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Effects of nicotine on the formation of osteocalcin and osteoprotegerin and synthesis of its mRNA in MG63 osteoblast-like cell

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The purpose of this study was to evaluate the correlation between nicotine and the activity of bone forming cell. MG63 osteoblast-like cells were used for this study. Several factors were examined including the proliferation of cell, alkaline phosphatase activity, the formation of osteocalcin and osteoprotegerin, and the synthesis of its mRNA. MG63 osteoblast-like cells were incubated for 1, 2, 3 and 6 days with nicotine added to the culture medium in 1.0 ㎛M, 1.0 mM, 2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM concentrations.

The proliferation of MG63 osteoblast-like cells was temporarily activated at the low nicotine concentrations. At high concentrations (>5.0 mM), however, it was suppressed. Alkaline phosphatase activity was suppressed in a dose-dependent manner as the concentration of nicotine increased.

Osteocalcin decreased in a dose-dependent manner at high nicotine concentrations of more than 7.5 mM and the same result was show when the osteoblasts were treated with low concentrations for longer than 3 days. There was a difference in the influence of nicotine on the synthesis of osteocalcin mRNA and formation of osteocalcin itself at 1 and 3 days.

Generally, osteoprotegrin notably declined in all experimental groups. However, the level of its mRNA increased at high nicotine concentrations of more than 7.5 mM after 3 days and more than 5.0 mM after 6 days.

Key words : Alkaline phosphatase, Osteocalcin, Osteoprotegerin, mRNA

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The adverse effects of tobacco smoking on the general health of the population have been well documented. However, the mechanism of the action of smoking on cell metabolism is still poorly understood. In the dental field, it has recently been reported that tobacco smoking is associated with periodontal disease and oral cancer.[1-4] Tobacco smoking is a risk factor for the resorption of alveolar bone.[1,5] Many other studies have concluded that those who smoke tobacco have a

higher prevalence of periodontal disease.

Concerning the effect of smoking on bone cells, it is generally known that smoking inhibits the healing of fractured bones with regard to orthopedics[2,6] and that smoking also aggravates osteoporosis, which usually occurs in females, especially menopausal females.[5,7] However, this knowledge is based not on scientific experimentation but on epidemiological investigation.

In the orthodontic field, bone remodeling has been the main focus with regard to the periodontal tissues when we apply forces on teeth. As we well know, osteoblasts are activated at the pressure site and osteoclasts are activated at the tension site.[8,9] Smoking has been receiving attention as one of the factors affecting the function of bone cells. However, there is a lack of data on the correlation between smoking and the meta-bolism of bone cells.

To evaluate the functions of a cell, we generally analyze the specific protein produced in the cell. Osteoblasts produce many types of proteins including collagenous and noncollagenous proteins. Notably, osteocalcin constitutes about 20% of the noncollagenous constituents in adult bone.[10] Osteocalcin is the most abundant noncollagenous protein in adult bone and constitutes about 2% of the total protein.[11] The serum levels of osteocalcin correlated well with bone turnover,[12] although the physiological role of osteocalcin is not yet known. The serum levels of osteocalcin have also been used as a clinical indicator of bone metabolism.[11]

Recently, two molecules produced by osteoblast-lineage cells were identified as playing important roles in osteoclastogenesis. One of these molecules is osteoprotegerin (OPG), which is identical to an osteoclastogenesis inhibitory factor.[13-15] OPG is a secretory protein belonging to the tumor necrosis factor (TNF)-receptor family. This protein inhibits not only formation of osteoclast-like cells in culture but also bone resorption both in vitro and in vivo. Therefore, it is called osteoclastogenesis inhibitory factor (OCIF).[15] The other molecule is the receptor activator of the NFKB ligand (RANKL), which is identical to the osteoclast differentiation factor (ODF), the osteoprotegerin ligand (OPGL), and TNF-related, activation induced cytokine (TRANCE).[14] OPG is a water-soluble receptor for OPGL and competes with RANKL, which is found on the osteoclast precursor and mature osteoclast[13]. It was clarified that OPGL-knockout mice exhibited typical osteopetrosis with the total occlusion of the bone marrow space within the endosteal bone. OPGL-knockout mice lack osteoclasts but have normal osteoclast progenitors that can differentiate into functionally active osteoblasts when co-cultured with normal osteoblasts/stromal cells.

