Original Investigation

The Combined Effects of Single Nucleotide Polymorphisms, Tobacco Products and Ethanol on Normal Resting Blood Mononuclear Cells

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Abstract

Introduction: Tobacco and ethanol consumption are crucial factors in the development of various diseases including cancer. In this investigation, we evaluated the combined effects of a number of single nucleotide polymorphisms (SNPs), with ethanol and tobacco products on healthy individuals.

Methods: Pure nicotine, cigarette smoke extract, and Swedish snuff (snus) extract were used. The effects were examined by means of in vitro cell cycle progression and cell death of peripheral blood mononuclear cells (PBMCs) obtained from healthy donors.

Results: After 3 days, in vitro, resting PBMCs entered the S and G2 stage in the presence of 100 μM nicotine. The PBMCs only proceeded to S stage, in the presence of 0.2% ethanol. The nicotine- and ethanol-induced normal cell cycle progression correlated to a number of SNPs in the *IL12RB2, Rad 52, XRCC2, P53, CCND3,* and *ABCA1* genes. Certain SNPs in *Caspases 8, IL12RB2, Rad 52, MMP2,* and *MDM2* genes appeared to significantly influence the effects of EtOH-, snus-, and snus + EtOH-induced cell death. Importantly, the highest degree of cell death was observed in the presence of smoke + EtOH. The amount of cell death under this treatment condition also correlated to specific SNPs, located in the *MDM2, ABCA1,* or *GASC1* genes.

Conclusions: Cigarette smoke in combination with ethanol strongly induced massive cell death. Long-term exposure to smoke and ethanol could provoke chronic inflammation, and this could be the initiation of disease including the development of cancer at various sites.

Introduction

Tobacco use, both of smoking and smokeless varieties, increases the risk for poor health and causes a burden on the health care system. Nevertheless, one-sixth of the world's population continues to smoke (Jha, Ranson, Nguyen, & Yach, 2002). In addition, people exposed to cigarette smoke by passive smoking can also be seriously affected (Mao, Hu, Semenciw, & White, 2002). These effects include an increased risk of cardiovascular disease, chronic obstructive pulmonary disease, certain metabolic disorders, and cancer (Vineis et al., 2004). The world-wide incidence of premature death attributable to smoking-related diseases is expected to rise from 5.4 million in 2005 to 6.4 million in 2015 (Foulkes, Brunet, Sieh, Black, Shenouda, & Narod, 1996; Mathers & Loncar, 2006). Why and how cigarette smoke plays a pathological role in so many diseases including cancer remains unanswered.

Cigarette smoke contains nicotine in addition to more than 4000 compounds, which bind to and chemically modify or damage DNA (Rodgman, Smith, & Perfetti, 2000). Genetic factors are also likely to play an important role because not all heavy smokers develop cancer. Interestingly, an increased cancer risk among the first-degree relatives of cancer patients has been reported (Foulkes et al., 1996). The human genome is characterized by large numbers of genetic variations known as single nucleotide polymorphisms (SNPs) in the germ-line

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DNA (Sherry et al., 2001; Wheeler et al., 2008). Although most SNPs are considered functionally neutral, a portion of them actually affect gene expression or protein function. Furthermore, associations of numerous SNPs with risk and prognosis in cancer patients have been reported (Shen et al., 2003; Xu et al., 2005).

A low incidence of tobacco-associated morbidity and mortality in Sweden has been suggested to be related to the low incidence of smoking and the habit of using local nonsmoking tobacco, known as Swedish snuff (snus). Chemical and toxicological analysis have suggested that snus contains less carcinogenic substances than other types of nonsmoking tobacco (Idris, Ahmed, Mukhtar, Gadir, & el-Beshir, 1995; Idris et al., 1998). The possibility of preventing the harmful effects of smoking by encouraging smokers who are unable or unwilling to stop smoking, to switch to less harmful, smokeless products has been discussed (Lee & Hamling, 2009; Rodu & Phillips, 2008).

