

Nicotine Down-regulates COL1A2 Promoter in Cultured Human Skin Fibroblasts

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Background : It has become generally accepted that cigarette smoking contributes to accelerated coronary and peripheral vascular disease, pulmonary fibrosis and periodontal disease. Moreover, it has been postulated that cigarette smoking causes skin-aging. Many of cutaneous manifestations of nicotine which is a major component of the particulate phase of tobacco smoke are related to its vasoconstrictive and thrombotic effects on the peripheral vascular system. However, direct effect of nicotine on extracellular matrix (ECM) proteins including collagens is not well established.

Objective : To evaluate the effect of nicotine on type I collagen gene expression in cultured human skin fibroblasts.

Methods : After exposure to different doses of nicotine on cultured human skin fibroblasts, we examined the expressions of $\alpha 1(I)$ procollagen gene and fibronectin gene by Northern blot analysis and chloramphenicol acetyltransferase (CAT) assay with CAT construct containing the 3.5 kb COL1A2 promoter.

Results : In Northern blot hybridization, steady-state levels of $\alpha 1(I)$ procollagen mRNA were decreased 0.8-fold at 1 $\mu\text{g}/\text{mL}$ of nicotine, 0.5-fold at 10 $\mu\text{g}/\text{mL}$ and 0.2-fold at 100 $\mu\text{g}/\text{mL}$, compared to untreated control. Those of fibronectin mRNA were decreased 0.9-fold, 0.7-fold, and 0.3-fold, respectively. In CAT assay, the relative COL1A2 CAT activity was 1.0 in the untreated control, 0.7 at a concentration of 1 $\mu\text{g}/\text{mL}$ of nicotine, 0.5 at 10 $\mu\text{g}/\text{mL}$, and 0.3 at 100 $\mu\text{g}/\text{mL}$.

Conclusion : These results indicate that nicotine is a down-regulator of collagen gene expression at transcriptional level in vitro. We speculate that nicotine may contribute to the skin-aging by modulation of extracellular matrix gene expression including collagen as well as by its vasoconstrictive and thrombotic effects. (*Ann Dermatol* 13(3) 153~157, 2001).

Key Words : Nicotine, Collagen, In vitro

Nicotine, the active ingredient of tobacco is a tertiary amine containing a pyrimidine and pyrrolidine ring. Nicotine causes increased platelet aggregation, endothelial cell damage, and alterations in

prostacyclin and thromboxane metabolism¹. Skin changes with nicotine include brown pigmentary staining of hands and nails, leg ulcers, cyanosis, decreased skin temperature, and other signs of arterial insufficiency². Moreover, it has been reported that tobacco smoking promoted skin-aging and when smoking and sun exposure coexisted, the effects on wrinkling were synergistic^{3,4}. In vivo effects of nicotine are as the result of its interaction with receptor systems such as cholinergic nicotinic gating sites, which in turn, causes the release of neurotransmitters such as serotonin and cate-

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Table 1. Steady-state levels of $\alpha 1(I)$ procollagen and fibronectin mRNA in cultured skin fibroblasts treated with nicotine

Nicotine ($\mu\text{g/mL}$)	$\alpha 1(I)$ procollagen/GAPDH	Fibronectin/GAPDH
0	980 \pm 30 (1.0)	294 \pm 21 (1.0)
1	784 \pm 25 (0.8)	264 \pm 18 (0.9)
10	490 \pm 20* (0.5)	205 \pm 15* (0.7)
100	196 \pm 12* (0.2)	87 \pm 8* (0.3)

The values are mean \pm SD and expressed as densitometric absorbance unit which are the percentage of the value of GAPDH.

* significantly different from control ($p < 0.05$)

() : fold difference

Table 2. CAT assay of COL1A2 promoter activity in cultured skin fibroblasts treated with nicotine

Nicotine ($\mu\text{g/mL}$)	% of acetylation	Fold difference
0	10.3 \pm 1.5	1.0
1	7.2 \pm 1.3	0.7
10	5.1 \pm 0.8*	0.5
100	2.0 \pm 0.6*	0.2

The values are mean \pm SD and expressed as fold induction, as compared to control.

