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MITOGENIC EFFECTS OF NICOTINE TO HUMAN PERIODONTAL LIGAMENT(PDL) CELLS *IN VITRO.*

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Nicotine is one of the major components of cigarette smoking which causes various systemic and local diseases to human body. Mitogenic effects of nicotine to systemic disease are interesting factors in the results of cellular proliferation especially to vascular and pulmonary tissue or cells. The study of local effects concerns with destruction of tissue and delayed healing rate after various surgical treatment.

Platelet-Derived Growth factor(PDGF) and Insulin-like growth factor(IGF) are known as major mitogens to human PDL cells.

The purpose of this study was to investgate the mitogenic effects of nicotine to human PDL cells. We studied the expression of PDGF-α receptor, PDGF-βreceptor, and IGF-1 receptor mRNA from the nicotine treated human PDL cells by northern analysis.

The experimental groups were divided into different serum(1%, 10%) and nicotine(100ng/ml,1000ng/ml) concentrations and each group was studied by time course.

The results of this study showed upregulation of PDGF-α, β receptor and IGF-1 receptor mRNA at 100ng/ml nicotine concentration and 10% serum group to the time course. These results suggest that physiologically attainable nicotine concentrations may stimulate the mitogenic gene synthesis to human PDL cells *in vitro.*

Key Words : Nicotine, PDL cell, Mitogenic effect, Northern analysis.

Nicotine, which is a major component of cigarette smoking, is one of few natural liquid alkaloids. It is colorless, voletile base(pKa=8.0-8.5) that turns brown and acquires the odor of tobacco on exposure to air. It possesses a biphasic action on the adrenal medular. Small doses evoke the discharge of catecholamines, which accelerates cardiac rates, vasoconstriction and raises blood pressure and larger doses prevent their release. Nicotine is readily absorbed from the respiratory tract, buccal membrane and skin. Absorption and excretion of nicotine depend on pH of biological membrane and urin. [1,2]

The effects of nicotine on cellular functions are dose dependent and various to the cell types. Thyberg[3] shows that nicotine stimulates the initiation of DNA synthesis *in vitro* cultivation of smooth muscle cells. In murine embryonic fibroblast, DNA synthetic rates were elevated under repeated exposure to fresh cigarette smoke condition[4]. Litwin et al. studied with human embryonic diploid lung fibroblasts. They show that nicotine affect the stimulation of growth according to concentration. The effect of nicotine in a concentration of 50ug/ml showed toxic and stimulated growth, in 10ug/ml, no effect on cell growth. On the contrary, in

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lower concentration such as 1ug/ml it inhibits their growth[5]. Steven et al. also shows the effect of nicotine to dose-dependent stimulation of epithelial cell growth and conclude that the selective mitogenic effect of nicotine may be related to an increased risk of squamous cell carcinoma[6]. But nicotine does not always stimulate the growth or proliferation of the cell. Konno et al. showed that nicotine inhibits the cellular proliferation on human promyelocytic HL-60 leukemia cells and BALB/ C3T3 cells and suggests that one of the main effect of nicotine is inhibition of protein synthesis and acts as a pleiotropic effector and is capable of inducing growth modulating factor[7,8]. In 1991, Fang et al. studies with osteoblast-like cells, they showed that nicotine surpress cellular proliferation and stimulates alkaline phosphatase activity in UMR 106-01 osteoblast-like cells. He suggests the cause of some different results from his is reflection of species cell culture conditions, differences and types of cell model used, degree of cell confluence, and concentration of nicotine studied, or interaction of nicotine or its metabolites with factors in the medium[9].

Platelet-Derived Growth Factor(PDGF) is one of the principle mitogens in human blood serum that exert its effect on mesenchymal cells, such as fibroblast and glial cells. PDGF has 3 dimeric isoform(AA,BB and AB) with the AB heterodimer as the most abundant species and two different PDGF receptor population, such as α and β, of which PDGF α-receptor binds all the PDGF isoform and the β-receptor binds only PDGF-BB dominantly[10]. PDGF-AA and -BB are reported to major mitogen for human PDL cells in vitro [11,12]. The β-type receptor for PDGF predominantly mediates a chemotactic response by means of ligend-induced activity of the receptor protein-tyrosine kinase [13] and PDL fibroblastic cells have predominantly the β-type of PDGF receptor[14].

Insulin-like Growth Factor(IGF) is growth-promoting peptides which is structually related to insulin and interact primarily with the IGF-1 receptor[15]. The combination of PDGF and IGF synergetically stimulates proliferation and chemotaxis of rat PDL fibroblastic cells and promote mitogenesis and protein synthesis in mesenchymal cells in culture[14,16].

