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<th><strong>Title</strong></th>
<th>A preliminary study of DNA damage in peripheral lymphocytes from lung cancer patients and healthy subjects</th>
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<td>This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.; A Preliminary Study of DNA Damage in Peripheral Lymphocytes from Lung Cancer Patients and Healthy Subjects by See King SHAM, Yim Tong SZETO, Iris Frances Foster BENZIE and Kian Cheng TAN-UN, Turkish Journal of Medical Sciences, v. 33 n. 3, Copyright©TUBITAK 2003.</td>
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Abstract: A pilot study was undertaken to investigate the differences in oxidative DNA damage and 2-h DNA repair in peripheral lymphocytes between lung cancer patients and healthy subjects. Twenty-four lung cancer patients and 23 normal controls were recruited from the Queen Mary Hospital, Hong Kong SAR, China. Single cell gel electrophoresis (comet assay) was performed to measure the oxidative damage, repair and baseline of peripheral lymphocytes in the subjects. There were no significant differences in baseline DNA damage and oxidative stress damage and DNA repair at 2 h among the two groups of lung cancer patients who had blood taken before and those who had blood taken after chemotherapy, and normal subjects. There were no differences in basal DNA, oxidative DNA damage or DNA repair between those with and without vitamin supplements in normal subject diets. Our results demonstrated that oxidative DNA damage in lymphocytes is not associated with the risk of lung cancer.

Key Words: Comet assay, DNA damage, Lung cancer, Lymphocyte

Introduction

In Hong Kong, lung cancer is the leading cause of cancer-related deaths in both men and women. An average of 3400 new cases were diagnosed each year during the period 1985-1994 (1). The mortality rate for lung cancer has been stable at around 56 per 100,000 for men and 23 per 100,000 for women in the past decade (1). As lung cancer occurs in older subjects, its incidence and mortality will remain high with increasing numbers of older subjects in the population in Hong Kong.

Smoking is the major cause of lung cancer. Although smoking causes the vast majority of lung cancer in women in other parts of the world, in Hong Kong two-thirds of cases occur in nonsmokers (2-4). Avoidance of environmental tobacco smoke (ETS) exposure for 15 years has been shown to reduce the risk of lung cancer (5). Exposure to air pollutants, indoor cooking fumes, and occupational exposures to certain chemicals such as arsenic, asbestos and uranium are also risk factors for lung cancer (6).

A diet rich in fat and cholesterol has been shown to be associated with a high risk for lung cancer (7-9). Fang and co-workers suggested that the interaction of lipid peroxidation products, such as malonaldehyde (MA), with DNA might explain the role of dietary fat in carcinogenesis (10). Processed and preserved meats have been found to increase the risk of lung cancer (8). A study by the International Agency for Research on Cancer (IARC) has shown that heterocyclic aromatic amines in fried meat were carcinogenic (8,11). In addition, nitrosamines in preserved meat products have been implicated as the causative agent for several types of cancer (12). Nitrosamines undergo cytochrome P450-catalyzed hydroxylation activation and are metabolized into the active form of carcinogens, α-hydroxy (NDMA) N-nitrosodimethylamine, which fragment to yield formaldehyde and DNA/RNA alkylating methanediazonium ion (12). Moreover, genotoxic DNA alkylation can occur by the conjugation of glutathione with nitrosamines (13). Previous studies demonstrated that increased intake of ascorbic acid and other nitrite scavengers can prevent N-nitrosamine-induced carcinogenesis (12,14,15).

Fruit and vegetable consumption may confer a protective effect against lung cancer among nonsmokers (16,17). Koo and co-workers (3,18) have shown that adequate intake of fruits and vegetables reduced the risk of lung cancer among nonsmoking women. The protective effect of diets rich in vegetables and fruits has
been substantiated by the results of intervention studies based on the use of a biomarker for oxidative DNA damage (19-22). The high content of antioxidants such as carotenoids, vitamin C, tocopherols, and various flavonoids found in vegetables and fruits may be responsible for the protective effect. Antioxidants act by quenching endogenous or exogenous reactive oxygen species (ROS) that damage DNA (19); and they also reduce the impact on DNA by electrophilic intermediates of procarcinogens (23).

Survival from lung cancer is dependent on the stage of the disease at the time of diagnosis. Unfortunately, most patients are diagnosed late because in the early stage of the disease they do not have symptoms. Early detection before the tumor has spread to other parts of the body is essential for a good prognosis.

Oxidative damage to DNA and the rate of DNA repair may serve as early markers of lung cancer. The single cell gel electrophoresis (SCGE) assay (24), also known as the comet assay (25), is a rapid, relatively simple, visual biochemical technique for measuring and quantifying DNA damage. Using this assay to evaluate the resistance of DNA damage (strand breaks) and rates of repair in lymphocytes may provide a good potential biomarker of lung cancer before its development.

The aim of this study was to detect DNA damage and repair in peripheral blood lymphocytes in lung cancer patients and healthy controls using the comet assay. We hypothesized that lung cancer patients have higher baseline DNA damage, decreased defense against oxidant challenge, and less efficient DNA repair capacity compared to healthy controls.

