Repellent Nicotine Exposure Leads to a More Malignant and Metastasis-Prone Phenotype of SCLC: A Molecular Insight into the Importance of Quitting Smoking during Treatment

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Cigarette smoking is strongly correlated with the onset of lung cancer. Nicotine, a major component in cigarette smoke, has been found to promote tumor growth and angiogenesis, as well as protect cancer cells from apoptosis. Among all lung cancer cases, small cell lung cancer (SCLC) is found almost exclusively in smokers; metastasis and chemoresistance are the main reasons for the high mortality rates associated with SCLC. Retrospective studies have shown that patients with tobacco-related cancers who continue to smoke after their diagnosis display lower response rates and a shorter median survival compared with those who stop smoking. In the current work, we examined the effects of acute and repetitive exposure to nicotine, in the concentrations found in the lungs of active smokers, on the malignant properties of N417 SCLC cells in vitro. We observed that repetitive nicotine exposure induced a neuronal-like appearance in N417 cells along with increased adhesion to the extracellular matrix and chemoresistance. These changes were accompanied by enhanced migration through collagen matrices and adhesion to and transmigration across lymphatic endothelial cell monolayers. SCLC differentiation reverted after cessation of nicotine exposure. Here, we provide evidence for the leading role of the CXCR4/CXCL12 axis in these phenomena. Finally, we show how nicotine-differentiated N417 cells produced bigger and more vascularized tumors in mice, with lower apoptotic rates, than their nondifferentiated counterparts. In short, these findings identify the mechanisms through which nicotine increases SCLC malignancy and provide further evidence that CXCR4 is a potential anticancer target for nicotine-associated SCLC.

Key Words: SCLC; CXCR4; nicotine; malignancy.

Lung cancer is a major health issue worldwide (Ian Bray and Weiderpass, 2010; Parkin et al., 2005); it causes approximately 1.2 million deaths per year. Small cell lung cancer (SCLC) accounts for approximately 20% of total lung cancer–associated deaths. This type of tumor is characterized by rapid growth and early dissemination, with a poor outcome. Tobacco consumption is the principal etiologic agent causing SCLC. In fact, this type of lung cancer is found almost exclusively in smokers (Koletsis et al., 2009).

Although cigarette smoke is a complex mixture of more than 4000 compounds, nicotine is one of the major constituents of tobacco and has been shown to be the main addictive element (Borgerding and Klus, 2005). Nicotine is not carcinogenic by itself; products derived from the metabolism of nicotine form DNA adducts, mutating genes during DNA replication (Upadhyaya and Hecht, 2008). Additionally, nicotine can contribute to carcinogenesis in the lung by inducing cell proliferation (Dasgupta et al., 2009), stimulating angiogenesis (Mousa and Mousa, 2006), and endowing tumor cells with survival mechanisms against chemotherapy (Dasgupta et al., 2006; Tsurutani et al., 2005).

The action of nicotine is mediated primarily via the nicotinic acetylcholinergic receptors (nAChRs) (Schuller and Orloff, 1998). These receptors were first described in neurons and neuromuscular junctions, but they have also been found to function in a variety of non-neuronal tissues, including several histological types of lung cancer (Di Matteo et al., 2007). In fact, there are an increasing amount of published data showing how the activation of these receptors contributes to tumor growth, and they have been proposed as new targets for lung cancer therapy (Paleari et al., 2009).

The effects of nicotine on cellular functions depend on the cell type, dose, and frequency of exposure (Di Matteo et al., 2007). Interestingly, it has been demonstrated that the association between lung tissue damage and tobacco is related to the duration of the smoking period, number of cigarettes consumed, type of smoke inhalation, and cigarette brand (De Stefani et al., 2005). The average nicotine content per cigarette ranges from 1.0 to 1.8 mg, but when inhaled, it is rapidly absorbed by the
respiratory tract, buccal mucosa, and skin, reaching the brain as rapidly as 10 s after smoking. In fact, nicotine concentrations in the bronchial mucosa can reach 0.5–1mM immediately after smoking, whereas steady-state serum concentrations between 200nM and 1μM have been reported (Hukkanen et al., 2005; Russell et al., 1980). Moreover, nicotine bioavailability depends strongly on its chemical state (Svensson, 1987); it is rapidly protonated (nicotine pKa = 8), rendering a molecule that is not able to bind to the cellular membrane in the acidic form. In addition to its low stability in base form, nicotine undergoes an extensive intracellular metabolism that occurs mainly in the mitochondria by the detoxifying action of cytochrome P450. For example, lung epithelial cells that express CYP2A13 oxidase are able to degrade nicotine to cotinine, its main metabolite, as it enters into the epithelial cell (Hecht, 2002), reducing the half-life of nicotine to 2 h.

