CYP2E1 mRNA EXPRESSION, GENETIC POLYMORPHISMS IN PERIPHERAL BLOOD LYMPHOCYTES AND LIVER ABNORMALITIES IN CHINESE VCM-EXPOSED WORKERS

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Abstract

Objective: To study the relationship between expression of cytochrome P4502E1 (CYP2E1) in human lymphocytes, variant CYP2E1 genotype, exposure to vinyl chloride monomer (VCM), and liver abnormalities in VCM-exposed workers.

Methods: A case-control study was performed on 90 male occupationally exposed workers and 42 matched male nonexposed controls. Data were collected based on health surveillance, workplace investigation and questionnaire survey. Total RNA and DNA were isolated from peripheral blood lymphocytes, and CYP2E1 mRNA expression was determined using RT-PCR, and the presence of CYP2E1 polymorphisms was identified based on PCR-RFLP.

Results: The mRNA expression of CYP2E1 in exposed workers (0.89±0.46) was significantly higher than in nonexposed controls (0.61±0.35) (P < 0.01). Logistic regression analysis demonstrated a statistically significant association between CYP2E1 mRNA expression levels and liver abnormalities in the VCM-exposed workers (OR = 3.66, P < 0.05). The genotype frequency for CYP2E1 variants among VCM-exposed workers was not significantly different between workers with liver abnormalities and those without.

Conclusions: Liver abnormalities in subjects exposed to VCM are positively associated with expression of peripheral blood lymphocyte mRNA, which is significantly increased in exposed workers compared to nonexposed controls. Therefore, CYP2E1 mRNA levels may be useful for health surveillance and protection of VCM-exposed workers.

Key words: Vinyl chloride, Lymphocyte CYP2E1 mRNA, Polymorphism, Liver abnormality

INTRODUCTION

Vinyl chloride monomer (VCM) is the principal starting material in the synthesis of polyvinyl chloride (PVC), a plastic resin used in various consumer and industrial products such as food and beverage containers, wrapping film, battery cell separators, electrical insulation, water pipes and hoses, flooring, and others. In 1974, three cases of hepatic angiosarcoma were reported in workers at a PVC production facility in Louisville, Kentucky. This report and subsequent studies led to the recognition of VCM as a definite human carcinogen that can cause angiosarcomas of the liver [1].

In workers, VCM is rapidly absorbed following respiratory exposure and is primarily metabolized in the liver to the electrophilic metabolites, chloroethylene oxide (CEO) and chloroacetaldehyde (CAA), by the
cytochrome P4502E1 (CYP2E1) system [2–3]. Although CYP2E1 is primarily a hepatic enzyme, recent evidence suggests that CYP2E1 is also expressed in the peripheral blood lymphocytes of rabbits [4], rats [5] and humans [6]. In addition, animal tests have demonstrated that certain exposures upregulate the expression of CYP2E1 both in the peripheral blood lymphocytes and the liver [4]. This suggests that the expression of CYP2E1 in peripheral blood lymphocytes may be a useful surrogate for hepatic expression of CYP2E1.

In the present study, we have taken advantage of this correlation to study the potential mechanism of VCM-induced liver damage by determining the level of expression of CYP2E1, as well as the presence of CYP2E1 polymorphisms, in the peripheral blood lymphocytes of VCM-exposed workers in relation to their exposure and the presence of liver abnormalities.

MATERIALS AND METHODS

Subject and samples

This study was performed on 90 male workers occupationally exposed to VCM and 42 male unexposed controls from the same company in China who were group-matched with respect to age, employment duration, education, social status, smoking, drinking, and personal and family history of disease. We took part in an occupational health examination in this company in 2003 and collected 2-ml samples of anti-coagulated peripheral blood. We also administered a questionnaire including the worker’s name, age, education, tobacco smoking, alcohol drinking, drug use, workshop section, duration of service in current job, use of personal protective equipment, and history of hepatitis and schistosomiasis. The workers with a history of hepatitis or schistosomiasis were excluded from the study. Data were also collected about liver structure or function abnormalities, including serum alanine aminotransferase (ALT) levels and B-type hepatic ultrasound results, from the workers’ medical records. Workers with an ALT level higher than 40 U or a defect on ultrasound were considered to have a liver abnormality.

