Nicotine reduces effectiveness of doxorubicin chemotherapy and promotes CD44$^+$CD24$^-$ cancer stem cells in MCF-7 cell populations

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Abstract. Breast cancer is the most common type of cancer in females and the second most common cause of cancer mortality after lung cancer. Cancer stem cells represent a novel approach to target cancer and reduce cancer recurrence and metastasis. Many patients with breast cancer continue to smoke after receiving their diagnosis. Nicotine is a key factor in tobacco addiction and also changes some cellular functions, such as activation of mitogenic pathways, angiogenesis and cell proliferation. In the present study, the impact of nicotine was assessed in a population of MCF-7 human breast cancer cells. Cluster of differentiation (CD)44$^+$CD24$^-$ cancer stem cell population of MCF-7 cells were evaluated using flow cytometry and scanning electron microscopy. Chemoresistance effects of nicotine were demonstrated in these cells. These findings demonstrated harmful effects of nicotine following metastasis of cancer, owing to the chemoresistance produced through uninterrupted smoking, which may impact the effectiveness of treatment.

Introduction

Breast cancer has a high incidence and poses a notable threat to human health, particularly in females (1). The worldwide recurrence and metastasis rate in patients with early breast cancer is estimated to be ~30% (1). Breast cancer metastasizes to the lungs, bone, liver and brain (2). Metastasis is a complex process that relies to a great extent on the interaction between primary tumors and the tumor-associated host stromal tissue (2). Surgical procedures, radiation-, endocrine- and/or chemotherapy are required for the treatment of the disease (3).

A frequently used chemotherapeutic drug in breast cancer is doxorubicin, which inhibits the replication of DNA by intercalating into and inhibiting the enzyme topoisomerase II. In doing so, the cell cycle stops and the cancerous cells undergo apoptosis (4). However, some types of breast cancer are known to develop resistance to anticancer agents such as doxorubicin (5). An increased risk of breast cancer development has been associated with cigarette smoking in epidemiological and clinical studies in which smokers and non-smokers were compared (5-7). Of the 4,000 components in cigarette smoke, the most important and effective is known to be nicotine (2) for breast cancer.

Long-term smoking of tobacco clinically contributes to the development of cardiovascular disease and also to the development of cancer (8). While smoking, 80-90% of inhaled nicotine (~1.0 mg per cigarette) is systemically absorbed (9). Nicotine is thought to have a role in the formation of various types of cancer and has been demonstrated to increase angiogenesis in previous proliferation model studies (10-15).

Cancer stem cells (CSCs) have become an important topic in oncology research. Malignant cancer stem cells were first identified and isolated from solid breast cancer tumor (16). Human cell surface antigen, cluster of differentiation (CD24), is a marker for normal mammary stem cells; however, when combined with other markers, CD24$^-$ breast cancer cells have the greatest tumor-initiating potential and may be successfully identified in stem cell populations (17).

Breast cancer stem cells are different from other breast cancer cells with regards to volume, size, membrane components, antigen expression, proliferation and metastasis (18). In the cancer stem cell model, a small group of tumor cells are responsible for cancer formation, progression and recurrence (11). A study by Honeth *et al* determined that the CD24 and CD44 cell surface proteins are putative markers for cancer stem cell populations in breast cancer (18).

In a previous study, breast CSCs were enriched in the minority fraction of CD44$^+$CD24^{low} lineage cells, with as few as 100 CD44$^+$CD24^{low} cells able to initiate tumor growth

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in immunocompromised mice (19). A study by Al Hajj *et al* was the first to isolate CSCs from human breast cancer. They prospectively identified and isolated the tumorigenic cells as CD44(+) CD24 (-/low) lineage (-) in 8/9 patients.

CSCs are hypothesized to be a subset of tumor cells with stem cell-like features that have the ability to self-renew and differentiate, which causes a heterogeneous tumor cell population (20). This variability led to diverse results in clinical studies (21). Previous studies reported that $CD44^{+}CD24^{-}$ breast CSCs enhance breast tumor cells because of their angiogenic potential (20-25).

The effects of daily exposure to chemical agents may be determined at a cellular level with advanced imaging techniques, including positron-emission tomography (26). These analyses of cancer cells and biotechnological advances are opening new horizons of molecular oncology and also provide major advances in our understanding of oncology.

