A low level of nicotine-induced chemoresistance in a KB cell line

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Abstract. Cigarette smoking is closely associated with the induction of head and neck squamous cell carcinomas (HNSCC). Nicotine is an important component in cigarette smoke that can activate the growth-promoting pathways and facilitate the development of cancer. As nicotine is currently used in replacement therapy for smoking cessation in different forms, from skin patches to the oral route, numerous studies have investigated the effect of nicotine on the body, but the results have been equivocal. The aim of this study was to investigate the effect of nicotine on cancer chemotherapy. The status of the cell death-related proteins after treatment with nicotine was determined and compared to the effect of anticancer drugs such as cisplatin and etoposide in the presence or absence of nicotine in the KB cell line. Nicotine induced Bad phosphorylation in association with the suppression of apoptosis. The inhibition rate of cells pre-treated with nicotine prior to anticancer drug exposure was significantly decreased when compared to cells exposed to anticancer drugs only ($P<0.01$). Collectively, this suggests that nicotine may alter chemo-resistance and carcinogenesis via the anti-apoptotic pathway in HNSCC, and chemotherapy for HNSCC should be performed in the absence of nicotine in patient blood.

Introduction

Head and neck squamous cell carcinoma (HNSCC) has a strong tendency for local invasion and cervical node metastasis and, in 30-40% cases, it shows distant metastasis and is hence difficult to treat (1,2).

Nicotine is a major component of cigarette smoke and is one of the important risk factors for the development of lung cancer; nevertheless, its accurate reaction mechanism has yet to be elucidated (3). Nicotine is a growth factor that activates growth-promoting pathways, and it has been reported that it can lower the treatment efficiency of anticancer agents in the treatment of lung cancer (4) and that adrenergic receptors have an important role in the nicotine signal transduction pathway (5,6).

The most important factor in the development of HNSCC is also smoking. Particularly in oral cancer cases, its association has been shown to be very high; nevertheless, studies on the accurate mechanism of the action of nicotine are not abundant in lung cancer cases (7). Among the various reactions of nicotine, studies on the death-related signal pathway of tumor cells have examined the alteration of diverse genes and proteins pertinent to apoptosis (8,9).

Most anti-cancer therapies, including chemotherapy, are focused on the induction of the apoptosis of tumor cells. Therefore, resistance to chemotherapy could be considered to be induced by a defect of the apoptosis program (10,11).

The Bcl-2 family consists of approximately 20 members in mammals; the family members commonly have at least more than one Bcl-2 homology (BH) domain (12) and, among these domains, the BH3 domain induces apoptosis through interaction with other Bcl-2 family proteins (13). In particular, the BH3-only proapoptotic proteins (such as Bad, Bid, and Noxa) accelerate apoptosis by releasing the protective function of Bcl-X_L (14). Bad is one of the BH3-only proapoptotic members and, with the induction of the phosphorylation of Bad, pro-apoptotic function is lost. Nevertheless, dephosphorylated active Bad neutralizes the anti-apoptotic function of Bcl-X_L by interacting with Bcl-X_L in the mitochondria, and consequently accelerates apoptosis (15).

The chemotherapeutic drugs cisplatin and etoposide are useful therapeutics in the treatment of HNSCC, and whether their effect on the induction of apoptosis is influenced by nicotine is very important to the treatment of cancer patients. During chemotherapy or during the follow-up period, blood nicotine supplied by direct or indirect smoking or through other pathways may mediate an effect on the efficacy of chemotherapeutic drugs.

We examined the effect of nicotine on the growth of oral squamous cell carcinomas and its mechanism using the oral squamous cell carcinoma cell line KB, which has the closest epidemiological relationship to smoking of the HNSCCs. In addition, based on the results, the effect of the supply of nicotine by direct or indirect smoking, or by other different pathways during chemotherapy or during the follow-up period of cancer treatment are discussed, and some guidelines for the treatment of heavily-smoking patients are suggested.

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Materials and methods

Materials. Nicotine, etoposide, cisplatin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342 and propidium iodide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Bad, phospho-specific Bad (Ser^{112} and Ser^{136}), anti-ß-actin and monoclonal anti-Bcl-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rnase A was purchased from Boehringer Mannheim (Ingelheim, Germany). Antibody detection was performed using enhanced chemiluminescence Western blotting detection reagents (Amersham, Piscataway, NJ, USA). The cell culture reagents RPMI-1640, Williams' medium E (WME), minimal essential medium (MEM) and gentamycin were obtained from Gibco/Life Technology (Burlington, ON, Canada). All other cell culture supplies were purchased from Gibco-BRL (Grand Island, NY, USA), and all other chemicals were of the highest purity available.

