Chapter 4

Effects of Nicotine Contained in Tobacco Mainstream Smoke on Vascular Smooth Muscle Cells

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Additional information is available at the end of the chapter

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Abstract

Cigarette smoking is a known risk factor for arteriosclerosis. In atheromatous plaques, the accumulation of vascular smooth muscle cells (VSMCs) have a phenotype differing from that of their normal contractile type. Nicotine is a major pharmacological agent in cigarette smoke. However, any direct effect of nicotine on VSMCs remains uncertain. We investigated the changes in the expression levels of differentiation markers and activity of mitogen-activated protein kinases (MAPKs) after nicotine exposure for 48 h using human aorta primary smooth muscle cells (HVSMC) differentiated with transforming growth factor-β. The results indicated that HVSMC phenotype changed to a synthetic-like phenotype after nicotine exposure. Nicotine is a factor that can change the expression of differentiation marker proteins in VSMCs. Thus, we proposed that nicotine directly affects the migration of VSMCs from the tunica media to atheromatous plaques in the vascular intima by inducing the transformation from a contractile-type to a synthetic-like type, which occurs before the development of atheromatous plaques. Nicotine is contained in nicotine patches and gums for smoking cessation. There may also promote atheromatous plaque formation. We anticipate that determining this mechanism will lead to new means of preventing and treating plaque formation and development in arteriosclerosis.

Keywords: nicotine, vascular smooth muscle, cell migration, proliferation, cigarette smoke

1. Introduction

According to the World Health Organization (WHO) World Health Statistics 2016, the world's highest cigarette smoking rates were 76.2% for men in Indonesia, and 52.0% for women in Nauru. Ranked second place was Jordan for men (70.2%), and Kiribati for women (40.9%),

third place was Kiribati for men (63.9%) and Serbia for women (39.7%). As of 2015, the gender smoking ratio was estimated as 33.7% men and 10.6% women in Japan [1]. Globally, an estimated 93.3 million people smoke, the majority of whom reside in developing countries, where smoking rates are estimated to be as high as 50% for men. It has been shown that men tend to use all tobacco products at a higher rate than women [2]. Atherosclerosis is more common in men than women [3]. It may be derived from this that men have more arteriosclerotic diseases.

Smoking, as well as second-hand smoke, induces circulatory diseases, heart attacks, strokes, cancers, and respiratory diseases [4–7]. Several studies have suggested that cigarette smoke has 7357 chemical compounds from different classes [8]. Nicotine is the most predominant alkaloid (approximately 90–95%), found in the tobacco plant, *Nicotiana tabacum* [9–10]. Plasma nicotine levels have been reported as 4–30 ng/ml after smoking a cigarette, 8–10 ng/ml after chewing nicotine gum, and 22 ng/ml after smoking a pipe [11, 12]. Nicotine is a toxic compound that should be handled with care, as it has been reported that more than 0.5 g of oral nicotine is required to kill an adult [13, 14].

Epidemiological studies show that cigarette smoking has long been known as a major risk factor for atherosclerosis [15–18]. In particular, nicotine in the cigarette smoke promotes atherogenesis [17–20]. However, little is known about the mechanism by which nicotine induces arteriosclerosis. Atherosclerosis is a specific form of arteriosclerosis in which an artery wall