Smoking in combination with ethanol consumption significantly increases the risk for heart disease and cancer (Altieri et al., 2004). Recently, we found that smoke extract in combination with ethanol induced massive cell death and abnormal cellular phenotypes in normal proliferating, human fibroblast and endothelial cells (Laytragoon-Lewin, Bahram, Rutqvist, Turesson, & Lewin, 2011). The present investigation provides evidence for a putative impact of SNPs, ethanol, and tobacco products on the cell behavior of healthy individuals. Both cell cycle progression and the degree of cell death in peripheral blood mononuclear cells (PBMCs) of healthy donors were used as indicators of the combined effects of SNPs and these substances.

Materials and Methods

Ethanol, Pure Nicotine, Snus Extract, and Cigarette Smoking Extract

Snus extracts were obtained from a portion of Swedish snus (Ettan; Swedish Match AB, Stockholm Sweden) by an orbital shaker. These snus extracts were aliquoted, protected from light, and stored at –80 °C. The cigarette smoke extract was prepared from filter cigarettes of an American blend type (Filter red pack, Marlboro USA) using a Borgwaldt RM 20/CS-smoking machine. Particulate phase from the cigarette smoke was collected on a 9-cm Cambridge filter. The filter was weighed before and after smoking to ensure that the amount of collected particulate phase was reproducible between sessions. The smoke components in the filter were extracted with ethanol and concentrated using a rotary evaporator without warming. The amount of nicotine in snus extract or cigarette smoke extracts was measured by LC-MS-MS. These extracts were aliquoted, protected from light, and stored at –80 °C.

The 0.2% ethanol (Kemetyl AB, Sweden) and 100 μM pure nicotine (Sigma Chemical Co., USA), snus extract or smoke extract containing 100 μM nicotine were freshly prepared before use in the test system.

Blood and DNA Samples

The ethical committee of Uppsala region gave their consent to this study. A total of 54 healthy blood donors (49 males and 5 females, mean age 52.6 years) were included. PBMC were obtained from 30-ml heparinized blood with Ficoll/Isopaque gradient (Pharmacia, Uppsala, Sweden) separation. High molecular weight DNA from the PBMCs was obtained by standard extraction with phenol and Chisam (Sigma Chemical Co.). The quality and quantity of DNA were determined by a spectrophotometer.

Control and Treated PBMC Cultured

RPMI media containing 10% fetal calf serum, 2 mM glutamine, 100 U penicillin, and 0.1 mg/ml streptomycin (Sigma Chemical Co.) was used. Under the control condition, PBMCs were cultured without any treatment (control). The treated PBMCs were cultured in the presence of 0.2% ethanol (EtOH), 100 μM pure nicotine (nicotine), snus extract containing 100 μM nicotine (snus), snus extract containing 100 μM nicotine and 0.2% ethanol (snus + EtOH), or cigarette smoke extract containing 100 μM nicotine and 0.2% ethanol (smoke + EtOH).

Cell Cycle and Cell Death

After 3 days, controls and treated PBMCs were analyzed for cell cycle progression and cell death as previously described (Castro, Heiden, Wang, & Tribukait, 1993). Briefly, these cells were fixed in 4% formaldehyde buffer and bare cell nuclei were directly stained with Subtilisin Carlsberg solution (Sigma Chemical Co.). The DAPI-stained nuclei were analyzed using PAS II flow cytometry at flow rate of <200 nuclei/second. A multicycle program for cell cycle analysis was applied for histogram analysis (Phoenix Flow Systems, San Diego, CA). At least 40,000 cell nuclei were analyzed per histogram.

SNP Analysis

The blood DNA was analyzed for SNPs in 30 candidate genes (Table 1). The identification of 29 SNPs was performed at the SNP & SEQ technology platform at Uppsala University, Sweden (www.genotyping.se). One SNP, *TP53* codon 72 (rs 1042522) was performed by PCR-RFLP, as previously described (Zainuddin et al., 2009).

Statistical Analysis

Differences in the proportions of each cell cycle stage, cell death, and correlation with each SNP was calculated using the Student's *t* test. All statistical analyses were carried out using Statistica version 9.1 (Stat Soft, USA).