The purpose of this study was to investigate the expression of these mitogenic growth factor receptor gene from the nicotine treated human PDL cells by northern analysis.

MATERIALS AND METHOD

CELL CULTURE

Human PDL cells were kindly received from Dr. NAOKI MATSUDA(JAPAN).

The cells were used in experiment between 8 and 13 passages and were cultured Dulbecco's modified eagles medium(DMEM), supplemented with non essential amino acids, 10mM sodium pyruvate, MEM vitamins, antibiotic and antimycotic solution and 10% fetal bovine serum.

Cells were cultured in 100mm culture dish(Falcon[R]) and maintained at 37°C in humidified atmosphere at 5% CO_2. Culture medium was changed every otherday. When cells were reached 90-95% confluence, the cell were collected by digestion with 0.15% trypsin and 0.5mM EDTA in Hank's balanced salt solution then plated into 100mm culture dish at 1 to 3 split ratio.

NICOTINE TREATMENT

(-)-Nicotine was purchased from SIGMA chemical company(St. Louis,MO USA). Experimental groups were divided into 0(control), 4, 8, 12, 24, 48, 96 hours nicotine treatment in 10% serum group and 0, 4, 8, 12, 24 hours in 1% serum group. Each time course group was subdivided by nicotine concentration at 0.01% (100ng/ml) and 0.1%(1000ng/ml).

In 1% serum group, when cultured cells were reached 80-90% of confluence, culture medium was changed with 1% serum concentration and incubate overnight then next morning nicotine was added to each concentration by time course as mentioned above.

In 10% serum group, the medium of 48 and 96 hours samples were changed everyday by half volume of nicotine contained medium for partly refreshing the nicotine concentration.

TOTAL RNA ISOLATION

Total RNA was isolated at each stage of time course from the sample using Guanidium thiocynate(GuSCN). Briefly described method, the cells were washed with cold PBS buffer immediately. 4ml of 4.2M GuSCN containing 25mM sodium citrate and β-mercaptoethanol(pH 7.0) were added for cell lysis and homogenization, and then it was transferred into ultratube. 25mM acetic acid and absolute ethanol were added and vortex completely and precipitated overnight at -20°C. Next day ultratube was centrifuged at 9,000 rpm for 15 min. at 4°C. Supernatant was carefully discarded and RNA pellet was suspended in 7.5M Guanidium hydrochloride (GuHCl) containing 25mM sodium citrate and 5mM Dithiothreitol (DTT; Fisher Biotech.) and precipitate with acetic acid and absolute ethanol overnight at -20°C. Next day RNA was precipitated at 9,000 rpm for 15 min. at 4°C then resuspended with half volume of GuHCl to second day procedure and transfer to 2.0ml tube and precipitate as same manner as mentioned above. On the final day RNA was precipitated at 14,000 rpm for 10 min. at 4°C. Final pellet of RNA was suspended in FORMAsol(Molecular Research Center, Cincinati,OH): then the OD 260/280 nm for total amount of RNA and purity were checked by using spectrophotometer.(SPECTRONIC GENESYS 5)

NORTHERN BLOTTING

Fifteen micrograms of total RNA from each sample was electrophoresed on a 1% agarous gel with 4.0mM 3-(N-morpholion) propanesulfonic acid, 10mM sodium acetate, 1mM EDTA with 0.37M of formaldehyde.

RNA was run at constant voltage of 5 V/cm for 3 hours with GIBCO BRL 0.24-9.5 RNA ladder.

RNA in gel was transferred to the $Nytran^+$ membrane(Schleicher & Schull, Keene, NH 03431) by downward capillary blotting using TURBOBLOTTER (Schleicher & Schull, Keene, NH 03431) with alkaline transfer solution -8mM NaOH-3M NaCl containing 2mM N-Laur sarkosine.

After 3 to 4 hours of capillary blotting, the membrane was neutralized in 0.5M Tris-HCl, pH 7.0, 1.5M NaCl for 5 min. and baked for 20 min. at 80°C for fixation.

DNA PROBE AND cDNA LABELING

DNA probes which were used in this study were as follows. Human PDGF-βR insert of 2.75kb purchased from ATCC. Rat PDGF-αR insert of 1.5kb from F. Hoffman-La Roche Ltd. Switzerland. Human IGF1R insert of 0.7kb from ATCC. Human GAPDH insert of 1.4kb from Dr. P. Bradford. Each probes were labeled with (α-^{32}p)-dCTP (>3,000 Ci/mmol, Du Pont NEN Research Products, Boston MA) using DECA prime DNA labeling kit(Ambion Inc. Austin Texas) for nothern analysis.