In order to examine the effects of nicotine on human osteoblast metabolism, varying concentrations of nicotine were used in this study. We examined osteoblast proliferation, alkaline phosphatase activity, the formation of osteocalcin and osteoprotegerin, and the synthesis of its mRNA, depending on the concentration of nicotine.

MATERIALS AND METHODS

Materials

Osteoblast cultures

MG63 osteoblast-like cells were obtained from the Korea Cell Line Bank. They were incubated at 37℃ in 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin. The medium was changed every 2-3 days. Cells were grown to confluence at 37℃. The MG63 cell cultures were washed with phosphate-buffered saline (PBS) to remove non-viable cells and debris.

Preparation of nicotine

Nicotine solution containing 50 mg/ml nicotine with ethanol was immediately added to the culture tubes to establish cultures with final nicotine concentrations of zero (control), 1.0 μM, 1.0 mM, 2.5 mM, 5.0 mM, 7.5 mM, and 10.0 mM.

Methods

Determination of cell proliferation

The effect of nicotine on the cell proliferation was determined by microtiter assay which uses the tetrazolium salt (MTT). A 100 ㎕ of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolum bromide were added to the cells after 1, 2, 3, and 6 days. After the cells were incubated for 4 hours at 37℃, their survival rates were determined according to the amounts of formazan crystal. Finally, the cell cultures were dissolved with dimethylsulfoxide (DMSO) and then their absorbance was measured by a microplate absorbance reader at 540 nm.

Determination of Alkaline phosphatase (ALPase)

Alkaline phosphatase yellow (pNPP) liquid substrate and p-nitrophenol were obtained from Sigma Co., St. Louis, USA. The concentration of protein was measured with Bradford reagent (Sigma Co., St. Louis, USA) The cell cultures were treated with 0.1% Triton X-100 and 300 ㎕ 50 mM Tris-HCl (pH 7.2) containing 2 mM of MgCl2 and then washed by sonicator for 60 seconds. The concentration of the supernatant, which was obtained after centrifugation (12,000rpm, 15 minutes, 4oC), was measured by Bradford reagent. A 100 ㎕ of ALP substrate(Sigma Co., St. Louis, USA) and 50 ㎕ of 3 N NaOH were added to the measured 100 l of protein (96 well, 37℃, 60minutes). Finally, the absorbance was measured by a microplate absorbance reader at 405 nm.

Determination of amount of protein

Osteocalcin

To measure the amount of osteocalcin, a Gla-type Osteocalcin EIA Kit (ZYMED Laboratories, Inc., South San Francisco, USA) was used. The supernatant, which was obtained after centrifugation of cultrued cells, was treated with PBS containing 0.5% Triton X-100 and 2 mM of phenylmethylsulfonyl fluoride (pH 7.2). The samples, which each containing distinct concentrations of nicotine, were incubated for 1, 3, and 6 days and stored for up to 24 hours at 4℃. A 100 ㎕ substrate solution was added to each well for 15 minutes and, finally a 100 ㎕ stop solution (1N H2SO4) was gently mixed into the well by hand. The absorbance was measured an by an ELISA reader at 450nm.

Osteoprotegerin

To measure the amount of osteoprotegerin, a human OPG/OCIF ELISA Kit (Oscotech Co., Cheonan, Korea) was used. After the MG63 cells were attached to a plate as in the ALP test, the 100 ㎕ supernatant, which was incubated with each concentration of nicotine for 1, 3, and 6 days, was maintained in an Elisa plate at room temperature. A 50 ㎕ stop solution was mixed into the wells. The absorbance was measured by an ELISA reader at 450-570nm.

Real time PCR

To measure the amount of mRNA, the polymerase chain reaction (PCR) using LightCycler-FastStart DNA Master SYBR Green I kit (Roche co., Germany) was carried out. The procedure was as follows.

Sample

The control groups which were not treated with nicotine and experimental groups which were treated with each concentrations of nicotine (1.0 ㎛M, 1.0 mM, 2.5 mM, 5 mM, 7.5 mM, 10 mM) were incubated for 1, 3, and 6 days. The total of 21 samples were prepared.

