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Investigation of micronucleus frequencies in lymphocytes of inhabitants environmentally exposed to chrysotile asbestos

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Abstract
Exposure to asbestos minerals has been associated with a wide variety of adverse health effects including lung cancer, pleural mesothelioma, and cancer of other organs. Many of the regions of Turkey have asbestos deposits. People in Doğanlı village – one of these regions – have been environmentally exposed to chrysotile asbestos since they were born. In this study the effects of asbestos on micronucleus (MN) frequencies of inhabitants exposed to chrysotile asbestos have been examined. Thirty subjects who had been environmentally exposed to chrysotile asbestos and living in Doğanlı village, and 25 controls were studied to assess the MN frequency. The control group was selected from healthy individuals with no exposure to asbestos and living in similar geographic conditions to Doğanlı village. Peripheral blood samples were collected from each subject and cultured for MN assay. Cytochalasin-B was added to lymphocyte cultures for evaluation of MN in binucleated (BN) cells. The differences between those exposed to chrysotile asbestos and controls were not statistically significant in terms of BN cells with MN ($p > 0.05$). There was not a significant relationship between MN frequencies and age, sex, smoking, both in chrysotile asbestos-exposed subjects and in controls ($p > 0.05$). Although the detection of calcified pleural plaques found in the inhabitants has indicated environmental exposure to chrysotile asbestos, our results show that chrysotile asbestos was not an inducer of MN in subjects exposed to chrysotile asbestos.

Keywords: *Calcified pleural plaques, chrysotile asbestos, lymphocytes, malignant pleural mesothelioma (MPM), micronucleus (MN)*

Introduction

In some areas of Turkey the presence of asbestos deposits is known (Barış 1987). Environmental exposure to asbestos causes endemic lung and pleural diseases. The soil, which contains asbestos fibers, is used as a white-wash material for the walls and floors of the houses and various other purposes such as insulating. This process is repeated every year.

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Thus, environmental or domestic exposure to asbestos occurs nearly 24 hours a day, beginning at birth and continuing for the entire life, unless the individual moves to an area without such exposure. In particular, inhabitants of the central and southeast regions of Turkey were reported to have a high incidence of malignant pleural mesothelioma (MPM) due to such exposures (Yazıcıoğlu et al. 1980; Barış 1987; Şahin et al. 1993; Çöplü et al. 1996; Demiroğlu 1998; Yılmaz et al. 1998; Metintaş et al. 1999; Müller & Fischer 2000; Şenyiğit et al. 2000a, 2000b; Dumortier et al. 2001). In addition, Selikoff et al. (1968) were the first to demonstrate the existence of a synergistic relationship between asbestos exposure and smoking for the formation of bronchial carcinomas. Some other studies implicate an enhancing effect of cigarette smoke on asbestos-induced toxicity (Eastman et al. 1983; McFadden et al. 1986; Kamp et al. 1998).

Asbestos has been shown to increase chromosomal aberrations in cultured human lymphocytes (Valerio et al. 1983), sister chromatid exchange (SCE) in cultured V79 cell lines (Trosić et al. 1997) and micronucleus frequency in cultured V79 cell lines (Lu et al. 1994), in cultured Syrian hamster embryo (SHE) fibroblasts (Dopp et al. 1995, 1997; Dopp & Schiffmann 1998) and in cultured human bronchial epithelial (HBE) cells (Kodama et al. 1993) *in vitro*. Still some other studies showed enhanced SCE in cultured human lymphocytes of asbestos-exposed workers (Rom et al. 1983; Fatma et al. 1991; Lee et al. 1999) and inhabitants (Dönmez et al. 1996). Atalay et al. (2000) found that the SCE frequencies in the pleural effusion cells of patients with malignant mesothelioma were significantly higher than in the controls.

In recent years, mutations in the K-ras, p53 and p21 genes have been investigated in the MPM patients with asbestos exposure. However, studies have shown that mutations in these oncogene and tumour suppressor genes do not seem to be significantly involved in the development of mesotheliomas (Cote et al. 1991; Mor et al. 1997; Kitamura et al. 1998; Ni et al. 2000; Işık et al. 2001; Olut et al. 2001).

Doğanlı village, one of the regions with asbestos deposits in Turkey, is located about 44 km north of Yıldızeli district in the Sivas. The inhabitants use chrysotile asbestos as a raw material for painting and plastering of their houses. MPM have not been reported in the village, but the prevalence of asbestos-related radiological findings – in particular, calcified pleural plaques – is more common in inhabitants who are environmentally exposed to chrysotile asbestos (Özesmi et al. 1991). This study is designed to investigate the effects of chrysotile asbestos on micronucleus (MN) frequencies of inhabitants without MPM environmentally exposed to chrysotile asbestos in this village.