HYBRIDIZATION AND AUTORADIOGRAPH

Prehybridization and hybridization of membrane was performed by hybridization solution (50% formamide, 5xDenhardt's reagent, 0.5% SDS, 5xSSPE with 100μg/ml denatured salmon sperm DNA) in the Hybaid mini hybridization oven (Labnet, Woodbridge NJ). Hybridization was done overnight at 42°C.

After hybridization, the membrane was started get washed with 2xSSPE and 0.1% SDS at 42°C for the removal of unincorporated labelled cDNA probe. After washing, membrane was autoradiographed on FUJI X-ray films at -70°C. Duration of autoradiograph was determined by the radioactivity of the menbrane. After film processing, each film was analyzed with

Densitometer(Phamacia, LKB Biotechnology S-75182 Uppsala, Sweden) for standadization.

RESULTS

Fig.1. demonstrates the expression of mRNA by autoradiograph. They were compared with GAPDH mRNA for standadization of loading amount to electrophoresis in gel, and then they were calculated to folds from the control as basic value.

Fig.2-A shows time coures expression of PDGF-βR mRNA in human PDL cells in 1% FBS medium. There was no significant changes at concentration of 100ng/

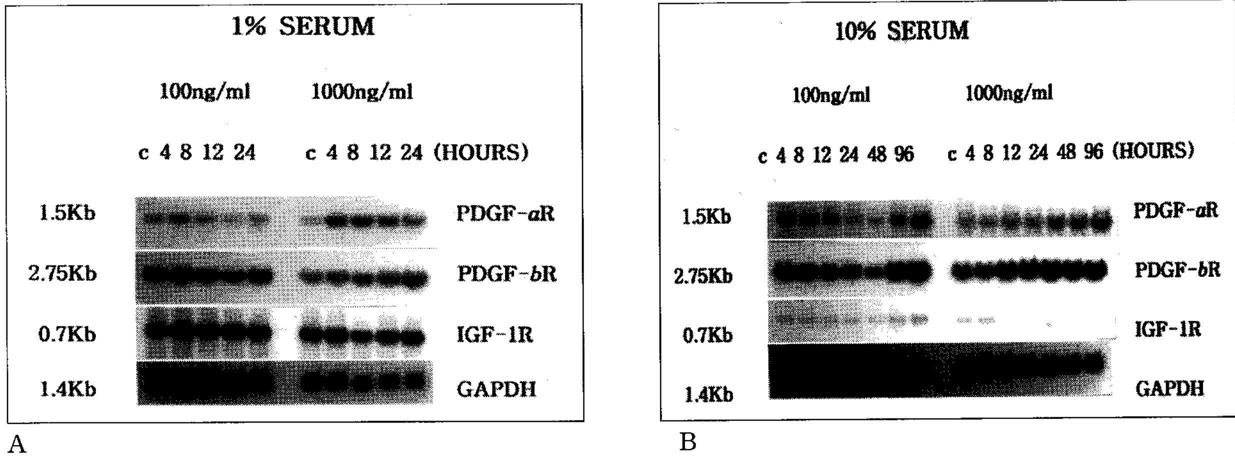

Fig.1. The expression of mRNA in 1%(A) and 10%(B) serum groups.

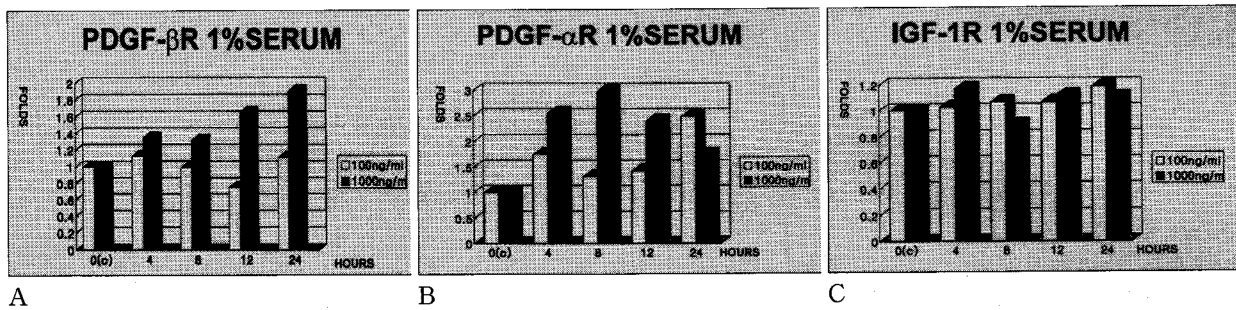

Fig. 2. The expression of PDGF-bR(A), PDGF-aR(B), and IGF-1R(C) in 1% serum experimental group.

