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Gelsolin is a potential cellular target for cotinine to regulate the migration and apoptosis of A549 and T24 cancer cells

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ABSTRACT

In the present work, we have investigated the effect of cotinine, the major metabolite of nicotine on the A549 and T24 cell lines in the context of structural and quantitative changes of F-actin, gelsolin and vimentin. The chosen cell lines constitute the established experimental models for lung and bladder cancers, respectively, in the case of which, smoking cigarettes is one of the key factor increasing their incidence rate significantly.

In order to evaluate the impact of cotinine on the viability and proliferation of A549 and T24 cells, the MTT assay was performed. The organization and distribution of F-actin, gelsolin and vimentin were examined using conventional and confocal fluorescence microscopy. The levels of F-actin and gelsolin as well as the percentages of apoptotic and dead cells were assessed using the image-based cytometer. The ultrastructural changes of cotinine-treated A549 and T24 cells were visualized under the transmission electron microscopy.

We have shown here that cotinine enhances the survival and proliferation rate of A549 and T24 cells. We have also found that in A549 cells, but not in T24 cell line, cotinine acted stimulating on the vimentin filament network. Furthermore, the increase in the fluorescence intensity of gelsolin upon the addition of cotinine to the T24 cells was found to be correlated with the lack of apoptosis induction as well as the increase of migration potential of these cells. On the other hand, the cotinine-induced decrease in the fluorescence intensity of gelsolin was associated with the increase in the percentages of apoptotic A549 cells and the decreased migratory ability of these cells.

Based on the obtained results, we propose that the gelsolin is an important cellular target for cotinine, through which this compound influences on the basic processes involved in neoplastic transformation and metastasis, such as migration and apoptosis.

1. Introduction

Smoking is being studied as one of the most important risk factor for cancer, especially lung cancer. This type of tumor is the most common cause of cancer death worldwide and about 85–90% belongs to the non-small cell lung cancer (NSCLC) (Dobruch et al., 2010). For this reason, the A549 cell line (human lung adenocarcinoma epithelial cell line) constituted the material for this study. The second cell line used in our study was T24 cell line, because smoking is associated with over a half of bladder cancer cases in men and one-third among women. The T24 cell line was established from highly malignant grade III and invasive human urinary bladder cancer patient, belongs to urinary bladder epithelial transitional carcinoma cells (Peng et al., 2006). Furthermore, it is not nicotine, but its metabolites are a direct cause of most tobacco-related diseases. Cotinine is one of primary metabolite of nicotine. In human, the conversion of nicotine to cotinine is a two-step process. The first step is the formation of the intermediate nicotine-$\Delta^{-1'(5')}$-iminium ion catalyzed by the cytochrome P450 and next its oxidation to cotinine by cytosolic aldehyde oxidase. Cotinine is detected in the blood, urine, saliva and hair. However, the concentrations of urinary cotinine are fourfold to six fold higher than those in blood or saliva (Bao et al., 2005; Hukkanen et al., 2005; Bramer and Kallungal, 2003; Benowitz, 2009). Blood and urine cotinine induces tumor promotion by inhibiting apoptosis and enhancing cellular proliferation. Nakada et al. have shown that in A549 cells

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cotinine suppresses caspase-mediated apoptosis induced by doxorubicin through the activation of the PI3K/Akt pathway (Nakada et al., 2012). The cytoskeleton plays an important role in proliferation, apoptosis and tumorigenesis. Actin filaments are involved in cell locomotion, membrane ruffling and the formation of lamellipodia. This type of cytoskeletal protein is connected also with proliferation, apoptosis and carcinogenesis (Desouza et al., 2012; Kabsch and Vandekerckhove, 1992; Grzanka et al., 2013). It has been suggested that F-actin connected with tropomyosin isoform (Tm5NM1) regulates cell proliferation (Lees et al., 2013; Schevzov et al., 2005) and during the apoptosis actin is very important in the processes such as membrane blebbing and margination of nuclear chromatin as well (Grzanka et al., 2013). Structure and functions of actin filaments are modulated by actin-binding proteins. One of them regulate the growth and stability of actin filament the other its disassembly (Dos Remedios et al., 2003). Gelsolin is a calcium-dependent actin-binding protein that modulates actin filament organization by its severing and capping activity toward actin filaments. It has been shown that gelsolin plays an essential role in regulating the migration ability of cells (podosome formation). This protein also protects against apoptosis by stabilizing the mitochondria and inhibiting cytochrome c release (Dos Remedios et al., 2003; Sun et al., 1999). Taking into account that cotinine is a direct reason of cancer, and gelsolin is an actin binding protein which are involved in cell death processes, the purpose of this study is to present the influence of cotinine on A549 and T24 cell lines in the context of structural and quantitative changes of F-actin, gelsolin and vimentin.

2. Materials and methods

2.1. Cell culture and treatment

The human urinary bladder carcinoma cell line T24 was purchased from CLS Cell Lines Service GmbH (Germany, Eppelheime). The human non-small cell lung cancer cell line A549 was kindly provided by Dr. P. Kopinski (Department of Gene Therapy, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Poland). The T24 cells were grown in DMEM:Ham's F12 medium (1:1 mixture; Sigma–Aldrich; St. Louis, MO) and in the case of A549 cells DMEM medium was used, supplemented with 5% and 10% fetal bovine serum (FBS; Gibco, Life Technologies Corp., Carlsbad, CA, USA) respectively. Both mediums were supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich, St. Louis, MO, USA). The cells were cultured in monolayer at 37 °C in a humidified CO_2 incubator (5% CO_2).