Extraction of RNA

First, the cell cultures were washed with HBSS and then mixed with RNAzolB^{TM} (WAK-Chemie Medical, Bad Homburg, Germany). Chloroform (1/10 times as big as the mixture) was added to the mixture and it was stored in ice for 15 minutes and then centrifuged at 12000 rpm, 4℃ for 20 mintutes. We transferred the supernatant to a new tube and mixed it with an eqaul size of ice-cold isopropanol. The mixture was maintained at 20℃ below zero for over 1 hour. After centrifuge was done again, we removed the supernatant and washed the pellet with diethyl pyrocarbonate (DEPC)-75%

ethanol and dried and finally, dissolved in 50㎕ of DEPC-dextrose in distilled in water (D/W). RNA level was measured at O.D 260nm. (Dissolved materials were stored at -70℃ until it was tested)

Reverse transcription

Before the test, we denatured RNA at 65℃ for 5 minutes and stored it in ice. we mixed each reagents, which are 5×RT buffer (Promega, Madison, USA), 200㎛ M dNTP, 0.5ug oligo (dT) 15 primer, 10 units AMV reverse transcriptase (Promega, Madison, USA), 40 units RNasin and 2㎍ RNA, in DEPC-D/W and made the total volume of mixture to 20㎕. Using Thermal cycler (2400. Applied biosystems) at 42℃ for 60 minutes and at 95℃ for 5 minutes, we synthesized cDNA.

Realtime PCR

For the realtime PCR, we used LightCycler-FastStart DNA Master SYBR Green I kit (Roche co., Germany). We mixed 12.6 ㎕ DEPC-D/W, 2.4 ㎕ 25mM MgCl2, 1㎕ 10 ㎛M primer, 2 ㎕ LightCycler-FastStart DNA Master SYBR Green I and 1.0 ㎕ cDNA. The PCR was done by 40 cycles at 95℃ for 60 seconds and at 72℃ for 15 seconds. The sequence of each primer was as follows.

primers		sequence
hOPG	sense primer	TGG AAC CCC AGA GCG AAA TA (20 mer)
	antisense primer	TGT GAG CTG TGT TGC CGT TT (20 mer)
osteocalcin	sense primer	GCT TTT GGC GRR TGT G (16 mer)
	antisense primer	GGA AGC GGG GAT CAG A (16 mer)
actin	sense primer	AGC CTC GCC TTT GCC GA (17 mer)
	antisense primer	CTG GTG CCT GGG GCG (15 mer)

Statistical analysis

The mean and the standard deviations of each variable were obtained. The changes of cell proliferation, alkaline phosphatase and the formation of osteocalcin and osteoprotegrin between control and experimental group were evaluated by paired t-test.

RESULTS

Effects of nicotine on the proliferation of MG63 osteoblast-like cells

In the MTT test, after 1 day, the concentrations of nicotine of less than 2.5 mM showed an increase in the proliferation and survival rate of the MG63 cells compared with the control group, but the concentrations of nicotine of more than 5.0 mM showed a notable decrease in those of the MG63 cells. After 2 and 3 days, the concentrations of nicotine of less than 1.0 mM showed an increase in the proliferation and survival rate of the MG63 cells, but the concentrations of nicotine of more than 2.5 mM showed a decrease in the same. Contrarily, when the MG63 cells were exposed to nicotine for 6 days, even at concentrations of 1.0 ㎛M, both their proliferation and survival rate decreased compared with the control group. Especially, when the MG63 cells were exposed at high concentrations (>5.0 mM) for more than 2 days, the survival rate was lower than 50% (Tables 1, and 2).

Effects of nicotine on ALPase

After 1 day, the concentrations of nicotine of less than 5.0 mM showed no change in ALPase activity and those of more than 7.5 mM showed a decrease. However, after 3 and 6 days, every experimental group showed notable decreases in ALPase activity compared with the control group. Generally, in every experimental group, ALPase activity decreased in a dose-dependent manner. Especially, as in the MTT test, ALPase activity significantly decreased when the MG63 cells were exposed at the high concentrations of nicotine (>5.0 mM) for more than 3 days (Table 3).