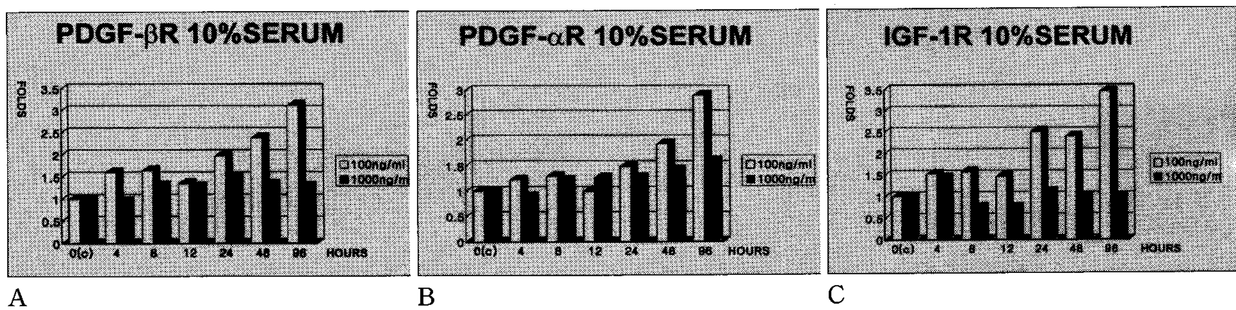

Fig. 3. The expression of PDGF-bR(A), PDGF-aR(B), and IGF-1R(C) in 10% serum experimental group.

ml nicotine treated group but gradually upregulated at concentration of 1000ng/ml.

The expressions of both mRNA of PDGF-αR and IGF-1R at 100ng/ml and 1000ng/ml nicotine concentrations in 1% serum groups show no more significant changes Fig.2-B,C.

But in all 10% serum groups, nicotine upregulated the expression of PDGF-αR, PDGF-βR, and IGF-1R

mRNA at 100ng/ml concentration according to time course, but at concentration of 1000ng/ml, no significant changes were shown. Fig.3-A,B,C.

DISCUSSION

There are many studies about the serum concentrations of nicotine in smokers. It is various from 10 to 100ng/ml according to human subject and study methods, but usually the average serum concentration of nicotine is less than 100ng/ml. In vitro study, toxic effects of nicotine is much higher than serum level. Steven et. al.[6] reported the toxic effect of nicotine at 100ug/ml to 10mg/ml to human cervical mucose cell, but in 100ng/ml to 10ug/ml no toxic effect eventhough stimulates proliferation. They concluded that in physiologically attainable concentration, nicotine does not impair and occasionally enhance the proliferation of human cervical cells in vitro. Fang et al[9] also reported toxic effect at 1uM(160ng/ml) to 10mM(1.6mg/ml) nicotine concentrations in UMR 106-01 osteoblast-like cells. For this study we decided the nicotine concentrations of experimental group as 100ng/ml and 1000ng/ml, which almost near the maximum serum concentration and ten fold of it.

The half-life of a drug is useful in predicting the rate of accumulation of that drug in the body with repetitive doses, and the time course decline after cessation of dosing. Usually, nicotine has a 2 hours of half-life in blood or serum level, and would predict accumulation over 6-8 hours of regular smoking. But smoking represents a multi-dosing situation with considerable accumulation while smoking and persistant levels for 24 hours of each day in habitual smokers[1].

In vitro study, as use of serum free or low-serum media, it is advantageous that it improves reproducibility between cultures and causes less protein interference in bioassay[17]. But starvation of cell is unfavorable for long term culture condition. That is the reason for limited treatment time in low-serum experiomental group within 24 hours.

As results showed in 1% serum PDGF-βR was upregulated at 1000ng/ml but no significant changes at 100ng/ml with control. On the contrary, in 10% serum group, both receptor of PDGF showed similar upregulatory patterns at 100ng/ml and no remarkable changes at concentration of 1000ng/ml. From the data of IGF-1R, there was no significant changes in 1% serum at both concentrations. But in 10% serum group showed similar upregulatory pattern with PDGF-R of 10% serum groups. These findings suggest that physiologically attainable concentration of nicotine could influence to mitogenic effect of human PDL cell.

