

Chronic cigarette smoke extract treatment selects for apoptotic dysfunction and mitochondrial mutations in minimally transformed oral keratinocytes

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Cigarette smoke demonstrates a carcinogenic effect through chronic exposure, not acute exposures. However, current cell line models study only the acute effects of cigarette smoke. Using a cell line model, we compared the effects of acute *versus* chronic cigarette smoke extract (CSE) on mitochondria in minimally transformed oral keratinocytes (OKF6). OKF6 cells were treated with varying concentrations of CSE for 6 months. Cells were analyzed monthly by flow cytometry for mitochondrial membrane potential (MMP), cytochrome *c* release, caspase 3 activation and viability after CSE exposure. At each time point, the same assays were performed after 24 hr of valinomycin (MMP-depolarizing agent) treatment. The mitochondrial DNA of chronically CSE-treated cells was sequenced. After 6 months of CSE treatment, the cells were increasingly resistant to CSE-mediated and valinomycin-induced cell death. In addition, chronic CSE treatment caused chronic depolarization of MMP, cytochrome *c* release and caspase activation. Cells grown in the presence of only CSE vapor also exhibited the same resistance and chronic baseline apoptotic activation. Mitochondrial DNA sequencing found that chronic CSE-treated cells had more amino acid-changing mitochondrial mutations than acutely treated cells. CSE treatment of normal cells select for apoptotic dysfunction as well as mitochondrial mutations. These findings suggest that chronic tobacco exposure induces carcinogenesis *via* selection of apoptosis resistance and mitochondrial mutation in addition to previously known genotoxic effects that were found by acute treatments. Chronic models of tobacco exposure on upper aerodigestive epithelia may be more insightful than models of acute exposure in studying head and neck carcinogenesis

Head and neck squamous cell carcinoma (HNSCC) is among the most morbid of human cancers with ~40,000 new cases yearly in the United States. The primary risk factor for HNSCC is tobacco exposure with alcohol as a cocarcinogen, with a latency period of several decades.¹ As early as 1964, there were published reports of the tumor-promoting activity of tobacco extracts in mice.^{2,3} Despite these early studies,

there is incomplete knowledge of how cigarette smoke induces the early cellular changes that lead to malignancy.

Impaired apoptosis is a central characteristic of neoplastic and malignant transformation.^{4,5} Mitochondria have a central role in the signal transduction and coordination of apoptosis.⁶⁻⁸ Apoptosis at the mitochondrial level is initiated by depolarization of the mitochondrial membrane and proceeds *via* release of cytochrome *c* and other apoptogenic factors from the intermembrane space of mitochondria.⁸ Valinomycin, a potassium ionophore, which facilitates the selective transport of K⁺ ions across the inner membrane of mitochondria,⁹ induces apoptosis in various mammalian cell lines^{6,10-12} by disrupting the $\Delta\Psi_m$.¹³ Previous study showed that HNSCC cell lines are resistant to $\Delta\Psi_m$ depolarization-induced apoptosis (*i.e.*, valinomycin treatment).¹⁴ This finding, in conjunction with the knowledge that mitochondria are uniquely susceptible to oxidative damage and that cigarette smoke mediates its effects through oxidative damage,¹⁵⁻¹⁹ implies that the effects of tobacco exposure on mitochondrial mechanisms of apoptosis are of significant interest in HNSCC carcinogenesis.

There are 2 canonical apoptotic caspase pathways involved in apoptosis: the mitochondrion (intrinsic) pathway and the

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