Workers’ cumulative VCM exposure was determined based on average VCM concentrations in the workshop, deriving from workplace monitoring data, and exposure duration; assuming an average daily exposure was 2 hours. Thus, the individual cumulative dose was calculated as follows:

\[
\text{cumulative dose (mg)} = \sum (\text{average monthly VCM air concentration} \times \text{days of exposure within that month} \times 120 \text{ minutes per day}) \times \text{lung ventilation} (= 6.5 \text{ l/min assuming 30% dead space}) \times 70\%.
\]

Lymphocyte isolation

Lymphocytes were isolated from the peripheral blood samples according to the method of Boyum [7]. Briefly, 4 ml of Histopaque-1077 (Sigma Chemical Co.) was added to a 10-ml conical centrifuge tube, the blood sample was layered onto it, and the tube was centrifuged at 400 g for 30 min at room temperature. After centrifugation, the opaque interface was transferred to a new tube with phosphate-buffered saline and centrifuged at 250 g for 10 min at 4°C. Trizol (Gibco BRL Products) was added to the cells and agitated 3–5 times. The mixture was then stored at −70°C until use.

Total RNA isolation and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the lymphocytes using Trizol reagent (Gibco BRL) and quantified by measuring absorbance at 260 nm; purity was assessed from the 260/280 nm absorbance ratio. RT-PCR was performed using one-step SuperScript kit (Gibco BRL) following the manufacturer’s instructions. A total reaction mixture volume of 25 μl contained 3 μg of total RNA. The RT cycle was 30 min at 50°C and 2 min at 94°C; 40 PCR cycles followed at 94°C for 45 sec, 54°C for 1 min, and 72°C for 1 min. The PCR mixture was extended at 72°C for 10 min in order to complete the process [8]. Expression of the housekeeping gene glyceraldehyde 3-phosphoric acid dehydrogenase (GAPDH) was used for normalization of CYP2E1 mRNA to enable cross comparisons between the samples. The GAPDH primer was designed using Primer Premier 5. GAPDH and CYP2E1 primers are shown in Table 1.
RESULTS

A total of 132 subjects (90 exposed workers and 42 non-exposed controls) were included in the final analysis. The demographic characteristics of the study population are summarized in Table 2. The exposed and nonexposed group did not differ significantly with respect to age, duration of employment, or proportion of smokers or drinkers (t-test or χ²-test, P > 0.05). The individual cumulative dose for VCM in exposed workers ranged from 930.27 mg to 45 260.51 mg. The VCM-exposed subjects were then divided into high-exposure group (n = 49) and low-exposure group (n = 41) according to the median dose (15 000 mg).

As shown in Table 3, the CYP2E1 mRNA expression was significantly higher in the exposed group than in nonexposed controls (P < 0.01).

Table 1. Gene-specific primers and PCR condition

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sense</th>
<th>Primer sequences 5’ to 3’</th>
<th>Nucleotide position</th>
<th>Target size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1</td>
<td>Forward</td>
<td>ACCTGCCCCCATGAAGCAACC</td>
<td>1108-1127</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAAACAACTCCATGCGAGCC</td>
<td>1353-1334</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CGGAGTCACAGGGATTTGGCTGTAT</td>
<td>93-116</td>
<td>307</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCCTTCTCCATGGTGTTGAAGAC</td>
<td>399-376</td>
<td></td>
</tr>
</tbody>
</table>

All the primers were synthesized using Sangon Biological Engineering Technology and Service Co. LTD (Shanghai, China). The GAPDH PCR cycles were as follows: 30 sec at 94°C, 1 min at 57°C, and 1 min at 72°C. The results were expressed as the ratio of optical density of PCR bands for CYP2E1 and GAPDH. All analyses were performed using Quantity One-4.01 image analysis software.

CYP2E1-PstI polymorphism
Genomic DNA was extracted from the lymphocytes by routine phenol-chloroform method. For CYP2E1 gene analysis, restriction fragment length polymorphisms (RFLP) were detected by differences in a PstI site in the 5'-flanking region following PCR amplification, after Hayashi et al [9]. The primers used were as follows: 5'-CAG TCG AGT CTA CAT TGT C-3’ and 5’-TTC ATT CTG TCT TCT AAC TG-3’. 0.5 μl of DNA was added to a 10x PCR buffer containing 200 ng or primers, 1.25 mM MgCl₂, and 0.2 mM dNTPs in a final volume of 25 μl. Reaction mixtures were heated for 5 min at 94°C, and 1.25 units of Taq polymerase was then added. Subsequently, 35 cycles of amplification were performed: denaturing at 94°C for 30 sec, annealing at 58°C for 45 sec, and extending at 72°C for 45 sec. The PCR products were digested with PstI for 16 h at 37°C and analyzed on 2.2% agarose gel. Individuals who carried homozygous c1c1 genotype had a 410 bp product fragment, whereas individuals with homozygous c2c2 genotype had 290 and 120 bp fragments, and those with heterozygous c1c2 genotype had all the three fragments.