Effects of nicotine on the formation of osteocalcin

Effects on the synthesis of protein

After 1 day, when the concentration of nicotine was below 5.0 mM, the amount of osteocalcin showed a

Table 1. Effects of nicotine on the proliferation of osteoblasts (by MTT assay at 540nm) (Mean ± S.D.)

concentratin of nictotine	Absorbance			
	1 day	2 days	3 days	6 days
Control	0.63 ± 0.06	1.05 ± 0.08	1.62 ± 0.07	3.34 ± 0.14
1.0μM	0.65 ± 0.03	1.09 ± 0.31	1.69 ± 0.10	3.25 ± 0.12
1.0mM	0.64 ± 0.07	1.09 ± 0.25	1.65 ± 0.07	2.88 ± 0.16**
2.5mM	0.65 ± 0.04	1.00 ± 0.14	1.51 ± 0.14	2.90 ± 0.23**
5.0mM	0.48 ± 0.03**	0.59 ± 0.12**	0.74 ± 0.05**	0.85 ± 0.06**
7.5mM	0.39 ± 0.03**	0.38 ± 0.06**	0.27 ± 0.02**	0.20 ± 0.01**
10mM	0.37 ± 0.02**	0.26 ± 0.06**	0.18 ± 0.01**	0.16 ± 0.02**

**$p<0.01$

Table 2. Effects of nicotine on the survival rate of osteoblasts

concentratin of nictotine	Survival rate of osteoblasts(%)			
	1 day	2 days	3 days	6 days
Control	100.00	100.00	100.00	100.00
1.0μM	103.95	104.04	103.91	97.40
1.0mM	102.35	103.65	102.58	86.30
2.5mM	102.91	95.20	92.74	86.70
5.0mM	77.21	55.98	45.35	25.60
7.5mM	62.78	36.32	16.32	5.90
10mM	58.92	24.50	11.05	4.70

Table 3. Effects of nicotine on the alkaline phosphatase(ALPase) activity of osteoblasts (at 405nm) (Mean ± S.D.)

concentratin of nictotine	ALP activity(um/protein g/hr)		
	1 day	3 days	6 days
Control	0.026 ± 0.0098	0.098 ± 0.0032	0.206 ± 0.0005
1.0μM	0.017 ± 0.0058	0.069 ± 0.0033*	0.140 ± 0.0011*
1.0mM	0.017 ± 0.0035	0.073 ± 0.0061	0.149 ± 0.0170
2.5mM	0.025 ± 0.0327	0.078 ± 0.0146	0.185 ± 0.0400
5.0mM	0.020 ± 0.0214	0.034 ± 0.0070*	0.017 ± 0.0048*
7.5mM	0.006 ± 0.0032	0.021 ± 0.0205*	0.008 ± 0.0117**
10mM	0.015 ± 0.0150	0.009 ± 0.0064**	0.004 ± 0.0011**

*$p<0.05$, **$p<0.01$

tendency to increase in a dose-dependent manner. Especially, at 1.0 mM, the amount of osteocalcin increased to two-times as that of the control group but showed a decrease at nicotine concentrations of more than 5.0 mM. After 3 days, the amount of osteocalcin somewhat increased at nicotine concentrations of 1.0 mM, but this was not a significant change. At concentrations of more than 1.0 mM, the amount of

Table 4. Effects of nicotine on the formation of osteocalcin of osteoblasts (by ELISA at 450nm) (Mean ± S.D.)

concentratin of nictotine	amounts of osteocalcin(ng/ml)		
	1 day	3 days	6 days
Control	0.0083 ± 0.0008	0.0141 ± 0.0021	0.0101 ± 0.0005
1.0μM	0.0124 ± 0.0012	0.0160 ± 0.0007	0.0073 ± 0.0005*
1.0mM	0.0180 ± 0.0005*	0.0092 ± 0.0005	0.0094 ± 0.0008
2.5mM	0.0142 ± 0.0020	0.0099 ± 0.0005	0.0080 ± 0.0008
5.0mM	0.0095 ± 0.0000	0.0060 ± 0.0003	0.0040 ± 0.0010*
7.5mM	0.0065 ± 0.0000	0.0041 ± 0.0005	0.0034 ± 0.0005**
10mM	0.0060 ± 0.0003	0.0042 ± 0.0003	0.0024 ± 0.0041**

*p<0.05, **p<0.01

Table 5. Effects of nicotine on the formation of osteoprotegerin of osteoblasts (by ELISA at 450-570nm) (Mean ± S.D.)