Breast cancer is managed through surgical treatment, radiation therapy, endocrine therapy and/or chemotherapy (21). Resistance to chemotherapy is a challenge for the successful cure of several types of cancer; breast cancer has evolved resistance to a number of anticancer agents, such as doxorubicin (23,24). There is limited experimental data that supports direct links between breast cancer and exposure to nicotine. The present findings demonstrated the harmful effects of nicotine following metastasis of cancer due to the chemoresistance produced through uninterrupted smoking, which may impact the effectiveness of treatment.

Materials and methods

Cell culture and treatment. The human breast cancer cell line MCF-7 (American Type Culture Collection, Manassas, VA, USA) used in the present study was grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories; GE Healthcare, Chicago, IL, USA), 2 mM glutamine, 10 U/l penicillin and 100 μg/ml streptomycin (all Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were cultured in a humidified incubator with a 37˚C in an atmosphere containing 5% CO_2.

For nicotine treatment, cells were washed twice with phosphate-buffered saline (PBS), dissociated with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and seeded near confluence ($2x10^5$ cells/well) in 6-well plates. Cells were cultured in complete medium at 37˚C in a 5% CO_2 incubator for 24 h prior to nicotine treatment (Sigma-Aldrich) at concentrations of 0.01, 0.05, 0.1, 1 and 10 μM at the same time to the wells.

Cell diameter. MCF-7 cells were analyzed with an Vi-Cell XR cell viability analyzer (Beckman Coulter, Inc., Brea, CA, USA). MCF-7 cell diameters were measured 24 and 48 h after treatment with nicotine using the Vi-Cell XR cell viability analyzer.

Scanning electron microscopy (SEM). MCF-7 cells were plated on 6-well plates ($7x10^3$ cell/well). The cells were cultured in DMEM (Clonetics; Lonza Group, Ltd., Basel, Switzerland), which consisted of 5% FBS, 0,1% penicillin strep. MCF-7 breast cancer cells were centrifuged at 1600 x g. Cells were collected and prepared in accordance with the method of Groebel *et al* (27) and analyzed with an FEI Quanta 450 FEG-EDS scanning electron microscope (Thermo Fisher Scientific, Inc.).

Transmission electron microscopy (TEM). MCF-7 breast cancer cells were centrifuged at 1600 x g, 5 min at room temperature after treatment trypsin-EDTA. Cells were collected and prepared in accordance with the method of Groebel *et al* (27) and analyzed using a Philips CM100 transmission electron microscope (Philips Medical Systems, Inc., Bothell, WA, USA).

CSC analysis by flow cytometry. Allophycocyanin (APC)-conjugated mouse anti-human CD44 monoclonal antibody (cat. no. BD 559942) and phycoerythrin/cyanine 7 (PE/Cy7)-conjugated mouse anti-human CD24 monoclonal antibody (cat. no. BD 561646) were purchased from BD Pharmingen (BD Biosciences, Franklin Lakes, NJ, USA). CD24 and CD44 expression was analyzed in cells derived from monolayer cultures following dissociation in trypsin-EDTA at 37˚C. At least $1x10^5$ cells were pelleted by centrifugation at 500 x g for 5 min at 4˚C. Cells were washed in PBS, resuspended with anti-CD24-PE/Cy7 (1:20 dilution) and anti-CD44-APC (1:20 dilution); samples were incubated for 30 min at 4˚C in the dark. The labeled cells were washed using PBS and analyzed using a BD FACSAria II flow cytometer and software DiVa (BD Biosciences). The negative fraction was determined using appropriate isotype controls.

Actin immunostaining. MCF-7 breast cancer cells were plated onto poly-D-lysine-coated 6-well glass chamber slides (7,000 cells/well) and incubated at 37˚C in a 5% CO_2 incubator for immunostaining. After 24 h incubation, cells were treated with 0.01, 0.05, 0.1, 1 or 10 μM nicotine for the indicated time points (24 and 48 h). Immunofluorescence microscopy was used to determine F-actin organization in MCF-7 breast cancer cells. The cells were fixed in 3.5% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized and blocked in PBS containing 0.1% Triton X-100 and 5% FBS for 30 min at room temperature. Actin filaments were visualized using rhodamine-phalloidin label, and nuclei were stained with DAPI (Invitrogen; Thermo Fisher Scientific, Inc.). All images were obtained using an Olympus BX51 Microscope equipped with a DP72 camera, controlled by Olympus DP2-TWAIN software (ver7.2; Olympus Corp., Tokyo, Japan) (28).