Methods

Subjects

Twenty-four lung cancer patients were recruited between February and July of 1999. They were diagnosed in 1998 and 1999. All patients had advanced non-small-cell lung cancer and were receiving treatment at the Department of Medicine, Queen Mary Hospital, Hong Kong. They were treated with chemotherapy (cisplatin 75 mg/m² and docetaxel 75 mg/m² every 3 weeks or carboplatin 75 mg/m² and docetaxel 75 mg/m² every 3 weeks). All patients had adequate hematologic, renal, and hepatic function before chemotherapy. The majority of recruited lung cancer patients had lung adenocarcinoma. A group of 23 healthy subjects were recruited from the hospital laboratory as controls.

The study was approved by the Medical Ethical Committee of the Queen Mary Hospital and the University of Hong Kong.

Blood sample collection

Peripheral blood samples were collected from patients and controls in sterile disposable syringes, transferred into heparinized tubes, kept in iced water and the comet assay was carried out within 2 h.

Preparation of reagents and slides for the comet assay

Eighty-five microliters of prewarmed (40 °C) 1% (w/v) standard agarose in phosphate-buffered saline (PBS) was immediately applied to a microscope slide that had been precoated with 1% (w/v) agarose in water. For each subject, three slides were made: one for the study of hydrogen peroxide-induced damage, one for 2-h repair and one for the baseline.

Hydrogen peroxide-induced DNA damage in isolated human lymphocytes

Heparinized blood was mixed with RPMI 1640 medium (GibcoBRL) supplemented with 10% (v/v) fetal bovine serum (GibcoBRL), and underlayered with Histopaque 1077 (Sigma) lymphocyte separation medium. It was then centrifuged at 200 g for 10 min at 4 °C. The “buffy coat” was removed after separation, washed in PBS (pH 7.4) and centrifuged as described before. The cell pellets were resuspended and then divided into three portions for studies on induced oxidative damage, repair and baseline levels. Cells for “induced damaged” and “repair” studies were incubated in 45 mM of hydrogen peroxide in PBS (pH 7.4) for 5 min on ice to suppress DNA repair. These cells were subsequently centrifuged, washed with cold PBS and centrifuged again. To assess DNA repair, pre-warmed RPMI 1640 medium (supplemented with 10% fetal bovine serum) at 37 °C was added to the cells for the “repair” studies after oxidant treatment (24). After 2 h, the cells were centrifuged at 4 °C, resuspended in PBS and mixed with agarose. The cell pellets were resuspended in 85 ml of 1% (w/v) low melting point agarose for the comet assay (26).
Single-cell gel electrophoresis (comet assay)

Cells were embedded in agarose, lysed and electrophoresed. Breaks in the DNA molecules hinder its supercoiling, allowing free DNA to migrate towards the anode. Cells before or after a repair period were suspended in 1% (w/v) low melting agarose in PBS, pH 7.4, at 37 °C. They were rapidly pipetted on the first layer of the precoated frosted glass microscope slides after gently removing the cover slips. The slides were kept at 4 °C for 5 min to solidify. After the removal of the cover slips, the slides were placed into cold lysing solution (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, 1% Triton X-100, pH 10.0) for 1 h to solubilize cellular proteins, leaving the DNA as “nucleoids”. After 1 h of lysis, all slides were placed into an electrophoresis chamber containing alkaline solution (0.3 M NaOH, 10 mM Na₂EDTA) for 40 min prior to electrophoresis to allow DNA unwinding. The current was switched on and electrophoresis was carried out at 25 V (0.83 V/cm), 0.3 A at 4 °C for 30 min after adjusting the buffer level in the tank. All of the procedures described above were conducted in the absence of ultraviolet light to prevent additional DNA damage. After electrophoresis, the slides were removed from the alkaline buffer, placed in a staining jar and washed three times at 4 °C (5 min each) with neutralizing buffer (0.4 M Tris, pH 7.5). Subsequently, the slides were stained with 35 ml of ethidium bromide solution (2 mg/ml in distilled water) and covered with cover slips (27).

Ethidium bromide-stained DNA on each slide was visualized at 200X magnification using fluorescence microscopy as “comets” with a fluorescent head and a tail (27) (see Fig.1). The fluorescent microscope was equipped with a 510-560 nm excitation filter, a 590 nm barrier filter and a CCD camera connected to an image analysis system for the comet assay. Evaluation of the images on the slides was performed using imaging software (Komet 3.0, Kinetics Imaging Ltd., Liverpool, UK). One hundred images were evaluated per slide and the average percentage of fluorescence in the tail (representing the fraction of DNA in the comet tail) on each slide was used. The repair proficiency was calculated by 1 - R/D x 100%, where D and R are the average percentages of tail DNA in 100 images in the damage and repair studies, respectively.