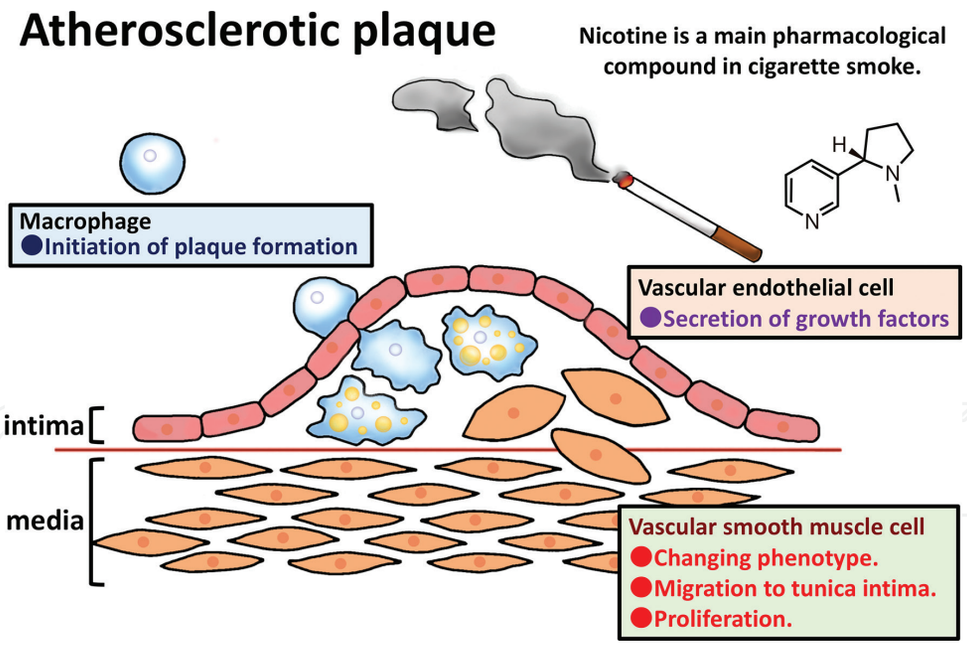

Figure 1. Atherosclerotic plaque is due to the cooperation of three types of cells. The first one is macrophages. Their invasion into tunica intima preludes the formation of plaque. The second is endothelial cells. It secretes some growth factors which affects the VSMC. The third is VSMCs. Under the stimulation, their phenotype changes before migrating to tunica intima and proliferating there. This phenotypic change is referred to as contractile-to-synthetic-type transition and it contributes to the development of plaque. This phenotypic change could be induced by different kinds of stimuli. Nicotine is one of them. Nicotine is a main pharmacological compound in cigarette smoke. It is reported to promote cell migration of rat and human VSMCs. However, little is known about whether nicotine promotes the phenotypic change of human VSMC.

thickens as the result of invasion, accumulation of white blood cells and fatty materials such as foam cells and cholesterol [22, 23] (**Figure 1**). It has also been known that accumulation of vascular smooth muscle cells (VSMCs) can be observed in atherosclerotic lesions (**Figure 1**). The proliferation of VSMCs with the subsequent formation of intimal thickening is a major event in the development of atherosclerotic lesions [24, 25] (**Figure 1**). Normally, the differentiated VSMCs constitute the tunica media of the artery and are responsible for the vasoconstriction function. However, why the VSMCs accumulate during arteriosclerotic plaque formation is not well understood (**Figure 1**).

In this chapter, we describe that nicotine in tobacco mainstream smoke causes dedifferentiation of VSMCs to migration-proliferation types via nicotinic acetylcholine receptors (nAChRs) expressed in the VSMCs, which is a cause of arteriosclerotic plaque formation.

2. The nicotinic acetylcholine receptors (nAChRs) on VSMCs

nAChRs are transmembrane ligand-gated ion channels expressed in the cell membrane of all mammalian cells, and their endogenous ligand is acetylcholine [26]. We were the first to report that nAChRs were expressed on VSMCs [27]. Also, we found that nicotine promotes cell migration of VSMCs GbaSM-4 cells isolated from basilar arteries of guinea pigs, and this cell migration is inhibited by methyllycaconitine, an antagonist of nAChRs [27]. That was the first report on the effect of nAChRs on VSMCs [27]. In subsequent studies of other groups, it was reported that nicotine promoted the chemotaxis and migration of VSMCs isolated from rats and humans [28, 29]. Thereafter, various types of nAChRs have been discovered and reported by real-time qPCR, Western blots, etc. in several tissues [30, 31].