Cell lines and cell culture. Human oral squamous cell carcinoma KB cells were maintained in RPMI-1640 medium and supplemented with heat-inactivated 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO_2, 95% air humidified incubator. For the preliminary studies of the nicotine-induced cell growth inhibition effect, cells were exposed to nicotine at increasing concentrations from 0 to 0.6 μg/ml during subculture. After an overnight subculture, cells were exposed to 0-200 μg/ml etoposide or 0-1000 μg/ml cisplatin, with or without pre-treatment with 0-0.6 μg/ml nicotine.

MTT cell viability assay. To analyze the sensitivity of cells to anticancer drugs, such as etoposide and cisplatin, and to nicotine, cells were suspended in RPMI-1640 at a density of $1x10^4$ cells/ml. A 100-μl sample of the cell suspension was seeded onto a 96-well plate. After an overnight culture, cells were exposed to etoposide, cisplatin and nicotine for 72 h, respectively. To define the sensitivity to anticancer agents of the nicotine-pre-treated cells, cells were exposed to nicotine for 24 h. After incubation with MTT (0.5 mg/ml) for 4 h, the medium was removed and 150 μl DMSO was added to dissolve the formazan crystals. Absorbance was measured at 540 nm using an ELISA microplate reader (Perkin-Elmer). Cell viability was determined relative to the untreated control cells. The data represent the mean values and standard deviations of triplicate assays of at least one experiment.

Metabolic labeling and immunoprecipitation. Cells were washed with phosphate-free RPMI medium 1640 and were metabolically labeled with [^{32}P]-orthophosphoric acid for 90 min. After treatment, the cells were washed with ice-cold phosphate-buffered saline and lysed in detergent buffer. Bad was immunoprecipitated using a Bad antibody, as described previously by Deng *et al* (16). Samples were subjected to SDS-12% polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and exposed to Kodak X-Omat film at -80˚C. Bad phosphorylation was determined by autoradiography.

Western blot analysis. After trypsinization, the cells were washed with PBS and underwent lysis in 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 5 mM EGTA, 50 mM glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin. The cell lysates were centrifuged and the protein content determined. Equal amounts of protein were separated on 6% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane overnight at 4˚C. The membranes were blocked with 5% skim milk in 0.1% Tween-20 Tris-buffer and sequentially incubated with monoclonal antibodies against Bad and Bcl-2, then reacted with goat-anti mouse IgG. The total density of the protein bands was calculated using GlykoBandScan analysis software (Glyko, Novato, CA, USA). To test for the amount of protein loading, the blots were stripped and reprobed with an antibody against ß-actin.

Propidium iodide and Hoechst 33342 nuclear fluorescence. Based on the nicotine level of heavy smokers, and to assess the interference effect of nicotine on the efficiency of chemotherapy, nicotine at a concentration of ~200 nM (0.0324 μg/ml), a value based on a study by Russell *et al* (17), was examined. The nicotine (0.0324 μg/ml) pre-treated KB cells were washed with MEM and supplied with L-15 medium containing IC_{30} of etoposide (8.3 μg/ml) or cisplatin (23 μg/ml) for 72 h, respectively. The cultures were then washed gently three times with PBS, and were fixed in neutral buffered formalin for 30 min. The cells were washed with PBS and stained with propidium iodide (PI) solution (50 μg/ml propidium iodide; 100 μg/ml RNase A) and Hoechst 33342 (5 μg/ml in PBS) for 10 min, respectively. The morphology of the cells was examined using a digital fluorescence microscope (Leica DM 5000 B, Wetzlar, Germany). The results were compared to those of the nicotine non-treated cells.

DNA fragmentation assay. Fragmented DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the protocol described by the manufacturer, with slight modifications. Briefly, at specific time-points after drug treatment, both floating and adherent cells were pooled in a 1.5 ml tube, where they underwent lysis for 1 h at 37˚C in a lysis buffer [10 mM Tris-HCl, pH 7.4, 10 mM ethylenediamine tetraacetic acid (EDTA), 0.5% SDS] with 0.5 mg/ml Rnase A. DNA was precipitated with ethanol overnight and was then resuspended in Tris-EDTA buffer (10 mM Tris, pH 7.4, 1 mM EDTA). The DNA sample (10 μg) was separated on 1.2% agarose gel containing 10 μg/ml ethidium bromide, then the DNA band pattern was visualized under UV light.