Doxorubicin resistance. A final doxorubicin (Sigma-Aldrich; Merck KGaA) concentration of 2.5 μg/ml was used in the present study. Cells were divided into a total of six groups (doxorubicin concentrations were 0.5, 1, 2, 4 or 8 μM) and in the control group MCF-7 cells received no treatment with doxorubicin. MCF-7 breast cancer cells were shaken every 15-20 min during their incubation with doxorubicin at 37˚C for 1 h. The cell viability test was performed using trypan blue (29).

Statistical analysis. Statistical analysis was performed and data were presented as the mean ± standard deviation, For

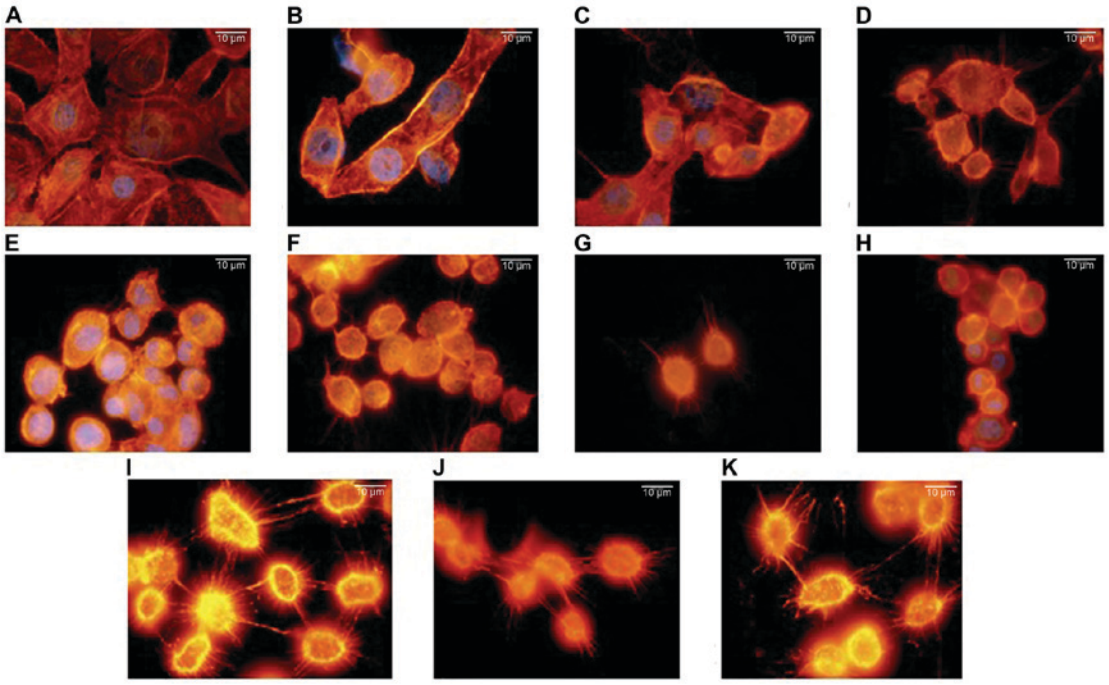

Figure 1. Effect of nicotine treatment on the actin cytoskeleton. MCF-7 cells were incubated in the presence of nicotine: (A) 0 μM (control) (24 h), (B) 0.01 μM (24 h), (C) 0.05 μM (24 h), (D) 0.1 μM (24 h), (E) 1 μM (24 h), (F) 10 μM (24 h), (G) 0.01 μM (48 h), (H) 0.05 μM (48 h), (I) 0.1 μM (48 h), (J) 1 μM (48 h) or (K) 10 μM (48 h). Actin filaments were visualized using rhodamine-phalloidin and nuclei were stained with DAPI for all conditions. Scale bar=10 μm (magnification, x100).

comparisons of two normally distributed groups, a Student t-test was used. All statistical analysis was performed using SPSS software for Windows (version 21; IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of nicotine on actin cytoskeleton in MCF-7 breast cancer cells. Lamellipodia formation on filamentous actin was investigated using fluorescence microscopy, in order to evaluate whether nicotine increases the metastatic potential in breast cancer (Fig. 1). MCF-7 cells appeared to have a diamond or polygonal shape. The F-actin filaments of the MCF-7 breast cancer cells treated with nicotine were altered to a rounded form with a smaller diameter. This effect was more pronounced as the concentration and duration of nicotine treatment increased.