We exposed cell line AC01 cells derived from mouse aortic smooth muscle to 0.1 μM nicotine [32], and performed exhaustive gene expression analysis using DNA microarray for gene expression after 48 h. As a result of whole gene expression analysis, α1, α2, α6, α7, α9, β1, β2, β4, δ, ε, and γ subunits of nAChRs were detected in AC01 cells (**Figure 2A**). After AC01 cells were exposed to nicotine for 48 h, a change was observed in the ratio of the fluorescence intensity of cy3 / cy5 indicating the amount of transcription of mRNA for each subunit. As a result, the α1, α6, α7, β2, and δ subunits increased by 2.6, 2.3, 2.4, 2.0, and 3.1 times, respectively, compared to the control (**Figure 2**).

Furthermore, we measured the expression levels of nAChRs in human vascular smooth muscle cells (HVSMCs) using real-time qPCR. As a result, α2, α6, α7, and β1 subunits of the nAChRs were detected. The expression level of the α2 subunit was relatively low, and it disappeared within 72 h of nicotine exposure. The expression level of the α6 subunit increased with time, to about 20-fold after 72 h as compared with the control (0 h). The α7 subunit was the most frequently expressed in the HVSMCs. The expression level of the β1 subunit was in trace amounts, and from this result, there was no clear influence of exposure to nicotine. Thus, it was discovered that nAChRs were expressed in response to nicotine in HVSMCs [33].

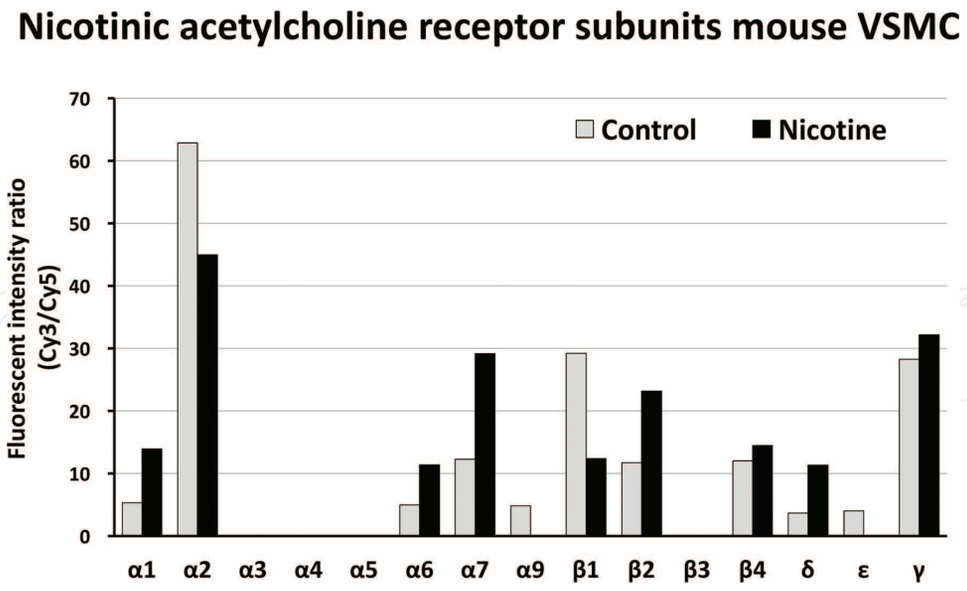

Figure 2. The mRNA levels of nAChR subunits in AC01 cells of mouse VSMC. AC01 cells were exposed to 0.1 μM of nicotine or not exposed for 48 h. Each sample was labeled with Cy3 (nicotine-treated cells) and Cy5 (non-treated cells), resulting in differently labeled samples. The labeled mixture of both samples was applied onto a 3D-gene™ mouse Oligo chip 25 K (Toray Industries, Tokyo, Japan), competitively hybridized, and washed. Scanned images were analyzed using GenePix Pro (MDS Analytical Technologies, Sunnyvale, CA, USA). All analyzed data were scaled by global normalization.