Materials and methods

Definition of the area and subjects

Doğanlı is a village with 270 inhabitants 44 km to the north of the district of Yıldızeli in Sivas in Turkey. The inhabitants are engaged in husbandry and agriculture. Asbestos deposits are abundant around Sivas. Chrysotile asbestos was detected in the soil as a raw material for painting and plastering the walls, floors and fireplace insulation of their houses in this village. Although cases of calcified pleural plaques were observed (2.7% in total people and 4.51% in people aged 20 and over), MPM has not been reported in this village (Özesmi et al. 1991). Today, all the inhabitants of Doğanlı village have stopped using asbestos and started to use modern building materials in the last decade. However, the inhabitants have been exposed to chrysotile asbestos for years.

Thirty subjects exposed to chrysotile asbestos and 25 controls were studied to assess the MN frequency. The experimental group (11.11% of inhabitants) were comprised of

22 women and 8 men who have lived in Doğanlı village since birth and used chrysotile asbestos in their houses. Their mean age was 59.97 years with a range of 32 – 80 years (27 non-smokers and 3 smokers).

The control group was selected from among healthy individuals with no exposure to asbestos, living in similar geographic conditions to Doğanlı, from the village of Kayseri, and engaged in husbandry and agriculture. The controls were of similar age, sex, habits, and socio-economic state as the subjects exposed to chrysotile asbestos. The control 25 subjects were made up of 15 women and 10 men, and their mean age was 54.80 years with a range of 33 – 77 years (22 non-smokers and 3 smokers).

A questionnaire containing the history of present and past illnesses, smoking habits, length of time in village, and records of family history was completed for each subject. All subjects were carefully examined and detailed clinical records of their history were kept by a chest physician. None of the subjects had taken any drug for at least three months previously for medical or other reasons.

The local ethics committee approved the study protocol. The study was conducted in accordance with the declaration of Helsinki and local laws, depending on which afforded greater protection to the patients.

Lymphocytes cultures

Heparinized blood samples (0.3 ml) were obtained from each subject and transferred to the Medical Biology Laboratory on the same day. The samples were immediately incubated for 72 h at 37°C in 5 ml of the culture medium Nutrient Mixture F-10 (Biol. Ind.) that was supplemented with 20% heat-inactivated fetal calf serum (Biol. Ind.), 1.5% phytohemagglutinin (Biol. Ind.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Biol. Ind.).

Micronucleus assay

After 44 hours of incubation, cytochalasin-B (Sigma Chemical Co, St Louis, MO, USA; 14930-96-2) was added to cultures to give a final concentration of 3 μg/ml, according to the method of Fenech and Morley (1985). The cultures were stopped at 72 h, treated with hypotonic solution (0.1 M KCl) by the method of Balasem and Ali (1991) for 3 min and fixed in two changes of methanol-acetic acid (3:1). The fixed cells were spread onto glass slides and stained with 5% Giemsa for 7 min. All the slides were coded and blind read. In order to determine intra-individual differences, different slides of two parallel cultures of one person were prepared. Cells with two macronuclei surrounded by cytoplasm and a cell membrane were scored for the presence of micronuclei. Published criteria for micronuclei determinations were followed (Fenech 1993, 2000) and 1000 binucleated (BN) cells were analysed for each case.

Statistical analysis

Statistical comparisons on frequency of BN cells with MN in subjects exposed to chrysotile asbestos and controls were made using Mann-Whitney U test from nonparametric tests. Differences between subjects exposed to chrysotile asbestos and controls were considered significant at $p < 0.05$. The effects of age, sex and smoking on MN frequencies in subjects exposed to chrysotile asbestos and controls were investigated by linear regression analysis.

Results

As stated in a previous study, the incidence of pleural calcifications associated with chrysotile asbestos found in the total population of Doğanlı village was 2.7%, and 4.51% in those aged 20 and over, but no lung cancer and MPM had been reported in the inhabitants of the village (Özesmi et al. 1991).

The percentage of BN cells with MN were studied in chrysotile asbestos-exposed inhabitants and controls. In addition, the percentage of BN cells with 1 MN, 2 MN, 3 MN, 4 MN and 5 MN were counted. No significant increase in the percentage of BN cells with all MN was observed in lymphocytes of chrysotile asbestos-exposed inhabitants (1.03 ± 0.52) when compared to controls (1.00 ± 0.49) ($p = 0.946$, Table I). No chrysotile asbestos was found to induce number of BN cells with all MN ($p = 0.946$) and BN cells with 1 MN ($p = 0.388$), 2 MN ($p = 0.235$), 3 MN ($p = 1.000$), 4 MN ($p = 1.000$) and 5 MN in subjects exposed to chrysotile asbestos when compared to controls (Table I). No significant relationship has been shown between MN frequencies and age, sex, and smoking both in chrysotile asbestos-exposed subjects and in controls ($p > 0.05$). Although smoking is known to increase MN frequency in lymphocytes (Tomanin et al. 1991; Fenech 1993), in this study the smoking did not cause any statistically significant alteration in MN frequency, probably owing to the small number of smokers (three subjects) in both groups.