Effect of nicotine on cellular surface morphology and ultrastructure. The surfaces of the nicotine treated MCF-7 cells (grown in DMEM with 5% FBS) as viewed under SEM were almost uniformly covered with an increased dense network of microvilli (Fig. 2A), whereas nicotine-treated cells typically exhibited an increase in the density and length of surface microvilli (Fig. 2). The SEM results supported our hypothesis in which an elevated number of filopodia were identified with a higher extensively in the nicotine-treated groups.

Nicotine increases the $CD44^+CD24^-$ cell population in MCF-7 cells. To investigate whether nicotine affects the size of a CSC population, MCF-7 human breast cancer cells were characterized using flow cytometry for surface expression of CD44 and CD24, which is used for the identification of CSCs. As demonstrated in Fig. 3, stimulation with 0.01, 0.05, 0.1, 1 or 10 μM nicotine increased the ratio of CSCs. Treatment with 1 μM nicotine after 24 h, increased $CD44^+CD24^-$ cells (~1.6-fold), suggesting that the effect of nicotine is due to the proliferation of $CD24^-$ cells. As demonstrated in Fig. 3, the effect of nicotine was observed in a dose-dependent manner until 1 μM nicotine treatment, after which the cell population was decreased at 10 μM, which indicated that maximal effects were achieved at a concentration of 1 μM. To confirm whether nicotine increases the CSC population, expression patterns of CD24 and CD44 in MCF-7 cells were analyzed by flow cytometry were determined. The results suggested that nicotine increased the proportion of CSCs in the cell population (Fig. 3B). Smaller changes between 24 and 48 h in cell diameter measurements were observed in nicotine-treated groups compared with controls (Fig. 4).

Nicotine treatment promotes cell viability during doxorubicin exposure. It would be beneficial to determine the specific compounds within cigarettes that may be responsible for promoting drug resistance because cigarettes contain a mixture of compounds. Therefore, the present study aimed to determine whether nicotine, a specific component of cigarettes, may be responsible for chemotherapy drug resistance. Trypan blue cell counting was performed to compare survival curves between control cells and 1 μM nicotine-treated cells in the presence of increasing doxorubicin concentrations (Fig. 5. The results demonstrated that nicotine significantly increased MCF-7 cell viability during exposure to 0.5, 2, 4 or 8 μM doxorubicin ($P<0.01$).