The T24 cells were treated with 1, 10, 100, 1000, 5000 ng/ml cotinine (Sigma–Aldrich, St. Louis, MO, USA) for MTT assay and 10, 1000, 5000 ng/ml for other experiments for 24 h. In turn, A549 cells were incubated for the same period of time with 1, 10, 100, 300, 500 ng/ml cotinine for MTT assay and 10, 100, 500 ng/ml for other tests. Control cells were incubated under identical conditions, without the addition of cotinine.

2.2. MTT assay

T24 and A549 cells were cultured in 24-well plates. A stock solution was prepared by dissolving thiazolyl blue tetrazolium bromide (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide MTT; Sigma–Aldrich; Milwaukee, WI) in PBS at 5 mg/ml. This MTT solution was mixed with medium (DMEM:Ham's F12 for T24 cell line and DMEM for A549 cell line respectively) without phenol red (Lonza; Verviers, Belgium) in the ratio 1:9 and added to each culture well. Cells were incubated for 3 h in 37 °C. After this time the MTT solution was removed and the MTT formazan crystals were dissolved in isopropyl alcohol. Next, the plates were placed in a 37 °C incubator for 10 min to dissolve the precipitate. Absorbance was measured at 570 nm using the spectrometer (Spectra Academy, K-MAC, Korea). The absorbance of control cells (subjected to the same procedure) was assumed as 100% and the statistical analyses were performed by using GraphPad Prism software.

2.3. Annexin V-FITC/PI assay

Tali™ Apoptosis Assay Kit–Annexin V Alexa Fluor® 488 and Propidium Iodide (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) was used to assess phosphatidylserine externalization. The nuclei were counterstained with propidium iodide. At first, the cells were harvested using 0.05% trypsin-EDTA solution (Sigma–Aldrich, St. Louis, MO, USA) and centrifuged (1800 g, 8 min). Next, the cells were resuspended in Annexin binding buffer (ABB) and incubated with Annexin V Alexa Fluor® 488 for 20 min at room temperature in dark. After centrifugation (300 × *g*, 5 min) and the removal of the supernatant, the cells were resuspended in ABB again and then incubated with propidium iodide (PI) staining solution for 5 min, in dark. The cell death was analyzed using the Tali™ Image-Based Cytometer (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA). The data were analyzed using Microsoft Excel 2010 and GraphPad Prism software.

2.4. Transmission electron microscopy

In order to observe the ultrastructure of A549 and T24 cells, a transmission electron microscope was used. After trypsinization, the control cells and cells treated with cotinine were fixed with 3.6% (v/v) glutaraldehyde (Polysciences; Warrington, PA) in 0.1 M sodium cacodylate buffer (pH 7.4; Roth; Karlsruhe, Germany) for 30 min at room temperature followed by three washing steps with 0.1 M sodium cacodylate buffer. Next, the cells were entrapped in fibrin clot and postfixed in 1% (w/v) OsO_4 (Serva) in 0.1 M cacodylate buffer for 1 h (RT). Then, the cells were passed through a series of ethanol and acetone solutions and embedded in Epon 812 (Roth, Karlsruhe, Germany) with the addition of hardeners (DDSA, MNA; Roth, Karlsruhe, Germany) and accelerator (Roth, Karlsruhe, Germany). Ultrathin sections were cut and double-stained with uranyl acetate and lead citrate. The samples were examined using a JEM 100CX transmission electron microscope (JEOL).

2.5. Fluorescent labeling of F-actin, gelsolin and vimentin

Equal number of cells for every treatments as well as in control cells was taken for fluorescence labeling. For immunofluorescence analysis, the A549 and T24 cells were fixed in 4% paraformaldehyde (Serva; Heidelberg, Germany) in PBS (30 min, 37 °C). Next the cells were washed three times with PBS, treated with 0.25% Triton X-100 (Serva; Heidelberg, Germany) in PBS for 10 min, washed again with PBS and blocked in 1% (w/v) BSA/PBS (Sigma–Aldrich, St. Louis, MO) for 10 min. The gelsolin was stained with gelsolin antibody produced in rabbit (GeneTex, 1:100) and anti-rabbit antibody-Alexa Fluor 555 (Invitrogen/Life Technologies, 1:500). The vimentin was visualized with a mouse monoclonal antibody specific for vimentin (Sigma, St. Louis, MO, 1:50) and a goat anti-mouse secondary antibody-TRITC (Sigma, St. Louis, MO, 1:85). The actin filaments were labeled with phalloidin conjugated to Alexa Fluor 488 (5 U/ml; Invitrogen, Molecular Probes; Eugene, OR). After 20 min of incubation at room temperature in the dark, the cells were washed with PBS (three times for 5 min). Counterstaining for nuclear DNA was performed using 4′,6′-diamidino-2-phenylindole dihydrochloride for 10 min (DAPI; 100 ng/ml, 10 min; Sigma–Aldrich; Buchs, Switzerland). The coverslips were mounted in Aqua-Poly/Mount (Polysciences; Warrington, PA). The stained cells were examined