論　文　内　容　要　約

Role of mitochondrial DNA damage in cigarette smoke extract-induced innate immunity and its clinical application to atherosclerosis screening

（タバコ煙抽出物によるミトコンドリア DNA 損傷と動脈硬化症スクリーニングへの臨床応用）

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Smoking is an independent risk factor for atherosclerosis, yet the mechanism by which smoking causes atherosclerosis remains unclear. Several reports suggest that DNA damage is involved in the development of atherosclerosis. We have previously reported that nuclear DNA damage was increased in peripheral mononuclear cells of smokers compared to those of non-smokers. The objective of this study is to investigate the effect of cigarette smoke extract (CSE) on both nuclear and mitochondrial DNA damage and the subsequent cellular response.

We prepared CSE by dissolving eight cigarettes smoke in 15 ml of PBS and administered to human umbilical vein endothelial cells (HUVECs). DNA double-strand breaks (DSBs), estimated by immunofluorescence staining using a phosphorylated histone H2AX antibody, was significantly increased at 72 hours by CSE, which is relatively slow compared to other DSBs-inducing stimuli such as H_2O_2. Oxidative DNA damage, estimated by immunofluorescence staining using 8-hydroxy-2'-deoxyguanosine (8-OXO-dG) antibody, was increased in both nucleus and cytosol by CSE addition. We speculated that the cytosolic staining might indicate oxidative damage to the mitochondrial DNA. Co-immunostaining using 8-OXO-dG antibody, Mitotracker, and DAPI, followed by confocal microscopy revealed that the mitochondrial DNA was oxidatively damaged. Mitochondrial membrane potential was evaluated by the JC-1 assay, and mitochondrial dysfunction was observed by CSE addition. It is known that stimuli that cause mitochondrial dysfunction increase mitochondrial outer membrane permeability called mitochondrial outer membrane permeabilization (MOMP) mediated by the activation of BAK/BAX, which results in the release of mitochondrial contents such as cytochrome c and activation of caspases. Activated caspase-3 cleaves inhibitor of caspase-activated deoxyribonuclease (ICAD), which induces translocation of caspase-activated deoxyribonuclease (CAD) to the nucleus, resulting in DNA fragmentation and apoptosis. However, recent reports have shown that this MOMP does not occur in all mitochondria depending on the stimulus, but only a part of mitochondria, called minority MOMP, and that the cells survive because of partial DNA fragmentation (i.e., DSBs). We hypothesized that the increase in DSBs as late as 72 hours might be due to this mechanism, and examined the downstream signaling of the apoptotic pathway. We found significant accumulation of active BAX in the mitochondria of cells treated with CSE, and CSE treatment significantly activated caspase-3. In addition, CSE treatment resulted in decreased cytosolic expression of ICAD and nuclear translocation of CAD.

We next examined whether the accumulation of DNA in the cytosol was occurred as a result of DNA damage using double-strand DNA (dsDNA) antibody, and found that dsDNA antibody was stained in the cytosol of cells treated with CSE. To know the origin of the

accumulated cytosolic DNA, real-time PCR analysis was performed using specific primers for nucleus or mitochondria. The results revealed that the accumulated DNA in the cytosol was derived not only from the nucleus but also from mitochondria. Accumulated cytosolic DNA is known to induce inflammation through the innate sensing pathways. We examined whether CSE activated cGAS-STING pathways. The production of cGAMP, a second messenger in cGAS signaling, was increased by CSE. In addition, TBK1, a protein downstream of the cGAS-STING pathway, was phosphorylated. Although CSE addition increased mRNA expression of IL-6, IL-1a, IFN-b, and MCP-1 within 3 days, we thought that cGAS-STING pathway may be involved in chronic inflammation. Therefore, we continued to administer CSE for 7 days and found that only the increase in IL-6 was maintained even at 7 days after the addition of CSE. We next examined whether the persistent increase in IL-6 mRNA expression was dependent on the activation of the cGAS-STING pathway. HUVECs were transfected either with siRNAs against cGAS or negative control siRNA. The increase of IL-6 mRNA expression was suppressed by siRNAs of cGAS, while IL-1a, IFN-b, and MCP-1 were not suppressed, indicating that CSE-induced persistent IL-6 expression was mediated by cGAS-STING pathway.

Cytosolic DNA is incorporated into extracellular vesicles and thus, cell-free DNA (cfDNA) in human peripheral blood has been reported to be altered in association with several diseases. We confirmed that cfDNA was increased in the culture medium after CSE administration, and then, measured cfDNA in the blood of smokers to support the significance of our in-vitro experiments. The cfDNA derived from not only nuclear DNA but also mitochondrial DNA were found to be increased in the blood of smokers compared to that of age-matched non-smokers. To further investigate whether cfDNA in blood could be a new biomarker reflecting the condition of vascular endothelium, we measured cfDNA in the blood of atherosclerosis patients. Both nuclear-cfDNA and mitochondrial-cfDNA were increased in patients with atherosclerosis. In conclusion, this study showed that persistent exposure to CSE induces not only nuclear but also mitochondrial DNA damage, which leads to cytosolic DNA accumulation, and evokes chronic inflammation via the cGAS-STING pathway. We also showed that damaged DNA is released not only into the cytosol but also into the extracellular space. Furthermore, we found that cfDNA was increased in the blood of smokers. Finally, our study suggests that cfDNA in blood may be a new biomarker reflecting the condition of vascular endothelium, because cfDNA in blood is also increased in atherosclerotic patients.