concentratin of nictotine	amounts of osteoprotegerin(ng/ml)		
	1 day	3 days	6 days
Control	90.32 ± 0.07	230.79 ± 0.00	251.31 ± 7.40
1.0μM	84.93 ± 0.00**	231.35 ± 0.00	240.54 ± 0.00
1.0mM	76.53 ± 0.00**	198.07 ± 0.00*	257.81 ± 0.00
2.5mM	63.70 ± 0.00**	201.56 ± 0.00*	276.51 ± 0.00
5.0mM	43.50 ± 1.68**	159.09 ± 3.81*	239.51 ± 2.80
7.5mM	34.86 ± 0.00**	102.84 ± 0.00**	142.77 ± 0.00*
10mM	22.50 ± 1.34**	43.10 ± 3.14**	53.00 ± 3.25**

*p<0.05, **p<0.01

osteocalcin decreased in a dose dependent manner. After 6 days, all the experimental groups showed a decrease in osteocalcin formation, and especially at high nicotine concentrations of more than 5.0 mM, there was a remarkable decreasing tendency (Table 4).

Effects on the synthesis of mRNA

After 1 day, the mRNA level of osteocalcin increased in a dose-dependent manner. However, after 3 days, there were no remarkable differences between the control group and the experimental groups. After 6 days, the mRNA level of osteocalcin decreased in a dose-dependent manner; particularly, when the concentration of nicotine was higher than 7.5 mM, the mRNA of osteocalcin was undetected (Table 6).

Effects of nicotine on the formation of osteoprotegerin

Effects of nicotine on the synthesis of protein

In general, the amount of osteoprotegerin decreased significantly in a dose-dependent manner in every experimental group. But, exceptionally, after 6 days, the amount of osteoprotegerin somewhat increased at nicotine concentrations of 1.0 mM, and 2.5 mM (Table 5).

Effects of nicotine on the synthesis of mRNA

A day after nicotine was added, the mRNA levels of osteoprotegerin in the control group and in every experimental group were almost the same, and even after 3 and 6 days, the mRNA level of osteoprotegerin showed

Table 6. The mRNA level of osteocalcin (by real time PCR)

concentratin of nictotine	mRNA level of osteocalcin		
	1 day	3 days	6 days
Control	1.24E - 03	1.64E - 03	1.97E - 03
1.0μM	1.17E - 03	2.01E - 03	2.15E - 03
1.0mM	2.07E - 03	8.92E - 04	1.18E - 03
2.5mM	1.26E - 03	1.78E - 03	1.24E - 03
5.0mM	1.90E - 03	1.42E - 03	1.22E - 03
7.5mM	2.31E - 03	1.73E - 03	0.00E + 00
10mM	2.32E - 03	1.75E - 03	0.00E + 00

*p<0.05, **p<0.01

Table 7. The mRNA level of osteoprotegerin (by real time PCR)

concentratin of nictotine	mRNA level of osteoprotegerin		
	1 day	3 days	6 days
Control	4.23E - 01	3.89E - 01	3.30E - 01
1.0μM	3.44E - 01	2.67E - 01	3.39E - 01
1.0mM	4.11E - 01	2.20E - 01	3.14E - 01
2.5mM	2.70E - 01	2.27E - 01	2.50E - 01
5.0mM	5.39E - 01	3.79E - 01	6.71E - 01
7.5mM	5.57E - 01	9.22E - 01	1.96E + 00
10mM	3.23E - 01	1.42E + 00	1.53E + 00

*p<0.05, **p<0.01

no change at nicotine concentrations of lower than 5.0 mM (3 days) and 2.5 mM (6 days). However, the mRNA level of osteoprotegerin increased at nicotine concentrations of higher than 7.5 mM after 3 days and higher than 5.0 mM after 6 days (Table 7).

DISCUSSION

Orthodontic tooth movement depends on the bone remodeling which is accomplished by alveolar bone resorption at pressure sites and alveolar bone deposition at tension sites.[8,9] The quality of bone remodeling depends generally on genetic variables but its extent could be affected by postnatal and environmental factors. Therefore, research on bone-cell growth and development and on bone metabolism is essential in the orthodontic field. Recently, smoking (nicotine), liquor (alcohol), coffee, green tea, and other environmental factors which affect the activity of bone cells, have been watched with keen interest. However, because most data are based on epidemiological investigation, little has been known about their accurate mechanisms.

Many research have shown that smoking could affect the periodontal tissues, teeth, and the tongue.[1-4] However, the research on how smoking affects the body is not yet conclusive.