The majority of the studies published on the effects of nicotine on cancer cells have used doses of nicotine similar to those present in the blood stream of smokers (10−8 to 10−7M) (Russell et al., 1980). In our study, we examined the effects of this compound when it was added to SCLC cells at concentrations found in the lungs of active smokers. The results of the current report support the importance of giving up smoking after being diagnosed with cancer. Specifically, we observed that nicotine-treated cells displayed a neuronal-like appearance that was accompanied by increased malignant features in vitro, such as anchorage-independent growth, resistance to chemotherapy, and adhesion to and transmigration across lymphatic endothelial cells (LECs). We also described how nicotine induced the expression of CXCR4 on SCLC cells, which plays a significant role in their migration and adhesion to the endothelium. Furthermore, in vivo experiments using xenografted nicotine-exposed SCLC cells demonstrated that SCLC cells exposed to nicotine promote bigger more vascularized tumors with less apoptosis. In summary, these results provide interesting scientific evidence to support the importance of smoking cessation in patients diagnosed with SCLC and point to the importance of developing nAChR-targeted therapy.

MATERIALS AND METHODS

Cell culture and pharmacological treatments. Nonadherent NCI-N417 SCLC cells were purchased from American Type Culture Collection (Bethesda, MA, USA) and grown in suspension in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 100 U/ml. All cell cultures were maintained at 37°C in a humidified 5% CO2 incubator. Cryopreserved human LECs were obtained from Lonza (Barcelona, Spain). They were cultured on collagen-coated 10-cm2 dishes in endothelial complete medium (EGM; Lonza) consisting of endothelial basal medium 2 supplemented with EGM-2 MV SingleQuots (Lonza) composed of FBS, hydrocortisone, epithelial growth factor, vascular endothelial growth factor (VEGF), fibroblast growth factor-2, insulin-like growth factor 1, ascorbic acid, and gentamicin/penicillin at the concentrations supplied by the manufacturer (not specified). N417 cells were treated daily with 1–500μM nicotine (Sigma-Aldrich, St Louis, MO) for 7 days, replacing the culture media every 48 h. To test the reversibility of the process, once the cells were differentiated, cell media was changed for growth media without nicotine and cells were maintained in culture for additional 7 days. To assess the role of nAChRs in nicotine effects, nicotine was added to cells pretreated for 30 min with or without a mixture of 10μM α-bungarotoxin (α-BTX; Sigma-Aldrich), 10μM mecamylamine (Sigma-Aldrich), and 10μM dihydro-β-erythroidine hydrobromide (DHβE; Sigma-Aldrich) that inhibit both α- and β-nAChRs. For chemoresistance experiments, several concentrations (0–100μM) of the following agents were used: etoposide, cisplatin, mitomycin, and taxol (Sigma-Aldrich).

Reverse transcription and complementary DNA amplification (RT-PCR). Total RNA was extracted with Trizol (Invitrogen, Barcelona, Spain). RNA (2 μg) was reverse transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase (Promega, Madison, WI) in a final volume of 20 μl following manufacturer’s instructions. Real-time quantitative PCR was performed using the SYBR green PCR master mix and the iCycler instrument from Applied Biosystems (Warrington, UK). Amplification conditions were the same as described previously (Martinez-Garcia et al., 2008). All samples were run in triplicate in a final volume of 25 μl that included 1 μl of cDNA and 20μM of each gene-specific primer. Fold changes in the expression of each target messenger RNA (mRNA) relative to β-actin were calculated based on the threshold cycle (Ct).

Immunofluorescence. N417 cells were grown on four-well chamber slides at a density of 5 × 104 cells per well in RPMI and exposed to nicotine, as described above. Cells were then washed with Tris-buffered saline (TBS), fixed in TBS containing 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 5 min, and then washed three times with TBS. Immunofluorescence against neuronal cell adhesion molecule (NCAM), neurofilament M (Nef-M), and Neu-N was performed as described previously (Martinez-Garcia et al., 2008).

Flow cytometric analysis of CXCR4. To analyze protein expression by flow cytometry, SCLC cells were resuspended in PBS and incubated with 1 μg/ml of the monoclonal anti-CXCR4 antibody (MAB172; R&D Systems, Minneapolis, MN) or the appropriate isotype control (MAB004; R&D Systems) at room temperature for 10 min. Cells were then washed and incubated with 1:500 fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Molecular Probes, Invitrogen Corp., Barcelona, Spain) at room temperature for 10 min. The cells were then washed twice with PBS and analyzed on a FACSscan flow cytometer using Cellquest software (BD Pharmingen, San Diego, CA).