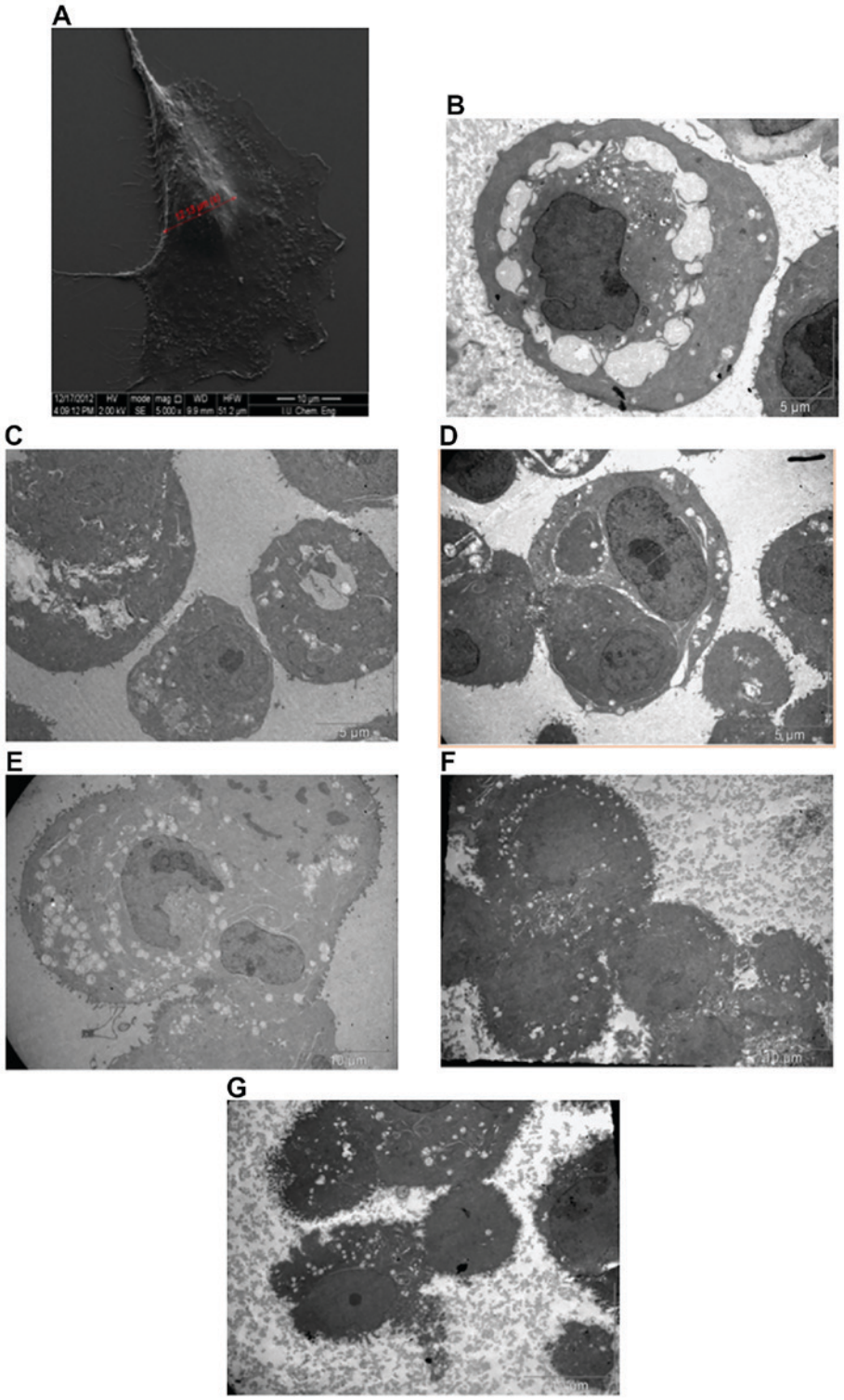

Figure 2. Effect of nicotine on cell surface morphology and ultrastructure. (A) Scanning electron micrograph of a MCF-7 cell treated with 1 μM nicotine for 48 h showing the direction of progress. Scale ba=10 μm (B-G) Transmission electron micrographs of MCF-7 breast cancer cells. (B) Control group (24 h): The spheroid cells typically generated pseudolumen. Scale ba=5 μm (C) 1 μM nicotine (24 h) group: Microvillus appendixes could be seen less frequently compare with the control group on the surface of cells. Scale bar=5 μm (D) 10 μM (24 h) group: Nucleolar organizing centers in the nucleoli were prominent. Scale bar=5 μm (E) Control group (48 h): Narrowing and closure in the pseudolumens of spheroid cells. Scale bar=10 μm (F) 1 μM (48 h) group: Cytoplasm/nucleus ratio decreased and protrusions on the surface of the cells increased. Scale bar=10 μm (G) 10 μM (48 h) group: Disintegration on the surface of the spheroid cells and microvillus transformation. Scale bar=10 μm (magnification, x100).

Discussion

Nicotine and its derivatives are well documented for their role in addiction and wide range of effects in cancer. Nicotine stimulates the cellular and molecular pathways in carcinogenesis throughout the gastrointestinal tract (28). Furthermore, nicotine affects the function of numerous systems in the human body (28). Nicotine is an established cause of numerous