A cigarette contains 0.1-2mg of nicotine and the blood concentrations of nicotine resulting from smokeless tobacco and cigarette smoking are comparable at approximately 15 mg/ml.[16] However, Hoffmann and Adams[17] have shown that concentrations of nicotine in saliva are about 1.56 mg/ml, which is more than 100,000

times higher than the blood level, so they are sufficient enough to affect the oral tissues. Also, Murray et al.[3] have reported that smokers show a large amount of nicotine on tooth root surfaces, which could affect periodontal tissue. The diseases of the oral cavity due to smoking include the diseases of the oral mucosa, gingivitis, periodontitis, cervical erosion, gingival erosion, and oral cancers. Pindborg,[18] Sheiham,[19] and Feldman[20] have reported on the relation of smoking to periodontal disease.

Tobacco smoke is a complex chemical mixture containing, among other things, N-nitrosamines and pyridine alkaloids such as nicotine, cotinine, and nornicotine.[17] The biologically important nitrosamines in tobacco smoke are N-nitrosodimethylamine (NDMA), N-nitrosonornicotine (NNN), and 4-(meth-ylnitrosa-mino)-1-(3-pyridyl)-1-butanone (NNK). Hoffman et al.[17] have reported that nitrosamines caused mutation through the activation of cytochrome P450 and that nicotine itself does not cause mutation in its meta-bolism.[21] Nicotine, which is the main ingredient in tobacco, has a harmful pharmacological effect on our health. Most nicotine is absorbed mainly through the alveoli of the lungs, and some are absorbed through the skin and oral mucosa. Similar to the pharmacological function of acetylcholine, nicotine is initially activated by bonding with the nicotinic receptor in the autonomic nerve ganglia and subsequently blocks up the nerves.[22]

There are nicotinic receptors on osteoblasts and other cells like fibroblasts, lymphocytes, and macrophages. The pharmacological effects of nicotine are various depending on the cell type.[6] For example, it suppresses the phagocytosis of neutrophil and the function of macrophages by the inhibition of the formation of protein, and RNA.[4] Nicotine also has different effects on the formation of DNA. For example, nicotine suppresses the formation of DNA in mouse brain cells and human leukemia cells, but has no effect on HeLa cells, human lung fibroblasts, or fetus fibroblasts. Peacock et al.[23] have reported that the DNA formation of fibroblasts increases at low concentrations of nicotine (<0.025 μM) and decreases at high concen-trations (>0.2 μM). As mentioned previously, the pharmacological effects of nicotine are various depending on cell types and research. This is due to the heterogenecity of the cell itself.[23,24]

Bone is made of mineralized tissues and most orga-nic materials are composed of collagen. Most collagens are of type I, which is composed of two 1(I) chains and one 2(I) chain, and the others are noncollagenous proteins containing ostecalcin, osteonectin, fibronectin, osteoprotegerin, etcetera.[25,26] In this study, we were mainly trying to observe the change of osteocalcin and osteoprotegerin by nicotine.

In this experiment, from day 1 to day 3, the survival and proliferation rates of the osteoblasts somewhat increased more than those of the control group, but did not show a significant change when the concentration of nicotine was below 1.0 mM. However, when nicotine was administered for more than 6 days at concen-trations of lower than 1.0 mM or over 5.0 mM, the sur-vival and proliferation rates of the osteoblasts were suppressed. Especially, when the osteoblasts were exposed for more than 2 days to concentrations of more than 5.0 mM, survival and proliferation rates decreased to under 50%. Walker et al.[26] have stated that the proliferation of osteoblasts increased at low nicotine concentrations between 0.01 μM and 10 μM, and decreased at high nicotine concentrations over 1.0 mM. This is similar to the results of our experiment. Fang et al.[25] also showed similar results, which used the UMR 106-01 osteoblastic osteosarcoma cells of a mouse. They assumed that when the cells are exposed to high concentrations of nicotine, inducing the change of cell shape, cell adhesion is inhibited, or vacuoles are formed in the cells, resulting in the occurence of necrosis. Weak organic bases, such as nicotine, are known to accu-mulate in lysosomes by membrane permeation and trapping by protonation. As the base concentration within the lysosome increases, water enters osmotically and the lysosomes swell into large vacuoles. Vacuolation of this type has been reported in the macrophages of a mouse, the fibroblasts of human skin, and osteoblast-like cells, and it may represent a toxic effect on cell metabolism.[16]