Cell adhesion to extracellular matrix proteins and to monolayers of LECs. SCLC cells (106 cells per milliliter) were labeled with 10μM calcine-AM for 10 min (Fluka, Steinheim, Germany) in adhesion medium (RPMI 0.5% bovine serum albumin [BSA] and 20mM N-2-Hydroxyethylpiperazine-N’-2-Ethanesulonic Acid buffer). Before the adhesion assay, 96-well plates were coated with concentrations of 5 μg/cm2 of fibronectin, collagen I, IV and 0.1 μg/cm2 of laminin. Adhesion of control or nicotine-exposed small cell lung cancer (SCLC) to these extracellular matrix (ECM) proteins was assayed as described previously (Irigoyen et al., 2007).

To measure SCLC cell adhesion to LEC monolayers, 4 x 104 endothelial cells were seeded onto fibronectin-coated 96 wells. Once the endothelial monolayer was established, 5 x 105 nicotine-treated or nontreated N417 calcine-labeled cells were seeded on top of each LEC monolayer. Tumor cells were allowed to adhere to LEC for 30 min at 37°C. After that time, adhered cells were washed and quantified by fluorimetric analysis as described (Irigoyen et al., 2007). For blocking experiments, 5 μg/ml of anti-CXCR4 antibody (MAB172; R&D Systems) or nonspecific IgG (MAB004; R&D Systems) was added to N417 cell suspensions 30 min before the adhesion assays.

Cell viability assays. For cell viability assays, 104 N417 cells were plated in 96-well plates (TPP, St Louis MO) in complete RPMI medium. When cells reached 50% confluence, increasing amounts of the four different chemotherapeutic drugs used were added in complete cell culture media. Forty-eight hours later, cell viability was assayed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich).
Briefly, MTT (Sigma-Aldrich) was dissolved in PBS at a concentration of 5 mg/ml and filtered. Afterward, 10 μl of stock solution was added to 200 μl of medium in each well. Cell culture plates were incubated for 3 h at 37°C. Subsequently, 100 μl of MTT lysis buffer (20% SDS and 50% dimethylformamide, pH 4.7) was added to each well and incubated for 24 h at 37°C. The absorbance was read at 540 and 690 nm in a TECAN sunrise microtiter plate reader (Hayward, CA).

**Soft agar colony formation assays.** Two milliliters of 0.6% agar in RPMI media was poured into each well of a six-well plate. Once solidified, a suspension of 5 × 10^5 SCLC cells in 1 ml of 0.3% agar in RPMI was added onto the previously formed agarose layer. Triplicate wells were plated in parallel to compare the growth of nicotine-exposed and nonexposed cells. Two weeks later, 0.5 ml of 10 mg/ml MTT (Sigma-Aldrich) was added to each well for 5 h at 37°C. The MTT was then washed out, and 0.5 ml of dimethylsulfoxide was further added to each well for 24 h at 37°C. Finally, colonies were counted in four high-powered fields per well using an inverted microscope.

**Cell chemotaxis assay.** SCLC migration was assayed using Boyden chambers of 8-μm pore size filters (BD Biosciences, San Diego, CA). A total of 5 × 10^5 N417 nicotine-treated or nontreated cells were seeded in 200 μl of migration medium (RPMI supplemented with 0.5% BSA) on the top membrane of transwell chambers. Complete media was used as chemoattractant on the bottom chamber. Migration was allowed to occur during 12 h at 37°C, and migrated SCLC cells on the lower chamber were counted using an ocular micrometer.

**Three-dimensional chemoinvasion assay.** To determine the influence of nicotine on the capability of SCLCs to migrate through the ECM and reach the lymphatic endothelium, we used Boyden chambers of 8-μm pore size (BD Biosciences). The bottom chamber was seeded with 2 × 10^5 LEC cells until a confluent monolayer was established. Afterward, the upper inserts were covered with 100 μl of type I collagen (2.0 mg/ml; BD Biosciences) containing 1 × 10^5 SCLC cells (treated or not with nicotine) in basal medium supplemented with 2% FBS. Twenty-four hours later, migrated SCLC cells in the lower face of the membranes were fixed and counted as described elsewhere (Irigoyen et al., 2007).