* significantly different from control ($p < 0.05$)

CAT : chloramphenicol acetyltransferase

cholamines⁵. Direct regulatory effects of nicotine on various cell types have been reported and seem to be dependent on the cell type. In cultured skin fibroblasts, tobacco smoking extracts lead to increased collagen production⁶. However, little is known about the molecular mechanism underlying direct effect of nicotine on extracellular matrix (ECM) proteins including collagens.

Consequently, we examined the effect of nicotine on type I collagen gene expression by Northern blot analyses, chloramphenicol acetyltransferase (CAT) assay in cultured human skin fibroblasts.

MATERIALS AND METHODS

Fibroblast Culture

Primary cultures of dermal fibroblasts were established from adult's skin (n=3, mean age: 28-years-old) left over from surgery and subcultivated on 100mm plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and amphotericin B (1 $\mu\text{g/ml}$). The cells were maintained in a humidified 5% CO₂ - 95% air incubator at 37°C. Analyses of

confluent fibroblast cultures were carried out at 3 - 6 passages of subcultivation.

Treatment of Skin Fibroblasts with different Doses of Nicotine

Nicotine was purchased from Sigma (St Louis, MO) and dissolved in 95% ethanol. We treated cultured skin fibroblasts with different doses of nicotine (1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$). Cell viability was determined by trypan blue exclusion 24h after treatment. Survival rates were more than 90% in all experiments presented. All experiments were performed in triplicate.

Northern Analyses

Total RNA was isolated by the method of Chomczynski and Sacchi⁷ from cultured human skin fibroblasts. Extracted RNA were analyzed by Northern hybridization with [³²P] labeled 1.8-kilobase pair (kb) $\alpha 1(I)$ procollagen cDNA probe⁸ and [³²P] labeled 1.3-kb fibronectin cDNA probe⁹, respectively. The [³²P] cDNA-mRNA hybrids were visualized by autoradiography, and the steady-state levels of mRNA were quantitated by laser densitometer (LKB Instrument, Inc., Bromma, Sweden). α

Fig. 1. Northern blot analysis of $\alpha 1(I)$ procollagen and fibronectin mRNA transcripts from cultured normal skin fibroblasts treated with nicotine. The relative mRNA levels were determined by scanning densitometry.

1(I) procollagen and fibronectin mRNA levels were standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the same samples¹⁰.

CAT Assay

The construct containing the 3.5-kb COL1A2 promoter fused to the CAT gene (pMS3.5/CAT) was derived from a 3.5-kb EcoRI/SphI genomic subclone that spans from position -3500 to +58 of the COL1A2 promoter¹¹. After conversion of the EcoRI site to BamHI, the eukaryotic insert was excised with BamHI and Hind III and subcloned in the similarly digested polylinker of the expression vector p8-CAT a derivative of the pEMBL plasmid¹². Thereafter deletion mutants were generated in the parental pUC18 subclones and then transferred into the expression vector. The transfections were performed with the calcium phosphate/DNA coprecipitation method¹³, followed by a 1 min (15%) glycerol shock. Four hrs after transfection, cells were treated with different doses of nicotine. After a total of 24hrs incubation, the cells were harvested, as described previously¹⁴. CAT activity was determined by incubating cell extracts with [¹⁴C] chloramphenicol, followed by separation of its acetylated and non-acetylated forms by thin-layer chromatography as described elsewhere¹⁵. CAT activity in the cell extracts was calculated by the radioactivity in the acetylated forms as a percent of the total radioactivity in each sample.

Fig. 2. Effects of nicotine on COL1A2 promoter activity in cultured normal skin fibroblasts. The figure shows the CAT assay depicting a separation of acetylated (AC) and unacetylated (C) form of [¹⁴C] chloramphenicol by thin layer chromatography.

Statistics

Statistical examination of the data was performed using Wilcoxon's rank sum test¹⁶. $p < 0.05$ was considered significant.