Usually, the activity of ALPase is used to evaluate the function of osteoblasts. ALPase is closely related to the activity of osteoblasts. Because ALPase induces the apposition of bone mineral, a decrease in ALPase reduces bone mineralization. In this study, nicotine dose-dependently inhibited the formation of ALPase. Especially when nicotine was administered for more than 2 days at >5.0 mM, ALPase activity was significantly inhibited. The study on the osteoblast-like cells of a chick embryo calvariae[16] and the MC3T3-E1 preosteoblastic cells of a mouse[56] showed similar results. Fang et al.[25] have reported that UMR 106-01 osteoblastic osteosarcoma cells showed increased ALPase activity in a dose-dependent manner at nicotine concentrations of 1 nM - 10 mM, and notably, this activity increased 189% at 1 μM . Henderson[27] said that the diluted 102-104 solution of smokeless tobacco extract significantly increased ALPase activity, bone nodule formation, and cell proliferation, but inhibited their mineralization.

Only osteocalcin has the Gla residues which can combine with calcium and is produced only by osteoblasts and odontoblasts.[12,28] Whereas a precise physiological role remains to be established, osteocalcin is a general indicator of osteoblast activity. Also, osteocalcin has an important role in the differentiation and acitivation of osteoclasts.[29,30] Osteocalcin mRNA is a very unique gene which is found in mature bone, dentin and calcified cartilage. The appearance of osteocalcin mRNA corresponds to the time of bone nodule formation, and activates the calcification of the bone substrate.

This experiment showed the somewhat different effects of nicotine on the formation of osteocalcin (as a protein) and osteocalcin mRNA. The effect of nicotine on the formation of osteocalcin at the protein level was as follows. A day after nicotine was added, the amount of osteocalcin increased at concentrations of <5.0 mM, and decreased at concentration of >7.5mM compared with those of the control group. After 3 and 6 days, the formation of osteocalcin decreased in a dose-dependent manner. Regarding the change in osteocalcin mRNA level, a day after nicotine was added, the mRNA level slightly increased as did that of protein, but also increased at concentrations of >7.5 mM unlike that of protein. After 3 days, there were no notable changes in mRNA level between the control and experimental groups. However, after 6 days, the osteocalcin mRNA level decreased in a dose-dependent manner as did protein. Laroche et al.[28] have reported that long-term smokers have lower blood concentrations of osteocalcin than do non-smokers, and that this was because of a hormone change or the restriction of osteoblast function. Ramp et al.[16] revealed that nicotine activates the DNA formation of osteoblasts, but with a slightly inhibited total DNA content, suggesting stimulated cell turnover.

Recently, two molecules produced by osteoblast lineage cells were identified as playing important roles in osteoclastogenesis. One of the these molecules is OPG, which is identical to the osteoclastogenesis inhibitory factor.[13-15] This protein inhibits not only the formation of osteoclast-like cells in culture but also bone resorption, both in vitro and in vivo. In this experiment, the formation of OPG was significantly decreased in a dose-dependent manner. However, after 6 days, it increased somewhat more than did the control group at nicotine concentrations of 1.0 mM and 2.5 mM. However, a day after nicotine was added, there were no remarkable differences in the mRNA level of osteoprotegerin, but the osteoprotegerin mRNA level increased at concentrations of >7.5 mM (after 3 days) and >5.0 mM (after 6 days). This means that although nicotine promotes the formation of osteoprotegerin (OPG) at the mRNA level, osteoprotegerin (OPG) decreases substantially when osteoblasts are treated with high concentrations of nicotine over a long period. According to these results, we may assume that the formation of protein is decided not just at the mRNA level but is also affected through different pathways. This question requires more thorough investigation.

From the above results, we know that nicotine directly affects the functions of osteoblasts (ALPase activity, osteocalcin and osteoprotegerin formation and its

mRNA). But, we require more research on the mechanism by which nicotine affects osteoblasts and interacts with hormones. Osteocalcin and osteoprotegerin are just parts of the protein made by osteoblasts; therefore, we need to study other unknown extracellular substrates.