Results

Cell Cycle Progression, Treatments, and SNPs

Of the 30 SNPs, only 10 SNPs correlated with cell cycling behavior and the degree of induced cell when cells were treated with ethanol and/or tobacco products (Table 1). The vast majority of the ex vivo healthy blood donor PBMCs were resting cells, indicated by the fact that 93% cells were in the G0/G1 stage of the cell cycle. After 3 days of in vitro culturing under control conditions, the cell population consisted of 93%, 2.6%, and 1.8% cells in the G0/G1, S, or G2 stage, respectively (Table 2). A significant number of G0/G1 cells progressed further into the cell cycle in the presence of

Table 1. List of Genes, Single Nucleotide Polymorphisms, and the Combined Effects with Ethanol or Tobacco Products on Human Normal Blood Mononuclear Cells. The Calculation for *p* Values were Performed with the Student's *t* Test and $p < .05$ was Considered Significant

Gene	RS-number	Significance
MDM2	***2279744***	***Yes***
GASC1	***2296067***	***Yes***
Caspases 8	***1045485***	***Yes***
Rad 52	***11571424***	***Yes***
ABCA1	***2230806***	***Yes***
IL12-RB2	***3790568***	***Yes***
MMP2	***243865***	***Yes***
CCND3	***3218086***	***Yes***
p53	***1042522***	***Yes***
XRCC2	***2040639***	***Yes***
Caspases 9	1052576	No
FGFR4	2011077	No
BRCA-1	4986850	No
Chk-2	17879961	No
CYP2A6	28399433	No
TNF	1800610	No
p16CDKN2A	3088440	No
Lig-4	1805388	No
*CYP2D6*4*	1800716	No
DNA-PK	1231204	No
HTR3C	676641	No
ATM	1801516	No
CCND1	602652	No
p21	7767246	No
RB1	2854344	No
EHBP1	721048	No
Rad51	1801321	No
CHEK1	521102	No
Ku70	2267437	No
XRCC3	861539	No

Table 2. The Number of Individuals Tested (*n*) and the Cell Cycle Distribution of Cells (%) in Control, Ethanol (EtOH), Nicotine, Snus, Snus + EtOH, and Smoke + EtOH-Treated Groups. The Significance in Cell Cycle Distribution was Calculated by Comparing the Control to Each Group

	Individual (*n*)	G0/G1 (%)	S (%)	G2 (%)
Control	37	93.2	2.6	1.8
EtOH	37	89.8**	6.1**	1.8
Nicotine	37	88.6**	7.1**	2.9*
Snus	17	95.4	3.2	1.6
Snus + EtOH	16	95.0	3.6	1.3
Smoke + EtOH	18	96.7	1.9	1.5

*$p < .05$.
**$p < .005$.

Table 3. The Impact of Genotypes and Treatment with Ethanol (EtOH) and Nicotine on S and G2 Stage of Cell Cycle

				Treatment condition	
Cell cycle phase	Gene	Rs-number	Genotypes	ETOH	Nicotine
S	*IL12-RB2*	rs3790568	AG GG	NS	.0025*
	Rad52	rs1157142	AG GG	NS	.040*
	ABCA1	rs2230806	AA AG	.028*	NS
	XRCC2	rs2040639	AA AG	NS	.025*
	P53	rs1042522	CC GG	NS	.030*
	CCND3	rs3218086	AG GG	.041*	NS
			AA AG	.00040*	.00094*
G2	*IL12-RB2*	rs3790568	AG GG	NS	.0051*
	Rad52	rs1157142	AG GG	NS	.040*
	ABCA1	rs2230806	AA AG	NS	.00076*
			AA GG	NS	.036*
	CCND3	rs3218086	AG GG	NS	.049*
			AA AG	NS	.000067*

*p value.
NS = Not significant.

ethanol (EtOH) or nicotine compared with the control. Nicotine treatment increased the number of cells in both the S and G2 stage, whereas EtOH treatment only increased the number of cells in the S stage. Despite the fact that similar concentrations of ethanol and nicotine were used, snus extract (snus), in combination with ethanol (snus + EtOH) and smoke extract in combination with ethanol (smoke + EtOH), did not show any influence on the cell cycle progression of these resting G0/G1 PBMCs.