RESULTS

Effect of Nicotine on the Steady State Levels of $\alpha 1(I)$ procollagen and fibronectin mRNA

To examine the effect of nicotine on $\alpha 1(I)$ procollagen and fibronectin genes expression at mRNA level, cultured human fibroblasts were exposed to different doses of nicotine, and the corresponding mRNA levels were determined by Northern hybridizations (Fig. 1). In both nicotine treated and untreated cell cultures, characteristic 5.8 and 4.8-kb mRNA transcripts for human $\alpha 1(I)$ procollagen and 8.0-kb mRNA transcripts for fibronectin were detected. The steady-state levels of $\alpha 1(I)$ procollagen mRNA were decreased 0.8-fold at 1 $\mu\text{g}/\text{mL}$ of nicotine, 0.5-fold at 10 $\mu\text{g}/\text{mL}$ and 0.2-fold at 100 $\mu\text{g}/\text{mL}$, compared to untreated control. Those of fibronectin mRNA were decreased 0.9-fold, 0.7-fold, and 0.3-fold, respectively (Table 1).

Transcriptional Regulation of the Type I collagen Gene by Nicotine

To examine the effect of nicotine on Type I collagen promoter activity, transient transfections were performed. The construct containing the 3.5-kb COL1A2 promoter fused to the CAT gene (pMS3.5/CAT) was derived from a 3.5-kb EcoRI/SphI genomic subclone that spans from position -3500 to +58 of the COL1A2 promoter. The pMS3.5/CAT were transfected into the cultured skin fibroblasts and CAT activities were measured. Following treatment with different doses of nicotine, COL1A2 promoter activities were decreased in a dose dependent manner (Fig. 2). The relative COL1A2 CAT activity was 1.0 in the untreated control, 0.7 at a concentration of 1 $\mu\text{g}/\text{mL}$ of nicotine, 0.5 at 10 $\mu\text{g}/\text{mL}$, and 0.3 at 100 $\mu\text{g}/\text{mL}$, respectively (Table 2).

DISCUSSION

We found that nicotine down-regulated type I collagen gene expression at the transcriptional level. The inhibition of type I collagen gene expression by nicotine was dose-dependent. The results of this study demonstrated down-regulation of collagen and fibronectin by nicotine in cultured human skin fibroblasts. Direct regulatory effects of nicotine on various cell types have been reported and seem to be dependent on the cell type. In many types of cells such as osteoblast-like cells¹⁷ and gingival fibroblasts¹⁸ nicotine inhibits growth of cells and their production of collagen and fibronectin, while also promoting collagen breakdown. Whereas in cultured skin fibroblasts, tobacco smoking extracts lead to increased collagen production⁶. Furthermore, little is known about the molecular mechanism underlying direct effect of nicotine on extracellular matrix (ECM) proteins including collagens. Tomek *et al.*¹⁹ found that treatment of cardiac fibroblasts with nicotine (10 $\mu\text{g}/\text{ml}$ equivalent to 62 μM) led to decrease in the abundance of mRNA for α -1(I) procollagen. Our results are well in accordance with these results. However, other investigations⁶ using cultured dermal fibroblasts have found that low levels of nicotine (35 μM) in aqueous tobacco smoke extract increased collagen synthesis. These results are in contrast to the inhibition of collagen production seen in the present study and other studies¹⁷⁻¹⁹. This discrepancy may

be the presence of certain non-nicotine compounds in the aqueous tobacco smoke extract and/or factors such as differences in the species or the tissue of origin or age of fibroblasts.

Tipton and Dabbous¹⁸ have reported that in the cultured gingival fibroblasts the inhibition of collagen production by nicotine was accompanied by increases in collagenase activity. The basis for the inhibition of collagen production was unknown at that time, because they did not study the abundance of mRNA and promoter activity for type I collagen. In the present study, we found that nicotine down-regulated type I collagen gene expression at transcriptional level *in vitro*. Therefore, the underlying molecular mechanisms for the inhibition of collagen production by nicotine may be increased collagenase activity and/or decreased collagen gene expression.

We found that nicotine is a down-regulator of collagen gene expression at transcriptional level *in vitro*. Taken together, we speculate that nicotine may contribute to the skin-aging by modulation of extracellular matrix gene expression including collagen as well as by its vasoconstrictive and thrombotic effects.