Table 2. Demographic characteristics of the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>VCM-exposed</th>
<th>Nonexposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>90</td>
<td>42</td>
</tr>
<tr>
<td>Age (year)</td>
<td>34.73±8.19</td>
<td>37.57±7.95*</td>
</tr>
<tr>
<td>Duration of employment (year)</td>
<td>12.90±6.49</td>
<td>11.88±7.40</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>32 (35.55%)</td>
<td>17 (40.48%)</td>
</tr>
<tr>
<td>Current alcohol user (%)</td>
<td>41 (45.55%)</td>
<td>22 (52.38%)</td>
</tr>
</tbody>
</table>

* Mean ±SD.

Table 3. Lymphocyte CYP2E1 mRNA expression in the study population

<table>
<thead>
<tr>
<th></th>
<th>VCM-exposed</th>
<th>Nonexposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>90</td>
<td>42</td>
</tr>
<tr>
<td>CYP2E1 mRNA expression</td>
<td>0.89±0.46**</td>
<td>0.61±0.35*</td>
</tr>
<tr>
<td>(relative density)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** P < 0.01, compared with nonexposed group.
Finally, the 33 exposed workers with liver abnormalities were group-matched with 30 exposed workers without liver abnormalities for CYP2E1 genotype comparisons; these two sub-groups were comparable with respect to age, employment duration, education, social status, smoking, drinking, and personal and family history of disease. The overall frequencies of the c1 and c2 alleles of CYP2E1 were 81% and 19%, respectively. There was no statistically significant difference in the genotype distribution between individuals with liver abnormalities and those without (data not presented). Furthermore, although CYP2E1 mRNA expression was higher in individuals with a c2 allele (0.93±0.53) compared to those with only c1 alleles (0.82±0.39), this difference was not statistically significant even after controlling for age, smoking, drinking, and VCM exposure.

**DISCUSSION**

In this study, we compared cumulative VCM exposure, based on a fairly precisely calculated individual dose, with the expression of lymphocyte CYP2E1 mRNA and the proportion of individuals with liver abnormalities. In addition, we investigated the relationship between CYP2E1 genotype and expression. The key objective of the study was to explore the possible molecular mechanism of VCM-induced liver damage.

Previous studies have shown that VCM is primarily metabolized by CYP2E1 to CEO under conditions of high-level exposure (> 1 ppm) [2,3]. Given the exposure levels of workers in this study, we presumed that CYP2E1 would play an important role in the process of their metabolism. CEO is a reactive intermediate that binds covalently to cellular macromolecules to form adducts. Many studies have confirmed that VCM exposure is hazardous to the liver.

Thirty-three of exposed workers and seven of controls showed a liver abnormality (serum ALT > 40 U and/or a defect on B-type hepatic ultrasound), as shown in Table 4. Thus, the proportion of workers with a liver abnormality was significantly higher in the study group than in controls (P < 0.05).

To explore the potential relationship between CYP2E1 expression and liver abnormalities, the expression was compared between all the subjects with and without liver abnormalities. Demographically, these two groups did not differ significantly except for the fact that the subgroup with the normal liver structure and function was significantly older. The relative densities for CYP2E1 mRNA expression were 1.04±0.32 in the subgroup with liver abnormalities and 0.70±0.46 in the group without liver abnormalities. This was found to be a statistically significant difference after controlling for VCM exposure and age (P < 0.01).