For blocking experiments, 5 ng/ml of anti-CXCR4 antibody (MAB172; R&D Systems) was added to the upper chamber or 5 ng/ml of anti-CXCL12 (AF-310-NA; R&D Systems) blocking antibody was added to the bottom chamber 30 min before the assay.

**Cell transmigration through endothelial lymphatic monolayers.** LECs (4 × 10^5) were seeded in complete endothelial medium (EMB2; Lonza) on the upper well of Boyden chambers and allowed to form a confluent monolayer. SCLC cells (5 × 10^5) previously treated with nicotine or left untreated were added to LEC monolayers in migration medium and allowed to transmigrate for 8 h. For blocking experiments, 5 ng/ml of anti-CXCR4 antibody (MAB172; R&D Systems) was added 30 min before the assay. After that time, migrated SCLC cells on the lower chamber were counted using an ocular micrometer as described previously (Irigoyen et al., 2007).

**Tumor xenografts in athymic nude mice.** Human N417 cells (2 × 10^6) treated or not previously with nicotine (500μM) for 7 days were injected sc in 100 μl of matrigel (BD Biosciences) into the back of 4-week-old female athymic nude mice (Harlan, Barcelona, Spain). Subcutaneous tumor growth was measured transcutaneously with a caliper from day 13 to day 29, and the tumor volume was then calculated using the formula (Tumor volume = 0.5 × length × width^2). All the animals were humanely sacrificed, and tumors were extracted and fixed in 4% formaldehyde for 24 h followed by 24 h in ethanol 70%. All mice were treated in accordance with the guidelines for the Animal Care Ethics Commission of our institution (University of Navarra) under an approved animal protocol.

**Immunohistochemistry.** Formalin-fixed paraffin-embedded 3-μm tissue sections were used. Endogenous peroxidase activity was quenched, and antigen retrieval was carried out by microwave exposure for Ki-67 and caspase-3 detection or treated 30 min with concentrations of 25 μg/ml of proteinase K to achieve CD31 antigen demasking. Nonspecific binding was blocked with 5% goat normal serum in TBS for 30 min. Sections were incubated with anti-Ki-67 (1:100; Dako, Glostrup, Denmark), anti-caspase-3 (1:50; Cell Signaling, Boston, MA), anti-CXCR4 (1:50; R&D Systems), anti-CD31 (1:50; BD Biosciences), and anti-podoplanin (1:50; R&D Systems) overnight at 4°C. Sections were then incubated with the Envision polymer (Dako) for 30 min at room temperature to increase signal intensity. Peroxidase activity was developed with diaminobenzidine and counterstained with hematoxylin before being mounted in DPX mounting medium (BDH Chemical, Poole, UK). Negative controls were carried out by omission of the primary antibody.

For immunostaining evaluation, the following criteria were applied: The immunohistochemical staining for Ki-67 and caspase-3 was evaluated as the percentage of cancer cells with nuclear immunoreactivity counting 10 representative light microscopic areas per section (magnification, ×20) and represented as the mean ± SE. CXCR4 staining extension was scored as percentage of positive cells (0–100%), and the intensity of staining was assessed as 1+, mild; 2+, moderate; and 3+, intense labeling. A final score (0–300) was established by multiplying the percentage of labeled cells and the intensity of staining.

Finally, the presence of lymphatic vessels was expressed as the mean number of microvessel counts in three fields of maximum vessel density (magnification, ×20).

**Statistical analysis.** For determining statistical significance, Student’s t-test was used. When more than two groups were compared, ANOVA test followed by pairwise orthogonal contrasts was used. All data are presented as mean ± SE. Data were considered significant when p < 0.05. Finally, for qualitative determinations of CXCR4 staining in which an arbitrary score was used, the Kruskal-Wallis and U-Mann-Whitney tests were applied.

**RESULTS**

**Recurrent Nicotine Treatment Induced Morphological Changes in N417 Cells in an nAChR-Dependent Manner**

In order to study the influence of repetitive nicotine exposure on SCLC cells, we determined the effect of a 1-week treatment with several doses of nicotine, administered daily, on the SCLC-derived cell line, N417. Nicotine treatment resulted in increased adhesion and differentiation toward a neuronal-like phenotype. Already at day 4 of treatment, nicotine-treated cells had adhered to the substratum and extended filopodia. The effects observed were dose dependent, with maximal cellular elongation occurring at nicotine concentrations as high as 1μM daily during a week. Importantly, these effects were reversible, and N417 cells returned to their nonadherent clustered appearance when nicotine was eliminated from the culture media (Fig. 1A).