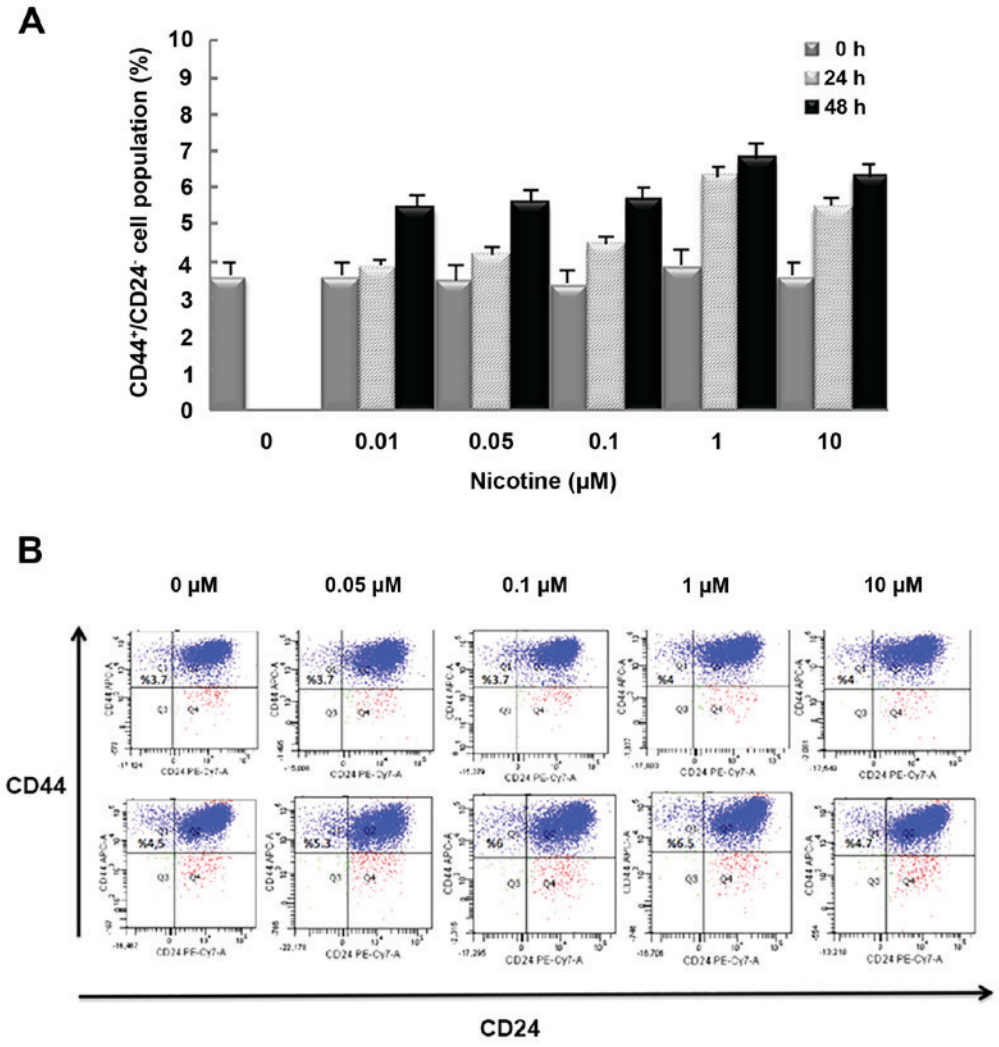

Figure 3. Effect of various concentrations of nicotine on $CD44^+CD24^-$ cell population. (A) Percentage of stem cells ($CD44^+/CD24^-$) in the MCF-7 cell population was determined by flow cytometry analysis. (B) Expression patterns of CD24 and CD44 in MCF-7 cells were analyzed by flow cytometry for 24 and 48 h. Anti-CD24 antibody labeled with PE-Cy7 and anti-CD44 antibody labeled with APC were applied to the analysis. Gates are based on the isotype control corresponding to each cell line. PE-Cy7, phycoerythrin-cyanine 7; APC, allophycocyanin; CD, cluster of differentiation.

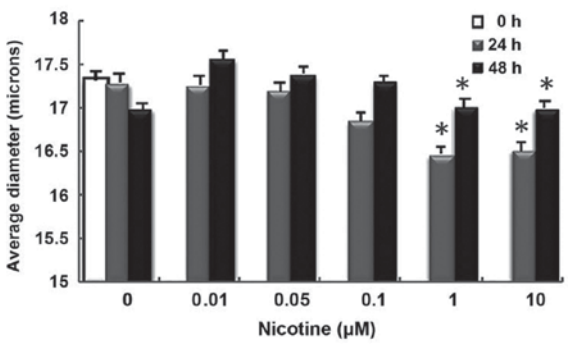

Figure 4. Effects of nicotine treatment on diameter. Average diameter of MCF-7 cells 48 h following the application of nicotine. Cell diameter of MCF-7 was significantly decreased compared with untreated cells after 24 and 48 h exposure at 1-10 μM nicotine. *P<0.05 as compared to the control group (0 μM nicotine).