To further examine the link between CYP2E1 expression and liver abnormalities, logistic regression analysis was performed for the exposed group, controlling for other potential confounders including age, smoking, drinking, duration of employment, and level of exposure. As shown in Table 5, increased CYP2E1 expression was significantly associated with an increased risk of liver abnormalities. Interestingly, older age was significantly associated with a decreased risk of liver abnormalities in this analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Smoking</th>
<th>Drinking</th>
<th>Age</th>
<th>Duration of employment</th>
<th>CYP2E1 expression</th>
<th>Level of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.945</td>
<td>0.956</td>
<td>0.092*</td>
<td>0.376</td>
<td>0.028*</td>
<td>0.751</td>
</tr>
<tr>
<td>OR (95%CI)</td>
<td>1.041</td>
<td>1.031</td>
<td>0.454</td>
<td>1.405</td>
<td>3.655</td>
<td>0.824</td>
</tr>
<tr>
<td></td>
<td>(0.330, 3.286)</td>
<td>(0.339, 3.140)</td>
<td>(0.181, 0.938)</td>
<td>(0.662, 2.979)</td>
<td>(1.147, 11.645)</td>
<td>(0.249, 2.724)</td>
</tr>
</tbody>
</table>

* P < 0.10, statistically significant for liver abnormality.
in several ways, including induction of hepatic angiosarcomas [10–12]. Our study revealed a significantly higher incidence of liver abnormalities among exposed workers than among unexposed controls. It is presently unclear how significant a role the CYP2E1 plays in the process of hepatic damage, both in terms of its level of expression and the presence of genetic variants.

We used RT-PCR, a semi-quantitative technique for determining mRNA expression, to examine CYP2E1 expression in lymphocytes in this study. Although lymphocyte expression of CYP2E1 is generally low, it is detectable using this approach and there is evidence implying that lymphocyte CYP2E1 is a reasonable surrogate for hepatic CYP2E1 [13]. In our study, CYP2E1 mRNA levels were significantly higher in VCM-exposed workers than in non-exposed controls. There was no significant difference in the expression levels between the workers with low and high levels of exposure. However, the presence of liver abnormalities was closely associated with CYP2E1 mRNA expression. These results may indicate that VCM mediates liver damage by the upregulation of CYP2E1 expression, possibly by generating more reactive intermediates at any given level of VCM exposure. Logistic regression analysis confirmed that high levels of CYP2E1 mRNA increased the risk of liver abnormalities, and further suggested that older workers were less likely to present liver abnormalities. The latter effect may be partially explained by the findings of Du et al. [14] which indicate that VCM exposure, rather than causing enzyme induction, might increase the breakdown of CYP2E1 in long-term exposure.

The presence of less susceptible workers, who are able to stay on the job for long periods of time, may also contribute to this effect.

The prevalence of the CYP2E1 c2 allele found in this study (19%) was similar to that reported in other studies of Chinese and Taiwanese populations (20%) [15]. CYP2E1 genotype has also been reported to be associated with VCM metabolism and related health effects [15–16]. The CYP2E1 c2 allele is considered to have a relatively higher transcriptional activity than the normal c1 allele [9], and this PstI polymorphism was associated with increased CYP2E1 mRNA in previous studies. It has also been reported that abnormally elevated ALT occurs in VCM-exposed workers with the c2 allele [17]. In this study, an association was found between CYP2E1 genotype distribution and VCM-induced liver abnormalities. Furthermore, although the workers with the c2 allele had higher CYP2E1 mRNA expression compared to those with only the c1 allele, the difference was not statistically significant. The inconsistencies noted between the findings of this and previous studies may be due to the relatively small number of subjects in these particular comparisons, which severely limited the statistical power of the analysis.

Further research with a larger number of workers will be necessary to more fully delineate the relationship among CYP2E1 genotype, CYP2E1 expression, and liver damage from VCM exposure.

In conclusion, this is the first report demonstrating a relationship between liver abnormalities and lymphocyte CYP2E1 mRNA expression in VCM workers, suggesting that VCM exposure can upregulate CYP2E1 expression. Since the subjects exposed to VCM had a higher prevalence of liver abnormalities, the elevated CYP2E1 mRNA may play a critical role in the induction of liver damage in VCM exposure. These findings need to be confirmed by a large-scale study because of the relatively small population sample analyzed in the present investigation.

ACKNOWLEDGEMENTS

We thank physician Shang-jian Chai for his assistance in medical examinations and collection of VCM exposure data. This research was supported by the National Natural Science Foundation of China (NSFC 30070650, 30671740), the China National Key Basic Research and Development program (2002CB512909), Scientific and Technical Supporting Programs of China (2006BAK05B01), as well as NIH grants P30-ES09089.

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