Moreover, using real-time PCR, we detected a highly significant increase in the mRNA expression of β2-nAChR in nicotine-treated N417 SCLC cells (Fig. 1B). We also phenotyped these changes by immunocytochemical analysis of the expression of the neuronal markers NCAM, Nef-M, and the transcription factor Neu-N in nicotine-treated and nontreated cells. Neu-N and Nef-M expression levels were significantly induced in differentiated cells, and NCAM expression was localized only on the surface of differentiated cells (Fig. 1C). Because nicotine-differentiated cells developed long filopodia, we used F-actin...
staining to determine whether they also had changes in their actin cytoskeleton and found that long F-actin filaments were distributed along the cytoplasm in nicotine-differentiated N417 cells (Fig. 1C). To our knowledge, this is the first time that a direct association between nicotine exposure and phenotypical changes in SCLC cells has been described.

Importantly, these effects were reversible, and N417 cells returned to their nonadherent clustered appearance when nicotine was eliminated from the culture media (Fig. 2). Besides, in order to ascertain if the effects observed were nicotine dependent, N417 SCLC cells were pretreated 30 min before the addition of nicotine with a cocktail of nAChR inhibitors, specifically the nAChR antagonists mecamylamine (α3-nAChR), DHBE (β-), and αBTX (α7-nAChR), resulting in nondifferentiated but viable cells (Fig. 2). This confirmed that the morphological changes observed in nicotine-treated SCLC cells were because of nicotine action through the nAcChRs.

As nicotine concentrations in the bronchial mucosa immediately after smoking can be 0.5–1 mM (Hukkanen et al., 2005), for the next experiments, we used 0.5 mM nicotine.

Nicotine Treatment Induced an Increase in Anchorage-Independent Growth, Adhesion to ECM Proteins, and Chemotactic Activity of N417 SCLC Cells

One of the main features of a tumor cell is its ability to dedifferentiate and, therefore, bypass all the lineage-specific growth controls. The finding of a trend toward a differentiated phenotype of the N417 SCLC cells after nicotine exposure led us to wonder if nicotine was detrimental or beneficial for tumor growth. Therefore, we determined if nicotine treatment of the N417 cells induced variations in anchorage-independent cell growth, adhesion to the ECM, and cell migration toward chemotactic factors.

Daily nicotine treatment considerably increased the number of colonies formed by N417 cells compared with that of untreated cells (Fig. 3). Notably, this increase was abrogated when nAChRs were blocked before the addition of nicotine (Fig. 3A). Changes in the adhesion of N417 cells to different ECM proteins were evident in nicotine-differentiated cells; there was a significant increase in the adhesion to collagen types I and IV, fibronectin, and laminin (Fig. 3B). Adhesion of nicotine-differentiated cells to the ECM returned to normal levels once the cells were cultured for 7 days in complete cell culture media depleted of nicotine, proving, again, the reversible effect of this drug on SCLC cells.

We also addressed whether nicotine exposure was relevant for the acquisition of the migratory properties of SCLC cells. We addressed this question using a Boyden chamber assay with 10% FBS complete medium as the chemoattractant. The results obtained demonstrated that nicotine treatment augmented the migratory capacity of N417 cells in an nAChR-dependent way (Fig. 3C). The results from these experiments point to nicotine as a promoter of a more malignant phenotype.

Nicotine Treatment Increases N417 Resistance to Chemotherapy

Nicotine has been recently studied for its capacity to inhibit apoptosis induced by chemotherapeutic agents such as cisplatin or taxol in non–small cell lung cancer (NSCLC) cells (Tsurutani et al., 2005). Taking this into account, we examined whether repetitive exposure to nicotine of N417 cells could promote an enhanced protection against apoptosis induced by cisplatin, mytomycin, etoposide, and taxol, which are all widely used drugs in the treatment of SCLC (Hann and Rudin, 2008). In fact, nicotine-treated N417 cells showed increased viability and a higher IC50 for all the drugs assayed, with more prominent effects observed for cisplatin and etoposide (Fig. 4A). We also tested whether nicotine-differentiated cells starved from nicotine for a week maintained their resistance to chemotherapy. Results in Supplementary figure 1 show...
a significant decrease in their resistance of N417 cells to etoposide once deprived of nicotine for a week.