3. Remodeling of vascular smooth muscle by nicotine

Nicotine did not induce any significant changes on the relaxation of tension in isolated VSMCs, despite its effects on the cardiovascular system [34]. However, Carty et al. proposed that nicotine was a mitogenic agent for VSMCs [35]. Previous studies, including our studies, reported that nicotine promotes the chemotaxis and migration of mammalian VSMCs [28, 29]. In addition, we reported that GBaSM-4 cells were promoted in their migratory ability after chronic exposure to nicotine [36]. Normally, the differentiated VSMC have contractile function, but do not migrate or proliferate. VSMC which migrate and proliferate and differentiated VSMCs which on exposure to nicotine start migrating and proliferating in the atherosclerotic plaque of patients with arteriosclerotic disease are different in phenotype from the contractile type VSMC. Apparently, nicotine has the effect of changing VSMCs from differentiated to dedifferentiated type, that is transformation from the contractile-type to the synthetic-like (proliferative) type.

Therefore, we examined the gene and protein expression after exposing HVSMCs to nicotine using human DNA microarrays, real time qPCR, and Western blots [33]. To the best of our knowledge, our study is the first to investigate the possibility that nicotine exposure for 48 h could induce a phenotypic change in HVSMCs (**Figures 3** and **4**).

Myosin II of motor proteins plays important roles in the contraction for smooth muscles and cell migration of non-muscle cells [37–41]. Myosin II isoform 11 is expressed in the contractile type of smooth muscle cells [42]. Myosin II isoform 10 is expressed during fetal development, as a synthetic-like non-muscle isoform [42]. Thus, the expression of myosin II isoforms 11

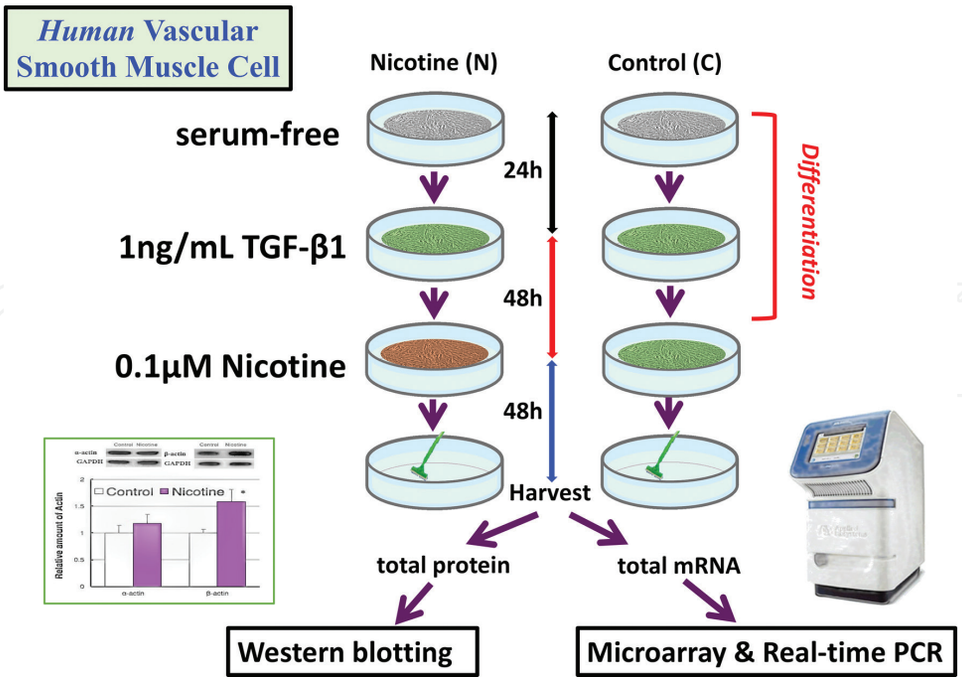

Figure 3. Primary human aorta smooth muscle cells were cultured under the differentiated condition. Upon reaching confluence, the cells were deprived of serum for 24 h. The differentiation was induced by TGF-β1 for 48 h. The cells of the nicotine group were then exposed to 0.1 μM of nicotine. In another 48 h, the total RNAs and proteins were purified for qPCR and immunoblotting, respectively.