CONCLUSIONS

In order to observe the effects of nicotine on human osteosarcoma cells (MG63 osteoblast-like cells), several factors were examined including the proliferation of cell, ALPase, the formation of osteocalcin and osteoprotegerin, and the synthesis of its mRNA.

The results were as follows.

1. At the low nicotine concentrations of less than 1.0 mM, cell proliferations were activated by nicotine up to the first 3 days. At high nicotine concentrations of more than 5.0 mM, however, cell proliferations were immediately suppressed after 1 day of exposure to nicotine. And it was also suppressed when exposed to nicotine at low concentrations of less than 1.0 mM for more than 6 days.
2. ALPase was suppressed in a dose-dependent manner as the concentration of nicotine increased. Particularly, the activity of ALPase notably declined when it was exposed to highly concentrated nicotine (>5.0 mM) for more than 3 days.
3. Osteocalcin increased one day after treatment with low concentrations of nicotine (<5.0 mM), compared with the control group. At the high nicotine concentrations of more than 7.5 mM, however, osteocalcin decreased as the concentration of nicotine increased and the same result was recorded when the osteoblasts were treated with low nicotine concentrations of less than 1.0 mM for longer than 3 days.
4. A day after treatment with nicotine, the level of mRNA increased as in the case of protein. But, unlike the case of protein, the level continued increasing at the high nicotine concentrations of more than 7.5 mM. Contrarily, there was no change in the level of mRNA after 3 days, and it decreased after 6 days.
5. The formation of osteoprotegerin notably declined in all experimental groups, compared with the control group, except that it increased somewat more than the control group at concentrations of 1.0 mM and 2.5 mM after 6 days.
6. The level of osteoprotegerin mRNA increased at high nicotine concentrations of more than 7.5 mM after 3 days and more than 5.0 mM after 6 days. However, there were no significant changes in the other experimental groups.

From the above results, when we treat the patient who have smoked tobacco for a long time, we need to pay attention to the correlation between nicotine and the activity of osteoblasts. Especially, the formation of osteoprotegerin is decreased at high concentrations of nicotine and this condition invreases osteoclast development. So, we have to pay attention to abnormal alveolar bone resorption which may occur in smokers.

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국문초록

니코틴이MG63조골세포주의오스테오칼신과오스테오프로테제린의 생성및mRNA 발현에 미치는영향

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흡연이 골형성 세포의 기능을 억제한다는 것은 이미 잘 알려져 있으나 대다수의 연구가 주로 역학조사에 기초하여 이루어져 왔다. 본 연구의 목적은 흡연과 골형성 세포의 상관관계를 실험적으로 평가하기 위한 것이다. 본 연구를 위해 인간 골육종 세포인 MG63 조골세포주를 이용하였으며, 니코틴이 조골세포의 증식, 염기성 인산분해효소의 활성, 오스테오칼신과 오스테오프로테제린의 생성 그리고 mRNA 발현에 미치는 영향을 관찰하였다. MG63 조골세포를 니코틴 농도가 각각 1.0 μM, 1.0 mM, 2.5 mM, 5.0 mM, 7.5 mM, 그리고 10.0 mM,이 함유된 배지에서 1일, 2일, 3일 그리고 6일동안 배양 실험을 하여 다음과 같은 결과를 얻었다.

MG63 조골세포의 증식이 저농도의 니코틴에서는 일시적으로 활성화되었지만 5.0 mM 이상의 고농도에서는 억제되었다. 염기성 인산분해효소의 활성은 니코틴 농도가 증가함에 따라 감소하였다.

오스테오칼신 생성양은, 배양 1일 후, 5.0mM 이하의 농도에서 증가하였으나 7.5 mM 이상의 고농도에서는 감소하였다. MG63 조골세포를 3일 배양한 경우, 오스테오칼신 생성양은 1.0 mM의 저농도에서도 감소하였다. 니코틴이 오스테오칼신 단백질 생성과 오스테오칼신 mRNA 생성에 미치는 영향은 1일과 3일에서 다소 차이가 있었다.

오스테오프로테제린의 생성양은 모든 실험군에서 니코틴을 함유하지 않은 대조군보다 감소하였다. 하지만 mRNA 수치는 단백질 생성과 상반되게 7.5 mM (3일), 5.0 mM (6일) 이상의 고농도에서 증가하였다.

주요 단어 : 염기성 인산분해효소, 오스테오칼신, 오스테오프로테제린, mRNA

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