Nicotine acts directly on growth plate chondrocytes to delay endochondral ossification through the alpha7 homopentameric neuronal nicotinic acetylcholine receptor

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INTRODUCTION

Though detrimental effects of cigarette smoking to the human body have been widely demonstrated to date, the effects for endochondral ossification are not well studied. Epidemiologically, maternal smoking reduces a height of newborns. Besides, smoking delays chondrogenesis in a mouse model of fracture healing. Cigarette smoking, thus, adversely affects endochondral ossification somehow in the course of skeletal growth and repair.

Among a great number of chemicals and physiological functions of cigarette smoking, nicotine is one of the candidates for the cause of delayed endochondral ossification. However, its possible mechanism remains unclarified. We hypothesized that nicotine affects growth plate chondrocytes directly to delay endochondral ossification. We here demonstrate that nicotine affected growth plate chondrocytes through alpha7 nAChR to decrease the matrix synthesis and to suppress hypertrophic differentiation, consequently to delay endochondral ossification.

METHODS

Human growth plate chondrocytes were isolated from epiphysis of extra fingers, which were surgically excised from patients with polydactyly. Ethical approval to perform this study was granted by the Institutional Review Board of the National Research Institute for Child Health and Development, Tokyo, Japan (88).

To detect the expression of nAChR, RTPCR and western blot analysis were done using primary chondrocyte culture. Expression of nAChR was also confirmed immunohistochemically in murine growth plate. The effect of nicotine on growth plate chondrocyte in vitro was determined in cultivation in agarose gel (four weeks) and in alginate beads (four months).

To study the intracellular signals after nicotine stimulation, we performed calcium imaging assay for primary chondrocyte cultures, since alpha7 nAChR has large Ca²⁺ permeabilities and also induces elevated intracellular free calcium by releasing it from intracellular calcium stores.

To study the effect of nicotine on endochondral ossification in vivo, ovulation-controlled pregnant C57BL/6J mice and alpha7 nAChR +/- mice (mated with alpha7 nAChR +/- male mice) were given drinking water with or without nicotine during pregnancy, and skeletal growth of their fetuses was observed histologically in every genotype of fetuses.

RESULTS

Western blot analysis revealed that chondrocytes produced alpha7 nAChR. Alpha7 subunit was detected at resting, proliferating and pre-hypertrophic chondrocytes of murine growth plate but not hypertrophic chondrocytes. These results suggest that the growth plate chondrocytes in their non-hypertrophic stage express alpha7 homopentameric nAChR.

In agarose gel culture, nicotine decreased the percentage of the colonies which produce matrix as revealed by alcian blue stains in a concentration-dependent manner. Similarly, nicotine suppressed Col X expression and enzyme activity of alkaline phosphatase (ALP) in a concentration-dependent manner. In contrast, nicotine did not affect the colony density and the number of cells per colony which are indicators for cell proliferation. These results suggest that nicotine decreases the matrix synthesis and suppresses hypertrophic differentiation of growth plate chondrocytes, but has little effect on cell proliferation in vitro. To investigate if nicotinic effect is mediated by alpha7 nAChR, we used MLA, the specific antagonist of alpha7 nAChR. MLA clearly reverted the nicotinic effect as assessed by Al-B-stained colonies, suggesting the involvement of alpha7 nAChR in nicotinic effect on growth plate chondrocytes.

In Alginate bead culture, nicotine dose-dependently suppressed Al-B- and Safranin-O-stained area at four months. Nicotine dose-dependently decreased the expression of Col II, aggrecan, Col X, Al-P, and Ihh gene at four months. These findings suggest that nicotine suppresses matrix synthesis and hypertrophic maturation of chondrocytes in alginate beads long-term culture.

Nicotine elicited a transient increase of intra-cellular calcium in a concentration-dependent manner. MLA, the specific antagonist of alpha7 nAChR, inhibited the calcium signals, implying that the effect of nicotine on chondrocytes is mediated through the alpha7 nAChR.

Maternal nicotine exposure significantly reduced the femur length (FL) and the hypertrophic length (HL)/FL of E15.5 fetuses, suggesting that nicotine delayed endochondral ossification. Nicotine significantly reduced FL and HL/FL in alpha7 nAChR +/- fetuses but not in alpha7 nAChR +/- fetuses. However, nicotine did not significantly affect body weight (BW) in both genotypes. Besides, scatterplot and correlation between the FL and the BW revealed that nicotine downwardly shifted the linear slope in alpha7 nAChR +/- fetuses but had no effect in alpha7 nAChR +/- fetuses. These findings suggest that maternal nicotine exposure decreased the fetal endochondral ossification through the fetal alpha7 nAChR in vivo.

DISCUSSION

Direct effect of nicotine on human growth plate chondrocytes

Cultured human growth plate chondrocytes derived from infant fingers serve a good model for analyzing whether nicotine has direct action to human growth plate chondrocytes. The present findings of nicotinic effect of decreasing matrix synthesis and suppressing hypertrophic differentiation but not proliferation on growth plate chondrocytes in vitro indicate the direct effect of nicotine to growth plate chondrocytes. The findings are consistent with the reports that maternal nicotine exposure has a negative effect on endochondral ossification in animal. Besides, the findings are reasonable considering the fact that longitudinal skeletal growth is partly brought by matrix synthesis and hypertrophic differentiation of chondrocytes.

Involvement of alpha7 nAChR in delayed endochondral ossification

The results with the usage of MLA, the antagonist to alpha7 nAChR, strongly suggest the involvement of alpha7 nAChR in nicotinic effect on chondrocytes in vitro. Such low-molecular weight substances may, however, have additional unclarified action in addition to the “specific” action. The proof of alpha7 nAChR involvement in delayed endochondral ossification was strengthened by the in vivo experiments with alpha7 nAChR gene-disrupted mice. Especially, considering the fact that maternal nicotine exposure caused delayed skeletal growth in only alpha7 nAChR +/- fetuses comparing with their alpha7 nAChR-/- littermates, fetal alpha7 nAChR but not maternal alpha7 nAChR is responsible for the mechanism of nicotine-induced delayed skeletal growth.

Since nicotine exposure has been reported to be epidemiologically and experimentally correlated with maternal effect, i.e., abnormal placental function and blood flow, physiological and pathological function of alpha7 nAChR in growth plate was confirmed by comparing “littermates” of alpha7 nAChR. This comparison confirms involvement of alpha7 nAChR on fetus, and eliminates a possibility of maternal effect. Furthermore, decrease of relative femur length and lack of nicotinic effect on body weight of alpha7 nAChR fetuses by maternal nicotine exposure hint specific effect of nicotine on bone growth rather than symmetrical or systemic effect. Therefore, the effect of smoking during pregnancy to endochondral ossification may be attributed to this direct action of nicotine on growth plate chondrocytes at least in part.

Our studies suggest that nicotine may cause delayed skeletal growth among a great number of cigarette chemicals, and indeed, amniotic fluid and breast milk has higher concentration of nicotine than maternal serum does. In addition, metabolism of nicotine in fetus and child is much slower than that in adult. We therefore should be careful for the effect of smoking, regardless of active or passive smoking, on growth plate chondrocytes. This nicotinic effect may also extend to the case of delayed fracture repair or generation of non-union in adults since process of bone repair also partly depends on endochondral ossification.