the soluble and particulate fractions were measured using the PKC enzyme assay system from Amersham according to the manufacturer's instructions. Total PKC activity represents the sum of soluble and particulate PKC activity. Protein concentrations were measured using the Bio-Rad protein assay kit.

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**References**