Additionally, to provide a biochemical basis for these results, we used Western blot analyses to measure Poly ADP (Adenosine Diphosphate)-Ribose Polymerase (PARP) cleavage in etoposide-treated and nontreated N417 SCLC cells that were either nicotine differentiated or not. As expected, following etoposide exposure, there was a diminution of PARP cleavage in the nicotine-treated cells compared with nondifferentiated cells (Fig. 4B). To further study if nicotine treatment conferred advantageous properties to tumor cells, we determined if nicotine-treated SCLC cells produce higher amounts of VEGF, a well-described proangiogenic and prosurvival growth factor (Byrne et al., 2005). Interestingly, nicotine-differentiated N417 cells produced increased amounts of VEGF compared with the non–nicotine differentiated cells (Fig. 4C).

Nicotine-Differentiated Cells Transmigrate More Intensely Across Monolayers of LECs in a CXCR4-Dependent Way

Compelling experimental evidence demonstrates that the mechanism used by cancer cells to metastasize is similar to those that regulate the trafficking of normal cells through the endothelium. In all these processes, chemokine-chemokine receptor interactions are of critical importance (Ruffini et al., 2007). First, we determined if nicotine-treated N417 SCLC cells had changes in the mRNA expression of the chemokine receptor CXCR2 that binds to CXCL5/Ena-78, interleukin (IL)-8, or GRO gamma; CXCR3 a receptor for CXCL9/MIG, CXCL10/IP-10, and CXCL11/ITAC, CXCR4 and CXCR7 both receptors for the chemotactic factor CXCL12. The results showed that the expression was altered for all the receptors except CXCR7, which is an alternative receptor for CXCL12 (Supplementary fig. 2). Because CXCR4/CXCL12 is the most important axis involved in NSCLC metastasis, we next examined if this chemokine signaling pathway was involved in the metastatic potential of nicotine-differentiated cells. Therefore, we first determined the expression of CXCR4 on the surface of nicotine-treated and untreated N417 cells by flow cytometry. We observed a prominent increase in the expression of CXCR4 on the surface of nicotine-treated cells, and this increase was partially impaired by treating cells with nAChR inhibitors or by depleting nicotine from the cell growth media (Fig. 5A).

LEC s are one of the main conduits for the tumor cells to metastasize. This endothelium has been described by us and others to produce CXCL12 (Irigoyen et al., 2007 and
Nicotine Induced SCLC Tumor Growth In vivo

To determine if nicotine exposure induced a more aggressive phenotype in vivo, we inoculated 10^6 control or 7-day nicotine-treated N417 SCLC cells sc into nude mice. Tumor growth was visually inspected every other day. Once the tumors were established, we extracted them and immunohistochemically analyzed their proliferation (Ki-67), apoptosis (anti-cleaved caspase-3), global vessel counts (anti-CD31), lymphatic vascularization (anti-podoplanin), and CXCR4 expression using specific monoclonal antibodies. As summarized in Figure 6, nicotine-differentiated cells formed bigger and faster growing tumors than their nontreated counterparts.

Immunohistochemical analyses of cell proliferation using Ki-67 expression demonstrated that nicotine treatment did not alter proliferation as Ki-67 expression was similar between the nicotine-treated and nontreated cells (Fig. 7A). In contrast, the percentage of cells positive for caspase-3, a marker for apoptotic cells, decreased significantly in the tumors obtained from the nicotine-treated cells (Fig. 7B). Additionally, global tumor vascularity, as determined by CD31 expression, was significantly higher in those tumors derived from mice inoculated with nicotine-treated cells (Fig. 7C), although no significant increase in the number of lymphatic vessels was found (Fig. 7D). Therefore, the increases observed in vascular density may be because of higher blood capillary density and could be a direct consequence of the augmentation observed in vascular endothelial growth factor-A production by nicotine-treated cells (Fig. 4C). We could not detect any tumor cells inside any of the lymphatic vessels analyzed. Finally, we evaluated CXCR4 expression in the nicotine-derived and non-nicotine derived tumors and found a dramatic increase in CXCR4 expression in the tumors treated with nicotine (Fig. 6E).

These findings provide in vivo data supporting the role of nicotine in the increased chemotactic activity of tumor cells.

DISCUSSION

It has been clearly established that patients with SCLC who cease smoking before chemotherapy or radiotherapy have higher response rates and longer survival curves than those who do not (Chen et al., 2010; Videtic et al., 2003). Moreover, smokers who continue smoking after diagnosis have additional health risks, which include the development of a second tobacco-related malignancy, possibly because of field carcinogenesis effects (Rubinstein, 1990). Smoking also produces impairment of vasodilation, potentially affecting tissue oxygenation and repeatedly exposed to nicotine displayed increased transmigration in a manner that was also dependent on the availability of CXCR4 on their cellular surface (Fig. 5D).