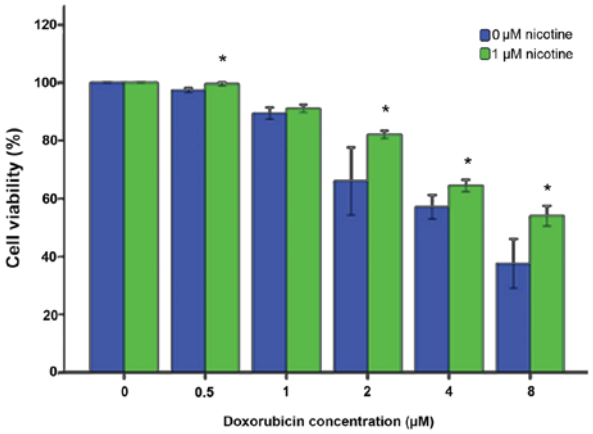

Figure 5. Effect of nicotine on the viability of MCF-7 cells during doxorubicin exposure. *P<0.01 vs. control (0 μM nicotine).

cancer types (10-13); however its role in breast cancer etiology is unclear. In the present study, the potential effects of nicotine in human breast cancer cells were evaluated.

In vivo studies have provided evidence of direct actions of nicotine, known as the dependency-inducing ingredient of cigarettes, regarding tumor development by increasing

cell proliferation and interfering with apoptosis (13-15). It is known that, aside from tumor development, nicotine also causes changes in the human immune system (30-33). Heeschen *et al* (13) has demonstrated proliferation, membrane transport, metabolic and hormonal changes in nicotine induced cells.

Nicotine in plasma was previously reported to be between 10 nM and 10 μM in smokers (33). Therefore, in the present study, these nicotine concentrations were used in accordance with previous reports. Nicotine is taken into the body by chewing nicotine gum, by sniffing tobacco or sucking it into the mouth through the use of cigarettes. Plasma concentrations of nicotine rise slowly, reach a peak level after 30 min and do not fall during the following 2 h (34). In nicotine gums, nicotine is secreted as the gum is chewed, therefore a complete extraction of nicotine from the gum does not occur (2 mg gum provides 53% secretion, while 4 mg gum ensures 72% secretion). In this circumstance, a significant amount of nicotine is swallowed through the mouth (34,35).

A previous study indicated that it took 15-30 min for 4-mg gum to raise the plasma nicotine by an average of 9-11 ng/ml compared with an average increase of 8-27 ng/ml within 2 min of completing each cigarette (36). Furthermore, nicotine is believed to be responsible for the occurrence and development of several cancer types, including breast cancer (37). Previous study models have also demonstrated that nicotine increases angiogenesis and proliferation (38). In the present study, when the results of the 24- and 48-h analyses of MCF-7 cells on an XR viability analyzer were evaluated between themselves, a nicotine dose-dependent increase in cell diameter was observed some changes in cell diameter measurements in the cells treated with nicotine compared with the controls. Cells were suspended with trypsin/EDTA during the sphere shape conducted using the cell XR viability analyzer.

CSCs, or tumor-initiating cells, have a major role in the onset of cancer and they have a resistance against chemotherapy and radiotherapy (39,40). In the present study, the presence of nicotine, these cells increase in number and invade, induced by changes in the dynamic values of other cells, thus providing mobility. This suggests that treatment would be hindered by nicotine intake, thereby deteriorating the treatment process. There is an increasing amount of evidence that suggests that breast cancer stem cells have a central role in the development of cancer (38). A clinical study has indicated that breast cancer metastases occur in the lungs of smokers (40). The findings of the present study suggested that in the presence of nicotine, the number of CSCs is associated with an increase in the $CD24^-$ cell population, which is active in metastasis. Like alcohol, nicotine is a facilitating risk factor in the metastasis process of breast cancer (38).

An investigation of the microcirculation of mammals has indicated that nicotine results in cell proliferation, membrane transport, metabolic and hormonal changes (41). The results of the present study are consistent with these suggestions. Gradually increasing dosages of nicotine, which were within the limits of nicotine in the blood plasma of smokers, stimulated the number of MCF-7 cancer stem cells. It was identified that 1 μM nicotine increased the number of $CD44^+CD24^-$ cells among the MCF-7 cells. The decrease in viability and ratio of $CD44^+CD24^-$ cells following treatment with 10 μM nicotine may be explained by a possible toxic effect.

Stimulation with nicotine may be the result of the increase in the percentage of $CD24^-$ MCF-7 cells, as identified by flow cytometry. Furthermore, the effects of nicotine on a CSC population were investigated with flow cytometry based on the $CD44^+CD24^-$ characteristics, and it was found that it also stimulates CSC proliferation. This finding suggested a direct relation of nicotine with disease development and progression in patients with breast cancer who smoke.