Data analysis

To study DNA damage and repair, lung cancer patients were divided into two groups: those whose blood was taken before the onset of first chemotherapy treatment and those whose blood was taken after chemotherapy (three treatments or less). The statistical analyses were performed using the statistical package of Excel 97 (Microsoft Corporation) and the statistical software, GraphPad Prism version 2.01 (GraphPad™ Software, San Diego, CA, USA). A chi-square test or one-way ANOVA was used in testing for statistical differences between the three groups. A P value of <0.05 was considered statistically significant.
Results

Table 1 shows that there were no significant differences in baseline DNA damage and oxidative stress damage among normal subjects and the two groups of lung cancer patients with blood taken before and after chemotherapy. The rate of DNA repair at 2 h was also not different among the three groups.

Table 2 demonstrates that among the control subjects there were no differences in basal DNA damage, induced oxidative DNA damage or DNA repair between those with and without vitamin supplements in their diet.

Discussion

Using the comet assay, oxidative DNA damage is correlated with the percentage increase in tail DNA found in lymphocytes, which was used as a marker of DNA strand breaks (28). Wei et al. (29) demonstrated that individuals with reduced DNA repair capacity were at increased risk of developing lung cancer. In this study, we were unable to find any difference between patients with and without chemotherapy and healthy controls. Since the sample size was small, further studies should be performed to confirm the effect of chemotherapy on oxidative DNA damage in the lymphocytes of lung cancer patients. As the turnover rate of lung epithelial cells is slower than that of lymphocytes, lymphocytes may not be ideal for studying the long-term effects of mutations (30). Baseline results showed individual differences (e.g., 7.4 ± 2.4 and 7.9 ± 2.0 of baseline tail DNA% in lung cancer patients) and it would be more pertinent to follow DNA damage in an individual over a period of time. Thus, lymphocytes may only give a fair indication of transient mutagen exposure. Lung cells are target tissues for investigations of the genotoxic effects of oxidant-producing compounds, such as components of cigarette smoke, pollutants and asbestos (31). Lung epithelial and pleural mesothelial cells (31,32) or nasal cells (33) that have a slower turnover rate may be used as target cells for measuring oxidant sensitivity and DNA repair.

In addition, antioxidant supplements before chemotherapy may have a protective effect on lung cancer patients as vitamins and trace minerals supplementation can ameliorate oxidative damage (34).

<table>
<thead>
<tr>
<th>Subjects/Treatment</th>
<th>Lung Cancer Patients</th>
<th>Healthy Controls</th>
<th>p value (ANOVA)</th>
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<tbody>
<tr>
<td></td>
<td>Pre-Chemo</td>
<td>Post-Chemo</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Baseline (mean ± SD)</td>
<td>7.4 ± 2.4</td>
<td>7.9 ± 2.0</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>45 mM H$_2$O$_2$ (oxidative stress) (mean ± SD)</td>
<td>42.2 ± 6.4</td>
<td>42.1 ± 9.4</td>
<td>44.0 ± 4.3</td>
</tr>
<tr>
<td>2-h repair (mean ± SD)</td>
<td>26.9 ± 9.1</td>
<td>27.8 ± 8.2</td>
<td>28.1 ± 7.7</td>
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<table>
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<tr>
<th>Subjects/Treatment</th>
<th>Tail DNA (%)</th>
<th>p value (t-test)</th>
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<tbody>
<tr>
<td></td>
<td>With supplement</td>
<td>Without supplement</td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>No oxidative stress (mean ± SD)</td>
<td>6.9 ± 0.6</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>45 mM H$_2$O$_2$ (mean ± SD)</td>
<td>44.0 ± 4.2</td>
<td>44.1 ± 4.6</td>
</tr>
<tr>
<td>2-h repair (mean ± SD)</td>
<td>26.2 ± 5.8</td>
<td>29.6 ± 9.7</td>
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Our result supports the hypothesis of Collins (1999) (13) that oxidative damage to DNA might not lead to cancer possibly because the mutation spectrum for oxidant treatment differs from that of spontaneous point mutations (35). Moreover, our result supports Collins et al. in that oxidative DNA damage in lymphocytes as a biomarker of oxidative stress may not reflect oxidative damage in other cell types that give rise to tumors (36). Collins suggested that endogenous oxidative damage to DNA might not be responsible for mutations occurring during tumorigenesis but deficiency in repair processes would be expected to be more important in promoting a significant mutational burden (13).

In this preliminary study, no significant difference in direct strand break has been found before or after chemotherapy. However, besides direct strand breakage and alkaline labile sites, DNA damage can be in the form of oxidized bases without breakage of the DNA strands. This type of DNA damage cannot be detected unless lesion-specific enzymes are added to create breaks at the sites of damage. The enzymes are introduced at the post-lysis stage in the assay, and this increases the sensitivity of the comet assay (37). Inclusion of the enzyme treatment step may help to detect DNA damage, e.g., oxidized nucleotides that have a lower rate of repair, other than direct strand breaks (38).

Conclusion
In this study, no significant differences in DNA damage and repairing efficiency were shown before or after chemotherapy or in lung cancer or normal subjects. However, further investigation on the other types of DNA damage besides direct strand break is essential. More accurate and precise information on the mechanisms of interactions between diet, environmental and occupational exposure in mutagenesis are definitely required.

Acknowledgments and Notes
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The study was approved by the Medical Ethical Committee of the Queen Mary Hospital and the University of Hong Kong.

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References