As all the in vitro results demonstrated a more aggressive phenotype for SCLC cells that had been repeatedly exposed to nicotine, we next addressed this idea in vivo.
local delivery of drugs (Conklin et al., 2009). Additionally, nicotine induces changes in natural killer–cell activity and cell-mediated immunity, both of which are linked to accelerated tumor progression (Lu et al., 2007).

Recently, research into the molecular mechanisms through which continued smoking affects SCLC progression pointed to the activation of the Akt pathway in the tumor cells as the leading underlying prosurvival mechanism (Tsurutani et al., 2005). In this respect, it was established that nicotine contributes to cell survival through the activation of the PI3K/Akt pathway that directly phosphorylates and inactivates the proapoptotic function of Bax and the mitochondrial apoptotic pathway (Xin et al., 2007). Nicotine also promotes anti-apoptotic signals through nuclear factor kappa B upregulation (Marrero and Bencherif, 2009), and it has been suggested that survivin and X chromosome-linked inhibitor of apoptosis play key roles in the anti-apoptotic activity of this component of tobacco (Dasgupta et al., 2006).

The biologic effects of nicotine are mediated by nicotine acetylcholine receptors (Di Matteo et al., 2007). Interestingly, differences in the expression of nACHRs between smokers and non-smokers have been reported (Zhang et al., 2009). Furthermore, chronic stimulation of nACHr by nicotine in smokers increased the proliferation of neuroendocrine cells and induced lung neuroendocrine tumors in animal models (Schuller et al., 2000). In agreement with these findings, we have observed that repeated treatment of SCLC cells with nicotine leads to changes in the mRNA expression of nACHr. In fact, we show that nicotine exposure induces a neuronal-like appearance in N417 cells along with increased adhesion to several ECM proteins, especially to fibronectin. We had previously observed similar phenotypical changes in lung primary epithelial cells exposed to a similar nicotine dose (Martínez-García et al., 2008). In addition, we also

![image](https://example.com/image1.png)

**FIG. 5.** Nicotine-differentiated cells display higher CXCR4 expression and increased chemotaxis, adhesion, and transmigration toward LEC. (A), Flow cytometric analyses of CXCR4 expression on the surface of untreated N417 cells (Control), nicotine-treated N417 cells (Nicotine, 7 days, 500µM), cells pretreated with nACHRs inhibitors (1+Nic) an hour before nicotine addition and cells differentiated for 7 days and allowed to dedifferentiated for another 7 days in nicotine-depleted medium (Nic + w/o). Gray histograms correspond to the isotype control. One representative experiment of three is shown along with its mean intensity. (B) Cell invasion through collagen gels was measured in Boyden chambers using LEC seeded on the bottom well as a chemoattractant. Control: untreated cells, Nic: nicotine exposed (7 days, 500µM). When indicated, anti-CXCR4 antibody (5 ng/ml) was added to SCLC cells an hour before seeding them, or anti-CXCL12 antibody (5 ng/ml) was added to the bottom chamber media 1 h before the experiment was started. (C) Cell adhesion of calcine-labeled N417 cells to LEC monolayers. Anti-CXCR4 antibody (5 ng/ml) was added to SCLC cells an hour before the adhesion experiments. (D) Transmigration of nicotine-treated (Nic) or untreated (Control) N417 cells through monolayers of LEC. Anti-CXCR4 or anti-CXCL12 antibodies were used for blocking experiments as in (B). Comparisons between control and treated cells were made using an ANOVA test, **p < 0.001 compared against non-treated cells, ##p < 0.001 compared against isotype.

![image](https://example.com/image2.png)

**FIG. 6.** *In vivo* xenotransplanted nicotine-treated N417 SCLC cells produced bigger tumors than nontreated cells. Tumor size was measured as $0.5 \times$ length $\times$ width$^2$. Growth rates were significantly increased in tumors transplanted with nicotine-treated N417 cells compared with nontreated tumors. Comparisons between tumor growth of control and treated cells were made using an ANOVA test, **p < 0.001.
demonstrated that prolonged nicotine treatment of SCLC cells makes them increasingly resistant to chemotherapeutic agents, such as etoposide, cisplatin, mitomycin and taxol.

There are reports in the literature that associate Akt-dependent tumor survival with integrin-dependent cell adhesion to the ECM (Ritzenthaler et al., 2008). This same mechanism may well be at work in our system. Significantly, all effects observed in our cells were abrogated when nAChR inhibitors were added or nicotine was depleted from the cell growth media. These results support the recent emphasis on the importance of developing nAChR inhibitors to efficiently treat lung cancer in active smokers (Paleari et al., 2009).