Statistical analysis. Data are expressed as the mean ± SD from at least three independent experiments. All values were calculated, graphed and then statistically analyzed using the Student's t-test and Sigma Plot 8.0 software. The level of significance was set at $P<0.05$ in all cases.

Results

Induction of Bad phosphorylation and an increase of the cell survival rate by nicotine. KB cells used in this study expressed a very low amount of Bcl-2 and a high level of endogenous

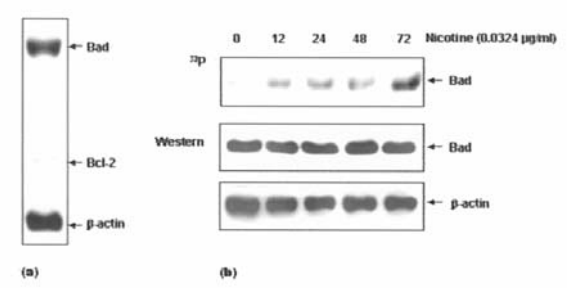

Figure 1. (a) KB cell line expresses a high level of endogenous Bad and a low level of Bcl-2 (faint band). (b) Nicotine induces Bad phosphorylation in the KB cell line. Bad was immunoprecipitated using a Bad antibody. Phosphorylation of Bad was determined by autoradiography (top). Western blot analysis was performed to confirm and quantify the level of Bad protein (bottom). Data shown are representative of three independent experiments.

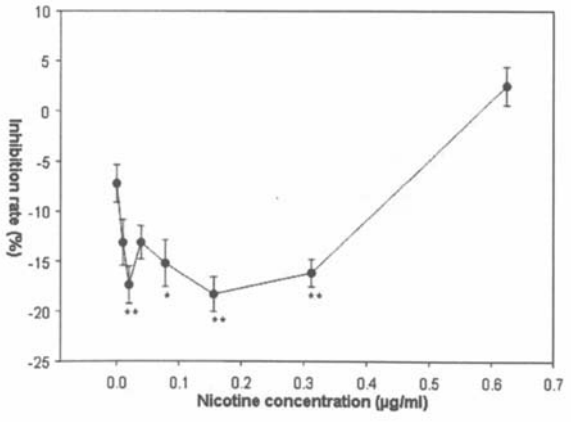

Figure 2. The effect of a low level of nicotine on the KB cell inhibition rate as determined by MTT assay. KB cells were incubated with different doses of nicotine for 72 h. Nicotine suppresses cell death, especially at concentrations <0.04 μg/ml; the inhibitory effect on cell growth was noticeably decreased. Asterisks indicate a significant difference from untreated cells (*P<0.05, **P<0.01). Data shown are representative of three independent experiments.

Bad (Fig. 1a). In the evaluation that assessed whether nicotine was involved in the increase in cell survival by the induction of phosphorylation of Bad in KB cells, with increasing time after treatment and with a low concentration (0.0324 μg/ml) of nicotine, the phosphorylation of Bad also increased (P<0.05) (Fig. 1b).

In addition, nicotine increased the cell viability of KB cells, and thus a low growth inhibition rate was seen. In cells treated with nicotine alone, the cell growth inhibitory effect by various concentrations of nicotine (0-0.6 μg/ml) was examined, and it was found that at concentrations <0.04 μg/ml, the inhibitory effect on cell growth decreased noticeably and thus cell growth increased (P<0.01) (Fig. 2).

The change in the levels of Bcl-2 and Bad with time was examined, and it was found that when cells were pre-treated with 0.0324 μg/ml nicotine, the levels of both Bcl-2 and Bad did not change (P>0.05) (Fig. 3).