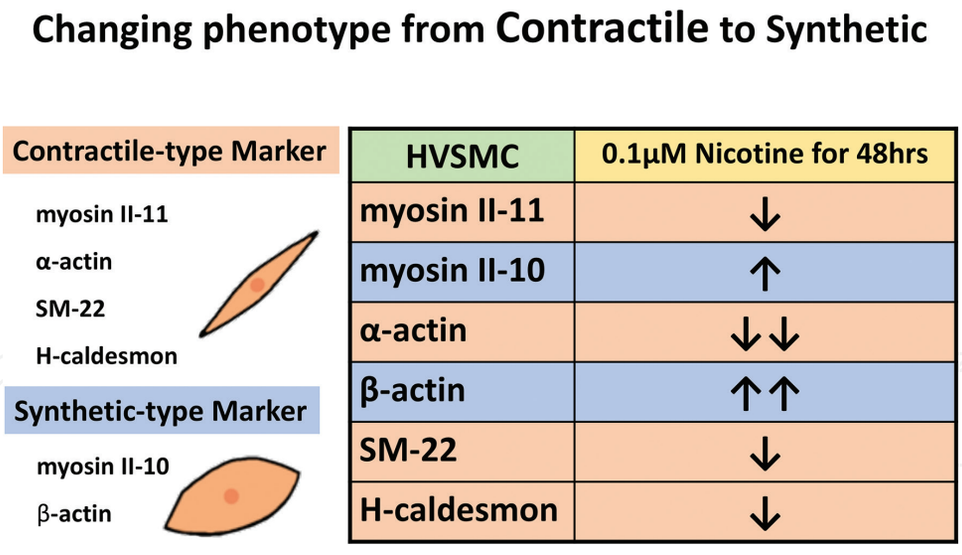

Figure 4. The change in mRNA levels upon nicotine exposure. HVSMCs induced by TGF-β were exposed to 0.1 μM of nicotine. The total RNA was extracted 48 h after exposure, and the cDNA corresponding to each time point of the cells were synthesized. Each gene was inspected using real-time PCR. The mRNA level of myosin II isoform 11, α-actin, SM-22, and H-caldesmon decreased 48 h after exposure to nicotine. On the contrary, the mRNA level of myosin II isoform 10 and β-actin increased after exposure of nicotine.

and 10 are indicative of the contractile and non-muscle (proliferative) types, respectively. In our study, myosin II isoform 11 mRNA level decreased by approximately 0.8-fold, 48 h after HVSMCs exposure to nicotine. In comparison, myosin II isoform 10 mRNA level increased in

a time-dependent manner to approximately 3-fold after 48 h [33]. During Western blot experiments using specific antibodies against each of the marker proteins, the protein expression of myosin II isoform 10 increased after 48 h exposure to nicotine. The amount of myosin II isoform 11 decreased by approximately 0.6-fold after the 48-h nicotine exposure. The myosin II isoform 10 level was increased to about 1.2-fold after exposure to nicotine [33]. These results indicated that the isoforms of myosin II had changed to the non-muscle (proliferative) type from the smooth muscle contractile type because of nicotine exposure (**Figure 4**).