The relative increase of PBMCs in either S/G2 or S stage in the presence of nicotine or EtOH only, correlated with the genetic background of the donor (Table 3). Within these 30 genes, the presence of certain SNP genotypes in *ABCA1* and *CCND3* genes correlated with a significantly increased number of normal resting cells in the S stage in the presence of EtOH. Certain SNP genotypes in the *IL12-RB2, Rad 52, XRCC2, TP53,* and *CCND3* genes correlated with a significantly increased number of S stage cells in the presence of nicotine. SNP genotypes in the *IL12-RB2*, *Rad52*, *ABCA1*, and *CCND3* genes also correlated with an increased number of cells in the G2 stage in presence of nicotine.

Cell Death, Treatments, and SNPs

Within 3 days of in vitro culturing, 11.1% of the PBMCs died under control conditions (Table 4). The amount of basal cell death number did not have any correlation with the SNP genotypes of the donors. There was no significant difference in the number of dead cells compared with controls, in the presence of EtOH alone, snus alone, or snus + EtOH. The highest degree of cell death, 19.8%, was observed in the presence of smoke + EtOH, and this differed significantly ($p < .005$) from the basal level in controls and from the other treatment conditions.

Table 4. The Number of Individuals Tested (*n*) and the Cell Death (%) in Control, Ethanol (EtOH), Nicotine, Snus, Snus + EtOH, and Smoke + EtOH-Treated Group. The Significance in Cell Death was Calculated by Comparing the Control to Each Treatment Group

Group	Cell death (%)	Test (*n*)
Control	11.1	45
EtOH	12.2	45
Nicotine	13.9	45
Snus	11.7	27
Snus + EtOH	12.2	25
Smoke + EtOH	19.8**	27

**$p < .005$.

Cell Death of PBMCs Correlated to Certain SNPs and Treatment Conditions

With these treatments, SNPs in the *Caspases 8*, *IL12-RB2*, *Rad 52*, and *MMP2* genes were correlated to cell death numbers in the presence of EtOH. SNPs in the *Caspases 8*, *IL12-RB2*, *Rad 52*, and *MDM2* genes were correlated to nicotine-induced cell death. SNPs in the *ABCA1* and *IL12-RB2* genes were correlated to cell death numbers in the presence of snus + EtOH, whereas only a SNP in the *ABCA1* gene was correlated to the proportion death cells in snus, respectively (Table 5).

Despite having similar amount of ethanol and nicotine as snus and EtOH, smoke + EtOH induced the highest amount of cell death ($p < .005$). This cell death number correlated with three SNPs located in the *MDM2, ABCA1,* and *GASC1* genes.

Discussion

Cigarette smokers and alcohol drinkers are a population that due to their lifestyle habits present a challenge to the health care system. By smoking 25 cigarettes/day (Feyerabend, Ings, & Russel, 1985; Jha et al., 2002), a smoker will accumulate approximately 100 μM of nicotine in the saliva per day. We show that under such nicotine concentrations, normal resting G0/G1 cells enter into the S and G2 stage of the cell cycle. In addition, 0.2% of EtOH-induced normal resting cell progression to the S but not the G2 stage. Normal cell cycle progression by nicotine and ethanol correlated to the presence of certain SNPs in the *IL-12RB2, Rad52, ABCA1, XRCC2, TP53,* and *CCND3* genes in healthy individuals and was, therefore, not a random event.

Certain SNPs in the *IL-12RB2, Rad52, ABCA1, XRCC2, TP53,* and *CCND3* genes have been reported to play an important role in cell cycle control, DNA repair, and cancer (Danoy et al., 2008; Yen et al., 2008; Zhou et al., 2007). Furthermore, a number of SNPs in the *ABCA1* and *IL12RB* genes are important in the response of normal cells to radiation-induced inflammation (Isomura et al., 2008; Schwarz et al., 2002). The SNP in *ABCA1* gene has also been reported to have a significant impact on cardiovascular disease and breast cancer (Attie, 2007; Hamon, Chambenoit, & Chimini, 2002; Lal et al., 2008). Normal resting cells from individuals with specific *ABCA1* or *IL12-RB2*, progress through the cell cycle in the presence of pure nicotine. Because abnormal cell cycle progression will influence the homeostasis of the body, cessation of smoking by nicotine replacement therapy might not be suitable in individuals carrying these "risk" SNPs.