Change in cell morphology due to nicotine treatment. It was confirmed that cell death induced by the administration of etoposide or cisplatin to KB cells was apoptosis, as determined

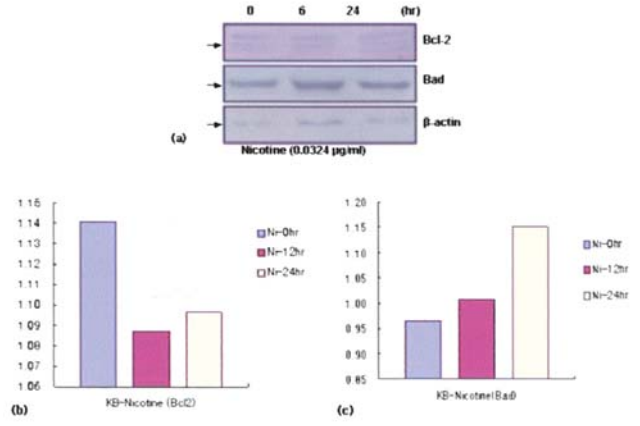

Figure 3. Western blot analysis of Bcl-2 and Bad in KB cells after pre-treatment with nicotine (0.0324 μg/ml) for 24 h. Representative Western blot (a) and densitometric analysis of Bcl-2 (b) and Bad (c) proteins. The levels of both Bcl-2 and Bad do not change significantly with time. Data shown are representative of three independent experiments.

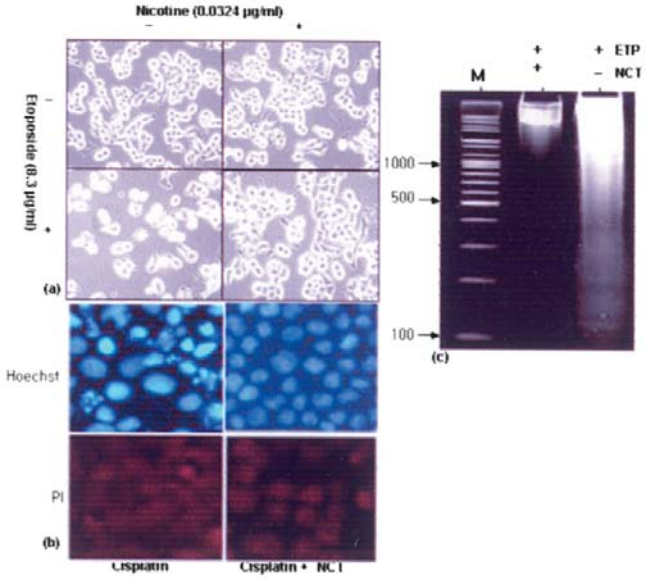

Figure 4. KB cells were pre-treated with or without nicotine (NCT) (0.0324 μg/ml) for 24 h, and the change in the cells in response to treatment with etoposide (ETP) (8.3 μg/ml) for 72 h was examined and compared. (a) Etoposide-induced apoptosis is suppressed by nicotine in the KB cell line. (b) Cells were treated with cisplatin with or without nicotine pre-treatment, and the cells were stained with PI and Hoechst 33342 and observed using a fluorescence microscope. Almost none of the Hoechst 33342-positive cells were stained by PI, indicating that they are apoptotic (not necrotic) in the cisplatin-treated KB cells, but not in the nicotine-pre-treated KB cells. (c) DNA fragmentation test shows a typical DNA ladder pattern in the etoposide-treated KB cells, but not in the nicotine-pre-treated cells. Lane M, DNA size markers; Middle lane, etoposide-treated KB cells with nicotine-pre-treatment; Right lane, etoposide-treated KB cells without nicotine-pre-treatment. Data shown are representative of three independent experiments.

by the DNA fragmentation test, PI staining and Hoechst staining. The induction of apoptosis was suppressed by nicotine pre-treatment for 24 h (Fig. 4).

The effect of chemotherapeutics on KB cells. Etoposide and cisplatin induced the cell death of KB cells efficiently, and

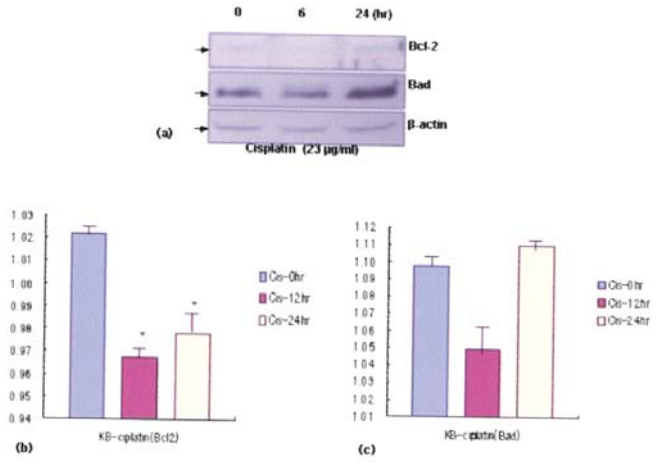

Figure 5. Western blot analysis of Bcl-2 and Bad in KB cells treated with cisplatin. Representative Western blot (a) and densitometric analysis of Bcl-2 (b) and Bad (c) proteins. The level of Bcl-2 decreased significantly with cisplatin treatment. Asterisks indicate a significant difference from untreated cells (*P<0.05). The level of Bad shows no significant pattern of change with cisplatin treatment. Data shown are representative of three independent experiments.