Subsequently, α-actin and β-actin were used as contractile-type and synthetic-like type marker genes, respectively [43]. After exposure of HVSMCs to nicotine, the α-actin mRNA level decreased by approximately 0.4-fold, whereas, the β-actin mRNA level increased to approximately 1.7-fold after 48 h, respectively [33]. Using Western blot experiments, the protein expression of α-actin levels did not significantly change. In contrast, β-actin levels significantly increased to approximately 1.6-fold after the nicotine exposure [33]. These results indicated that the actin isoform also changed to the synthetic-like type from the contractile-type after nicotine exposure (**Figure 4**).

SM22 and high-molecular-weight caldesmon (H-caldesmon) are major smooth muscle differentiation markers [44, 45]. The SM22 mRNA level decreased by approximately 0.9-fold after the 48-h exposure of HVSMCs to nicotine. The mRNA level of the H-caldesmon, a smooth muscle contractile-type marker protein, was about 0.7-fold after 48 h [33]. Using Western blot experiments, H-caldesmon and SM22 levels, significantly decreased by approximately 0.4- and 0.7-fold, respectively after nicotine exposure [33]. The decreased H-caldesmon and SM22 expression levels also indicated the transformation to the synthetic-like type from the contractile-type after nicotine exposure (**Figure 4**).

Notch receptors are intimately involved in HVSMC differentiation. Activation of Notch receptors by cell-cell adhesion induces the expression differentiation marker proteins of contractile-type on smooth muscles [46]. However, when HVSMCs at 100% confluence were exposed to nicotine in our study, the expression of Notch receptors did not increase [33]. This indicated that nicotine had suppressed the expression and function of the Notch receptors.

Mitogen-activated protein kinases (MAPKs) play an important role in cell proliferation and migration [46, 47]. MAPKs are also intimately involved in VSMC growth and migration [48, 49]. It has been reported that nicotine induces the production of growth factors such as vascular endothelial growth factor (VEGF), Platelet-derived growth factor (PDGF-BB), and Fibroblast growth factor (FGF-2) from VSMCs, and that PDGF-BB and FGF-2 promoted the proliferation of VSMCs [29, 50–52]. Nicotine-induced VEGF production was mediated by nAChRs via activation of the VEGF and its receptor as well as the extracellular signal-regulated kinase (ERK)1/2 pathway [27]. PDGF-BB caused cytoskeletal protein remodeling, enhanced the proliferation, and migration of VSMCs [51]. In our study, the phosphorylation levels of the p38 MAPK, ERK1/2, and c-jun N-terminal kinase increased after 48 h of nicotine exposure [33]. Activation of MAPKs signaling indicated that the characteristics of VSMCs changed to migration-type cells after nicotine exposure.

Our results suggest that nicotine can decrease the expression of differentiation marker proteins in HVSMCs, and change these cells from the contractile-type to synthetic-like type,

thus, promoting cell migration [33]. Therefore, we considered that nicotine facilitated the formation of intimal lesions characteristic of atherosclerosis. Recently, it was reported that nicotine upregulated the transcription of miR-200b in VSMCs [53]. The miR-200b-mediated down-regulation of Rho-specific guanine nucleotide dissociation inhibitor A facilitated the migration and proliferation of VSMCs in a Rho GTPase-dependent manner [53].

4. New challenges on HVSMC exposure to nicotine

Regarding the influence of nicotine on HVSMCs, a new problem was found during our research. It was about how nicotine works as a signal in HVSMCs. It has been shown that nicotine binds to nAChRs, and opens the ion channels in these receptors to significantly increased intracellular Ca^{2+} levels [54, 55]. We measured the changes in intracellular Ca^{2+} level in HVSMCs upon nicotine stimulation. Our results indicated that nicotine stimulation significantly increased intracellular Ca^{2+} levels in HVSMCs. In addition, mecamylamine, a non-selective nAChR blocker, effectively blocked the nicotine effect in the nicotine-treated HVSMCs. However, mecamylamine did not exhibit complete inhibition of the nicotine stimulation. This suggests that nicotine is involved in intracellular signal transduction through receptors other than nAChRs. From the results of our comprehensive gene analysis, several receptors whose gene expression were increased by nicotine exposure have been discovered. In the future, it would be expedient to clarify the functions of these novel nicotine receptors (**Figure 5**).