REFERENCES

1. Benowitz NL: Pharmacologic aspects of cigarette smoking and nicotine addiction. *N Engl J Med* 319: 1318-1330, 1988.
2. Moy JA: Cutaneous manifestations of drug abuse. In Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds): *Dermatology in General Medicine*. McGraw-Hill Inc, New York, 1993, pp1811.
3. Weiss W: Smoker's wrinkles. *JAMA* 226:788-789, 1973.
4. Lee SH, Chung JH, Yoon CS, Eun HC: Photoaging in asian skin: New Grading system and the influence of environmental factors. *Kor J Invest Dermatol* 7 (suppl 1) : 66, 2000.
5. Richard JL, Ducimetiere P, Bonnaud G, Claud JR, Lellouch J, Schwartz D, Dimatteo J: Incidence et evaluation du risque de maladie coronarienne. *Arch Mal Coeur Vaiss* 70:531-540, 1977.
6. Chamson A, Frey J: Effects of tobacco smoke extracts on collagen biosynthesis by fibroblast cell cultures. *J Toxicol Env Health* 9:921-937, 1982.
7. Chomczynski P. and Sacchi N: Single step method of RNA isolation by guanidium thiocyanate-phe-

- nol-chloroform extraction. *Anal Biochem* 162:156-159, 1987.
8. Chu ML, de Wet W, Bernard MP, Ding JF, Morabito M, Myers J, Williams C, Ramirez F: Human pro α 1(I) collagen gene constructure reveals evolutionary conservatism of a pattern of introns and exons. *Nature* 310:337-340, 1984.
 9. Oldberg A, Linney E, Ruoslahti E: Molecular cloning and nucleotide sequence of cDNA clone coding for the cell attachment domain in human fibronectin. *J Biol Chem* 258:10193-10196, 1983.
 10. Fort P, Marty L, Piechaczyk M, El SS, Danz C, Jeanteur P, Blanchard JM: Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multi-gene family. *Nucleic Acids Res* 13:1431-1442, 1985.
 11. Dickson LA, de Wet W, DiLiberto M, Weil D, Ramirez F: Analysis of the promoter region and the N-propeptide domain of the human pro alpha2(I) collagen gene. *Nucleic Acids Res* 13: 6117-6125, 1985.
 12. Dente L, Sollazzo M, Balderi C, Cesareni G, Cortese R: *DNA Cloning*. IRL Press, Washington, 1985, Vol I pp 101-107.
 13. Graham F. and Van der EB: A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467, 1973.
 14. Quinones S, Buttice G, Kurkinen M: Promoter elements in the transcriptional activation of the human stromelysin-1 gene by the inflammatory cytokine, interleukin 1. *Biochem J* 302:471-477, 1994.
 15. Sambrook J, Maniatis T, Frisch EF: *Molecular cloning in laboratory manual*, 2nd Edn., Cold Spring Harbor Laboratory, New York, 1989, pp 172-184.
 16. Armitage P, Berry G: *Statistical methods in medical research*. 3rd Edn., Oxford Blackwell Scientific Pub, Oxford, 1994, pp453-454.
 17. Ramp WK, Lenz LG, Galvin RJS: Nicotine inhibits collagen synthesis and alkaline phosphatase activity, but stimulates DNA synthesis in osteoblast-like cells. *PSEBM* 197:36-43, 1991.
 18. Tipton DA, Dabbous MK: Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts in vitro. *J Periodontal* 66:1056-1064, 1995.
 19. Tomek RJ, Rimar S, Eghbali-Webb M: Nicotine regulates collagen gen expression, collagenase activity, and DNA synthesis in cultured cardiac fibroblasts. *Mol Cell Biochem* 136:97-103, 1994.
 20. Sams WM Jr: Sun-inducing aging. Clinical and laboratory observations in man. *Dermatol Clin* 4:509-516, 1986.
 21. Fisher GJ, Datta SC, Talwar HS, Wang ZQ, Varani J, Kang S, Voorhees JJ: Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature* 379:335-339, 1996.