the IC_{50} was determined as 12.5 and 48 μg/ml, respectively. KB cells were treated with the IC_{30} concentration for etoposide (8.3 μg/ml) and cisplatin (23 μg/ml), and the change in levels of Bcl-2 and Bad with time was examined. It was found that with both etoposide (data not shown) and cisplatin, the level of Bcl-2 significantly decreased with treatment (P<0.05). However, the level of Bad showed a pattern that was slightly increased with treatment (P>0.05) (Fig. 5).

The effect of chemotherapeutics dependent on the presence or absence of pre-treatment with nicotine. KB cells were pre-treated with or without nicotine (0-5 μg/ml) for 24 h (data not shown), and the change in the cells in response to the treatment with etoposide (8.3 μg/ml) or cisplatin (23 μg/ml) for 72 h was examined and compared. It was found that nicotine elevated cell survival rate after treatment with chemotherapeutic drugs and thus, after treatment with cisplatin or etoposide, cell survival was markedly elevated in comparison to those cells untreated with nicotine (P<0.01). It was observed that pre-treatment with 0.0324 μg/ml nicotine resulted in a noticeable increase in cell survival (P<0.01). A similar response was also observed when cisplatin and etoposide were administered as a combination treatment (P<0.05) (Fig. 6).

Discussion

The opinion that smoking is deeply involved not only in the development of lung cancer but also in the development of various head and neck cancers, including oral cancer, has been suggested continuously, and it is known that among the diverse chemical components of cigarette smoke, nicotine has an important role in the carcinogenic process. The understanding of the theory of the action of nicotine pertinent to the carcinogenic process has been supported by various experiments; nevertheless, it has yet to be elucidated (3,18).

In addition to the reaction mechanism of nicotine involved in the development of cancer, it is very important to understand the effect of smoking during the treatment of cancer patients and, after treatment or the follow-up period, on the treatment efficacy of tumors and their recurrence. In cases in which patients are heavy smokers, and in both cases treated with surgical treatment only and those treated with a combination of chemotherapeutics and/or radiation therapy after surgical treatment, the cessation of smoking is difficult, even during the cancer treatment period, for the majority of patients. In such cases, for the treatment of withdrawal symptoms, nicotine is supplied through the skin or oral mucosa by skin patches, gum, lozenges and other methods of administration. However, based on the involvement in the carcinogenic process of nicotine, the interference of nicotine in the efficacy of various anticancer treatments can be anticipated and should be considered worrisome.

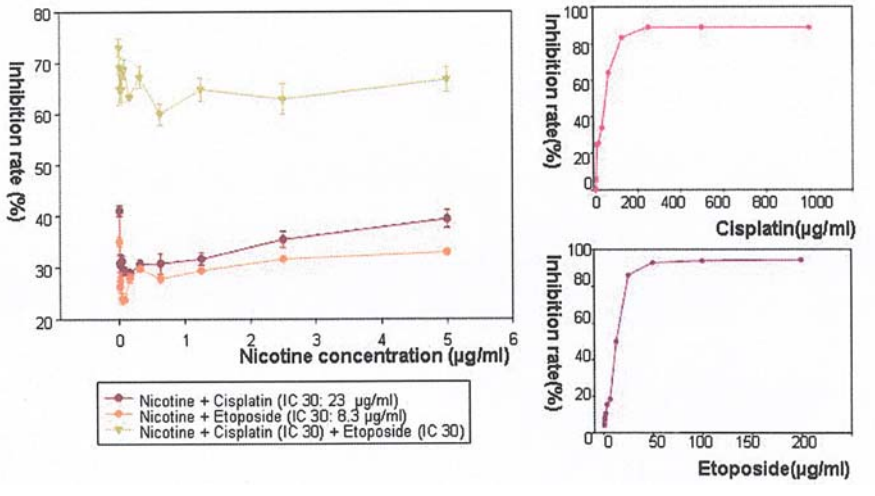

Figure 6. Cell growth inhibition curves of KB cells treated with cisplatin and etoposide in the presence of various concentrations of nicotine as determined by the MTT assay. Nicotine pre-treatment induces suppression of apoptosis by cisplatin, etoposide, or the combination of cisplatin and etoposide in the KB cell line (P<0.05). Cell growth inhibition curves for cells treated with various concentrations of cisplatin or etoposide are shown on the right. Data shown are representative of three independent experiments.