Furthermore, the transformation of VSMCs by nicotine shown in our study suggested that nicotine itself promoted arteriosclerosis. In addition to cigarettes, nicotine is also contained in

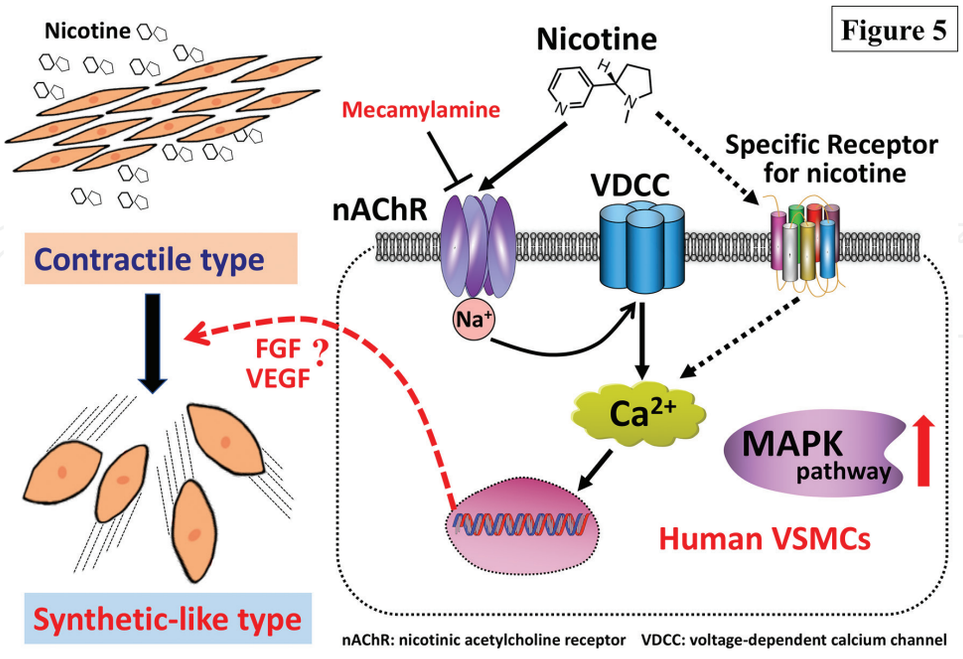

Figure 5. A schematic diagram showing the relationship between nicotine exposure and the phenotypic change in HVSMCs. The solid line arrows indicate an effect based on our results. The break line arrows indicate an effect based on our speculation.

therapeutic nicotine patches and gums used for smoking cessation. Thus, there is a possibility that these nicotine patches or gums promote atheromatous plaque formation. Moreover, smokeless tobacco contains large amounts of sodium, which enhance nicotine absorption [56]. These problems should also be considered sufficiently because nicotine used even during smoking cessation treatment and avoidance of tobacco sidestream smoke induces arteriosclerosis.

5. Conclusion

Several data have widely suggested nicotine as one of the factors responsible for the formation of atheromatous plaques in the vascular intima. Numerous studies so far, including our research, indicate that nicotine induces intracellular Ca^{2+} influx in HVSMCs via nAChRs and possibly via another nicotine-specific receptor. Consequently, HVSMCs are transformed from the contractile-type to the synthetic-like type, which occurs during the development of atheromatous plaques. Aside from cigarettes, nicotine is also contained in nicotine patches and gums used for smoking cessation. Thus, there is a possibility that these nicotine patches or gums promote atheromatous plaque formation. Therefore, we hypothesize that elucidating the mechanism of action of nicotine will lead to new means of preventing and treating atherosclerotic plaque formation and development of arteriosclerosis.

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