The morphological changes described in nicotine-treated N417 SCLC cells were also accompanied by an enhanced migratory activity in vitro. Invasion and metastasis are complex multistep processes that involve alterations in cell adhesion to ECM proteins as well as migration to surrounding tissues in order to reach the circulatory system and metastasize to distant organs. In these processes, it is believed that metastatic cancers co-opt signals that normally control leukocyte trafficking, mainly chemokine-mediated cell migration (Rollins, 2006; Ruffini et al., 2007). Among the chemokines involved in cell migration, CXCR4 and its natural ligand, the chemokine stromal cell–derived factor-1 (SDF-1/CXCL12), have been consistently reported as key mediators that regulate tumor migration and metastasis in a variety of cancers (Gelmini et al., 2008). Furthermore, it has been widely described that SCLC cells express high levels of functional CXCR4, and its activation by binding to CXCL12 leads to the upregulation of integrin-mediated adhesion of SCLC cells to the ECM and to endothelial cells (Burger et al., 2003; Hartmann et al., 2004).

Consequently, in the present study, we observed that nicotine treatment increased the expression of several CXC chemokine receptors on SCLC cells. We observed increased expression of CXCR2 receptor in nicotine-treated N417 cells. This chemokine receptor binds to the Glu-Leu-Arg (ERL+ CXC chemokines, CXCL3/GRO gamma, CXCL5/Ena-78, and CXCL8/IL-8, all of them being potent angiogenic factors (Keane et al., 2004)). The expression of the chemokine receptor CXCR3 also resulted elevated. This cell surface molecule binds to its ligands CXCL9/MIG, CXCL10/IL-10, and CXCL11/ITAC1, all of them are chemotactic factors for neutrophils and T cells, have been related with increased metastasis (Walser et al., 2006), and contribute to tobacco-induced inflammation (Luppi et al., 2007). More importantly, we observed that the increase in CXCR4 expression on the cell surface was dependent on the activation of nAChRs, providing a direct link between nicotine exposure and increased migration of SCLC cells. Additionally, nicotine-treated cells migrate more strongly toward SDF-1/CXCL12 produced by stromal cells, such as LEC, which we and others have described previously (Burman et al., 2005; Schober, 2008).

The lymphatic endothelium has been of great interest recently as an important route for the spread of cancers toward
the lymph nodes and, from the nodes, to distant organs. These vessels are endowed with structural features that make them amenable for tumor cell spread (Stacker and Achen, 2008).

Here, we also show evidence for increased chemotactic migration, adhesion, and transmigration of nicotine-treated N417 SCLC cells through monolayers of LECs. Furthermore, we show that these new features are dependent on both the activity of CXCR4 on tumor cells and the secretion of CXCL12 by the lymphatic endothelium. Interestingly, high amounts of CXCL12 are widely expressed in the lymph nodes, which are the primary sites to which lung tumors metastasize (Uchida et al., 2003). Therefore, higher expression of CXCR4 on the surface of tumor cells makes them more responsive to lymph node–derived chemotactic cues. A sound outcome of these facts is the abundant and still growing number of preclinical models showing how in vivo neutralization of the CXCR4/CXCL12 axis results in the inhibition or attenuation of metastases (Otsuka and Bebb, 2008). In this study, we present data to support the use of CXCR4 and/or CXCL12 inhibitors as adjuvants in the treatment of SCLC in patients who continue smoking after diagnosis.

The particular role of CXCR4 in lung cancer development is not restricted to fostering the metastatic potential of the tumor. Interestingly, Hartmann et al. (2005) reported that adhesion of SCLC cells to the ECM or to accessory cells within the tumor microenvironment confers resistance to chemotherapy via the activation of the CXCR4/CXCL12 axis. This provides a link between two of the most prominent effects observed in this work on nicotine-treated SCLC cells, namely increased chemoresistance and migration.

The results obtained by in vitro analyses were supported by the in vivo findings of more vascularized and less apoptotic tumors in mice that had been inoculated with nicotine-differentiated cells compared with the animals that were given nontreated cells. The higher CXCR4 expression seen on the nicotine-treated tumors may well be indicative of a more aggressive phenotype. Animal models other than the matrigel–loaded cells should be used to further investigate this point.

Taken together, these results provide strong in vitro and in vivo data supporting the importance of the CXCR4/CXCL12 axis in the reduced response to chemotherapy and worse prognosis of patients diagnosed with lung cancer who cannot stop smoking.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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