In cigarette smoking, the examined change in blood nicotine depends on the level of smoking. Russell *et al* (17) reported that, when an average of 6.7 cigarettes were smoked during the five hours between 10 a.m. to 3 p.m., the average blood nicotine concentration was detected to be 180±90 nM. In addition, the withdrawal symptoms were initiated within 2 h, reached a peak between the initial 24-48 h and persisted for several weeks or months (7,17). In this study, although smoking levels may vary depending on the individual, nicotine at an approximate level of 200 nM (0.0324 μg/ml) was used, based on a value of the Russell *et al* study (17) and on the general level of heavy smokers, to assess the interference effect of nicotine on the efficiency of chemotherapy. However, in this study, the growth inhibition rate of KB cells dependent on the concentration of nicotine (0-5 μg/ml) was examined by an MTT assay. It was found that at low concentrations of <0.04 μg/ml, a concentration similar to the blood nicotine concentration of heavy smokers, the anti-apoptotic effect was prominent and cell growth was suppressed.

Numerous studies have been conducted on the carcinogenic mechanism of nicotine; however, many factors interact with each other in a complex manner so that it was not possible to elucidate an accurate reaction mechanism. The effect of nicotine on anticancer therapy has been anticipated to be similarly very complex, and thus studies were focused on the change of factors associated with apoptosis. According to a study conducted by Jin *et al* (9), in cases of lung cancer the expression of Bad is more frequent than of Bcl-2, and it has been anticipated that the role of Bad in apoptosis is more significant than the role of Bcl-2.

It is possible to speculate that Bad may have a more important role in nicotine-induced survival and chemoresistance, which may be more significant in cases with or without a low level of Bcl-2. The result of the measurement of the level of Bcl-2 in a KB cancer cell line developed in the oral cavity, which was used in this study, indicated a low level of Bcl-2, and the level of Bad was detected to be high. This finding suggests that the change in the level of Bad has a greater role in apoptosis, similar to that of the lung cancer cases.

Upon phosphorylation of Ser^{112}, Ser^{136} and Ser^{155} of Bad, Bad loses its proapoptotic function (15,19). In the KB cell line it was found that, when treated with nicotine, the phosphorylation of Bad was induced. Based on a DNA fragmentation test and PI staining, it was confirmed that the cell death induced by etoposide or cisplatin was apoptosis. It was inferred that the reduction in cell death induced by nicotine might be the suppression of apoptosis. In addition, the increase of the phosphorylation of Bad without the alteration of endogenous Bad or Bcl-2 was detected, and it was considered that, in the KB cells, nicotine at least partially contributes to the generation of anti-apoptotic function through the increase of Bad phosphorylation.

It was confirmed that the KB cells were sensitive to cisplatin or etoposide and, to confirm the efficiency of the cell growth inhibitory effect of chemotherapeutics by nicotine, KB cells were treated with nicotine and the cell growth inhibition effect of cisplatin and etoposide was examined by MTT assay. As expected, a noticeable decrease in the efficiency of cell growth inhibition was confirmed, which was particularly noticeable when the concentration of nicotine was low. The concentration of nicotine exerted such a noticeable decrease on the efficiency of cell growth, that inhibition was equivalent to the blood nicotine concentration of heavy smokers.

If nicotine is supplied at a concentration that is comparable to that obtained through casual smoking, similar to the cases in our study, the cell growth inhibition effect of chemotherapeutics could be severely decreased. For patients undergoing chemotherapy and during the follow-up period after treatment, for heavy smokers that smoke unavoidably directly or indirectly or when nicotine is supplied through the skin or the oral mucosa, blood nicotine levels will become elevated and, due to the anti-apoptotic function of nicotine, the treatment efficacy of chemotherapeutics will decrease. In the case of nicotine administration, an improvement in treatment methods, such as the augmentation of the efficacy of chemotherapeutics by raising the dose of chemotherapeutics, the use of combination therapy or some other improvement in treatment methods will be required.

Acknowledgments

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