Discussion

In one of the first reports in Turkey, the incidence of pleural calcifications associated with chrysotile asbestos was found to be 2.6% for the inhabitants of some villages and towns around Diyarbakır (Yazıcıoğlu 1976). Also, the incidence of calcified pleural plaques related to asbestos was found to be between 5 and 10% in other regions in Turkey (Barış et al. 1979). These findings are in agreement with the results of our previous study, which found 2.7% of the total population of Doğanlı village and 4.51% in people aged 20 and over (Özesmi et al. 1991).

The exposure to environmental mutagens may result in genetic damage. MN frequencies in cultured human lymphocytes provide an index of accumulated genetic damage occurring during environmental exposures. The MN test is considered to be a useful new genotoxicity assay and designed as an alternative for the *in vitro* chromosome aberration test. Micronuclei arise during cell division from either chromosomes that are lagging in anaphase or from chromosome fragments. MN may thus contain a whole chromosome(s) and/or acentric fragment(s). For human lymphocytes use of cytochalasin-B is recommended to ensure that

Table I. MN frequencies in lymphocytes of inhabitants environmentally exposed to asbestos and controls (mean ± SD).

	No. of BN cells scored	Percentage of BN cells with					
		1 MN	2 MN	3 MN	4 MN	5 MN	all MN
Exposed subjects ($n = 30$)	1028.53 ± 20.92	0.84 ± 0.37	0.14 ± 0.07	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	1.03 ± 0.52
Controls ($n = 25$)	1015.76 ± 9.55	0.72 ± 0.29	0.20 ± 0.12	0.10 ± 0.00	0.10 ± 0.00	–	1.00 ± 0.49
p	–	0.388	0.235	1.00	1.00	–	0.946

cells analysed for MN have divided once (Kirsch-Volders et al. 2000). In the present study, our data show that no MN frequency depending on the percentage of BN cells with 1 MN, 2 MN, 3 MN, 4 MN, 5 MN and all MN is induced in lymphocytes of chrysotile asbestos-exposed inhabitants when compared to controls ($p > 0.05$; Table I).

Indeed, no reports have been found that can be compared one to one with those obtained from the present study for MN frequencies, because most of the existing studies are done on the frequency of SCE in human lymphocytes of workers occupationally exposed to asbestos (Rom et al. 1983; Fatma et al. 1991; Lee et al. 1999). But in respect to asbestos-exposed inhabitants, it has been shown that the rates of SCE were higher in exposed inhabitants compared to controls from Turkey (Dönmez et al. 1996; Atalay et al. 2000). However, the asbestos-induced MN frequency in cultured V79 cell lines (Lu et al. 1994), in cultured SHE fibroblasts (Dopp et al. 1995, 1997; Dopp & Schiffmann 1998) and in cultured HBE cells (Kodama et al. 1993) has been shown *in vitro*.

All forms of asbestos are carcinogenic. However, it is widely believed that the carcinogenic potency of tremolite is much higher than that of chrysotile. Crocidolite seems to be 2–4 times more potent than chrysotile in its capacity to induce mesothelioma (Demiroğlu 1998; Müller & Fischer 2000). The exposed asbestos fiber types, as well as factors such as cigarette smoking and genetic susceptibility, may play an additive or synergistic role in the development of malignant pleural and peritoneal mesotheliomas. Also, significant increases in MN frequency related to smoking were reported (Tomanin et al. 1991; Fenech 1993). In this study, the number of smokers was rather low in both groups, and smoking did not induce MN frequencies in chrysotile-exposed inhabitants and controls ($p > 0.05$).

Although the use of asbestos is abandoned today, Doğanlı villagers have been exposed to chrysotile asbestos for a long time. In this village, however, increased MN frequencies in inhabitants were not found when compared with asbestos non-exposed controls. Therefore, our results have shown that the MN frequencies in inhabitants environmentally exposed to chrysotile asbestos are not induced, and may indicate that the increased MN frequency is not related to calcified pleural plaques, even if extensive, in subjects exposed to chrysotile asbestos. Also, an explanation for the lack of effect of chrysotile asbestos exposure on MN frequency; it is possible that repairing mechanisms occur after exposure has terminated.

Conclusion

Although asbestos is known to be a complete carcinogen for the production of mesothelioma, its mechanisms of action are still in question. In a cellular response to asbestos-induced DNA damage, the cell can either arrest to permit DNA repair or initiate apoptosis (programmed death of the cells). The precise mechanism by which asbestos activates DNA repair pathways in eukaryotic cells is complex and not well established (Upadhyay & Kamp 2003). Fung et al. (1998) showed that crocidolite asbestos induces the DNA repair enzyme in mesothelial cells. In this study, we may suggest that the DNA repair pathway can preferentially occur in lymphocytes of inhabitants after exposure to chrysotile asbestos. Further studies are required to support that chrysotile asbestos did not induce MN frequency in human lymphocytes.

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