The mitogenic effects of nicotine is interesting in relation to lung cancer, which demonstrates a strong epidermiological link with cigarette smoking.

Schuller et. al.[18] suggest that nicotine may selectively stimulate proliferation of neuroendocrine lung cells in smokers on the base of his data, which demonstrate dose related mitogenic effect of nicotine.

There are many clinical reports of nicotine effects to periodontal disease such as alveolar bone destruction [19,20], or soft tissue pathogenesis like stomatitis nicotina, hyperkeratosis mucosa and gingiva. Also it is reported to delay the healing rate after periodontal surgery[21], extraction socket[22] and related to dental implant failure[23]. These effects are explained by inflammatory reaction of nicotine as stimulation of production PGE or IL-1, which is a mediator of those reactions.

Peacock et al.[24] studied about the effect of nicotine on reproduction of human gingival fibroblasts(HGF) in vitro by mitochondrial dehydrogenase activity for determining the cell numbers. They concluded that low concentration of nicotine had a stimulatory effect on cell replication, while higher concentrations of nicotine appear to have no significant effect on HGF reproduction. But Tipton and Dabbous[25] reported the effect of nicotine which inhibits the proliferation of human gigival fibroblast by the incorporation of [^{3}H]-thymidine into DNA *in vitro*. The origin of gingival fibroblast(GF) and periodontal fibroblast(PF) is different. The former is developed from the oral epithelium which originate from ectoderm and the later develop from ectomesenchyme reaction mainly from mesenchymal origin.

And also they have different role to adapt their function to the environment.

There are some report about the different characteristics between GF and PF. Hou and Yaeger[26]

concluded GF and PF contain functionally heterogenous subpopulations and synthesis and expression of extracellular matrix molecule of GF may be essentially different from that of PF. Ogata et al.[27] also reported the difference between human gingival fibroblast(HGF) and human periodontal ligament cells(HPDL). They found HPDL had a sharper spindle shape and exhibit a higher growth rate than HGF morphlogically, and HPDL cell have higher proliferation rates than HGF which shows higher levels of cAMP production and greater ALPase activity. From these results, we can suggest the different response to nicotine stimulation between GF and PF.

In northern analysis, we can detect the expression of mRNA from the experimental cell. The pathway or mechanism of nicotine effect to the cells are not clear yet, but it stimulates the cell through certain way. The mRNA can be replicated by transcription from the genetic information of coded gene or signals from extracellular stimulations. It can make protein through translation from ribosome and affect the cell function by the autocrine or paracrine. But in some cases, certain mRNA or protein may be inactiveted by mRNA degradation control factor or protein activity control factor in cytosol. For this reason, further study will be needed to confirm the mitogenic results like cellular proliferation assay or morphologic changes by electronic microscope.

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국문초록

In Vitro에서 니코틴이 치주인대세포에 미치는 세포분열효과에 대한 연구

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흡연의 주성분중의 하나인 니코틴은 인체내에 다양한 전신적 및 국소적인 질환의 원인으로 보고 되어지고 있다. 전신적인 질환에 있어 특히, 호흡기와 순환기 조직세포에 대한 세포분열효과가 많은 연구의 초점이 되어왔으며, 국소적인 효과에 대한 연구에서는 조직파괴나 치료후 치유지연에 대해 보고하고 있다.

Platelet-Derived Growth Factor(PDGF)와 Insulin-like Growth Factor(IGF)는 치주인대세포의 세포분열을 촉진하는 주요 성장인자로 알려져있다.

본 연구의 목적은 니코틴이 사람의 치주인대세포에 미치는 세포분열효과를 알아보기 위하여 니코틴 처리된 치주인대세포로부터 추출한 PDGF-α and β receptor 및 IGF-1 수용기의 mRNA 변화를 Northern 분석을 이용해 확인해 보고자 함이다.

실험군은 각기 다른 농도의 니코틴(100ng/ml, 1000mg/ml)과 배양액내 혈청농도(1%, 10%)로 나누었으며 이를 각각 니코틴 처리 시간에 따라 분류하였다.

본연구의 결과로 10% 혈청의 배양액과 100ng/ml 니코틴 농도군에서 모든 성장인자 수용체의 mRNA가 증가됨을 보였으며 이는 흡연자의 체내 축적 가능한 니코틴 농도에서 치주인대세포의 세포분열을 촉진한다는 추측을 가능케 한다.

주요 단어 : 니코틴, 치주인대세포, 세포분열효과, Northern 분석