Cell morphology findings of the present study demonstrated that nicotine increased cell invasion, although the results with electron microscopy and florescent microscopy indicated the orientation of cells but did not provide quantitative information about the invasive ability of breast cancer cells without characteristics of CSCs. Flow cytometry results suggested that nicotine had a proliferation-inducing effect on the $CD44^+CD24^-$ cell population. This difference was not observed in all cells in the study areas but it was identified in higher numbers in the nicotine groups. This may be explained by the heterogeneous quality of the population. Previous research has demonstrated the effects of nicotine on cell morphology, motility and the cytoskeleton, which leads to degradation of the actin cytoskeleton and finally to cell destruction (42,43).

In the present study, re-characterization of F-actin, a basic cortical skeletal component of *in vitro* cancer models, was also identified to be among the potential effects of nicotine on breast cancer cells. Nicotine-administered MCF-7 breast cancer cells exhibited changes in cell structure, including becoming rounder in form with decreased diameters, which was in direct proportion with the increase of nicotine concentrations. Based on these results, it is hypothesized that nicotine also has a role in the regulation of cellular cascades. It is possible that sensors of cytosolic mechanisms send signals to cell membrane proteins and to the nucleus, initiating mechanical events such as clustering and interactions between the cortical cell and skeletal structure.

In the present study, actin filaments were located at the region underneath the nuclear membrane. Actin filaments were also observed to increase around the inner part of the cell membrane. Thus, the accumulation of F-actin was evaluated, which was most likely related to the cellular motility. Thus, nicotine has an effect on cell motility and associates with the metastatic process.

In the present study, as a result of administration of varying concentrations of nicotine to MCF-7 breast cancer cells, morphological changes occurred in the structure of the cytoskeleton and, at the same time, cells experienced stress depending on the concentration of nicotine. A previous study demonstrated that one of the most important obstacles in chemotherapy is the resistance that cancer cells develop against apoptosis (44). One of the current approaches in this context is to use various drugs along with anticancer medications in order to ensure sensitivity of cancer cells for chemotherapy and to break the resistance (45). Sak (44) identified that this resistance is developed in cancer patients who do not cease smoking and continue their exposure to nicotine. The results of a doxorubicin experiment, which was performed because nicotine-administered MCF-7 cells

had an increased number of stem cells and these cells were resistant to chemotherapy, it was demonstrated that MCF-7 breast cancer cells developed resistance against this chemotherapeutic agent (44).

To evaluate whether nicotine increases metastatic potential in breast cancer, lamellipodia formation on F-actin was investigated using TEM and fluorescence microscopy. In light of these parameters, the present findings regarding mobilization were confirmed by SEM and fluorescence microscopy. Although these results are considered to be associated with metastasis, they may provide a basis for further studies on cancer development.

It is necessary to determine molecular and genetic targets that would be helpful in destroying CSCs without damaging normal cells, and to detect genetic and epigenetic events that trigger cancer. To the best of our knowledge, no previous studies have been conducted on the effect of nicotine on drug resistance. Inhibition of signal pathways that are involved in the resistance mechanism of CSCs would create novel treatment possibilities and therefore further studies on signal pathways are required. In order for gene sequence analyses and molecular markers that inhibit or control the interaction of cells with nicotine to be realized in the near future, studies addressing the genetic and molecular basis of nicotine effects are required.

Resistance against doxorubicin may be explained by an increased number of CSCs due to the presence of nicotine. However, some transcriptional factors that may modulate resistance to doxorubicin may be responsible, which was based on the present result that no correlation was identified between the resistance against doxorubicin and the elevation of the stem cell-induced cancer.

In the present study, it was identified that the population of CSCs increased in the presence of nicotine in MCF-7 cells. Nicotine was also indicated to increase the proliferation of MCF-7 breast cancer cells and actin filaments. The present results have aided in the understanding of drug resistance in breast cancer cells.

In conclusion, it is believed that nicotine, which is effective in the occurrence of breast cancer, is also one of the factors that has a role in the development and metastasis of this cancer. The results of the present study suggested that smoking and other forms of long-term nicotine intake, such as through nicotine gums, electronic cigarettes and nicotine pastilles, which are used as aids for smoking cessation during the cancer process, also contribute to the mobilization of cancer cells and affect the progression and relapse of the disease.

Acknowledgements

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