Neither snus extract nor smoke extract, which contained similar concentration of nicotine and ethanol, induce cell cycle progression of normal resting G0/G1 cells. Our results indicate that additional components in snus or smoke extracts have dominant effects over the cell cycle modulation effects induced by ethanol or nicotine. Identification of these components would require further investigation.

Cell death occurred to a similar degree in PBMCs in controls and in EtOH, nicotine, snus, or snus + EtOH. It is important to note that basal cell death in control conditions was random events, without any correlation to any of the SNPs in the 30 candidate genes investigated. The proportion of cell death in the presence of ethanol, nicotine, snus, and snus + EtOH was found to be related to certain SNP genotypes within DNA repair and cell death pathways.

One SNP in codon 72 of the *TP53* gene, rs 1042522 has been suggested to influence cell death and diseases such as cervical cancer and head and neck cancer (Jarrell et al., 1992; Meek, 2009; Schneider-Stock et al., 2004). In our material, we could only find SNP of *TP53* being involved in the increase of the number of S stage cells when the cells were treated with nicotine.

Table 5. The Impact of Genotypes and Treatment Conditions on Cell Death

Gene	Rs-number	Genotypes	EtOH	Nicotine	Snus	Snus + EtOH	Smoke + EtOH
Caspases 8	1045485	CG GG	.03530*	.003107*	NS	NS	NS
IL12-RB2	3790568	AG GG	.04739*	.0006*	NS	.0456*	NS
Rad 52	1157142	AA AG	.01909*	.003136*	NS	NS	NS
MMP2	243865	AA AG	.0094*	NS	NS	NS	NS
MDM2	2279744	AA AC	NS	.02124*	NS	NS	.043497*
ABCA1	2230806	AA AG	NS	NS	.031791*	.036766*	.037534*
GASC1	2296067	AA AG	NS	NS	NS	NS	.032076*

**p* value.
NS = Not significant.

However, we could not detect any influence of the *TP53* codon 72 on EtOH-, snus-, snus + EtOH-, or smoke + EtOH-induced cell death in normal PBMCs. Thus, normal resting cell death resulting from these treatments might involve SNPs at various genes but not the SNP at codon 72 of the *TP53* gene.

An interesting observation was that only smoke + EtOH induced massive cell death in combination with SNPs, yet did not affect cell cycle progression in resting normal cells. This treatment condition was also found to induce a development toward abnormal cells, alter gene expression, and provoke massive cell death in proliferating normal human cells (Laytragoon-Lewin et al., 2011). As a consequence of massive cell death, chronic inflammation could occur and this could be the initiation of various inflammation-related diseases including cancer (Smith, Perfetti, & King, 2006).

Individual variations in cell death from cigarette smoke + EtOH was significantly related to SNPs in the *ABCA1, MDM2,* and *GASC1* gene. Because the name SNP in the *ABCA1* gene is related to cardiovascular disease, cigarette smokers and ethanol drinkers with this SNP genotype may have an increased risk for the developed of cardiovascular disease. The SNPs in *MDM2* and *GASC1* have been identified as drivers of ontogenesis in squamous cells (Alhopuro et al., 2005; Chen et al., 2009; Jarrell et al., 1992). This might explain why the vast majority of cancer in smokers and drinkers are squamous cell carcinomas (Chin, Boyle, Porceddu, Theile, Parsons, & Coman, 2006). Future investigations on the mode of pathogenesis or carcinogenesis in smokers and drinkers carrying "high-risk" SNPs are, therefore, needed.

In conclusion, cigarette smoke and ethanol induced massive cell death without any direct influence on the cell cycle progression of human normal resting cells. The degree of cell death was significantly related to certain SNP genotypes in the *MDM2, ABCA1*, and *GASC1* genes of individual donors. These potentially relevant SNPs might predict the individual risk of developing diseases and cancer induced by cigarette smoke and ethanol drinking.

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Declaration of Interests

LER is an employee at The Swedish Match Company. None of the remaining authors have competing interests.

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