Note

Vitamin Levels in Lung Tissue of Rats with Bleomycin Induced Pulmonary Fibrosis

Handan Mert\(^1\), Ibrahim Yoruk\(^2\), Ali Ertekin\(^3\), Semih Di
de\(^1\), Yeter Deger\(^3\), Fatmagul Yur\(^1\) and Nihat Mert\(^1\)

\(^1\)Department of Biochemistry, Faculty of Veterinary Medicine, and \(^2\)Department of Chemistry, Faculty of Art and Science, University of Yuzuncu Yil, 65080 Van, Turkey

(Received September 12, 2008)

Summary

Bleomycin (BLM) is a chemotherapeutic agent against different carcinomas, one dose of which causes dependent pulmonary fibrosis. The present study was taken up in order to measure the retinyl ester, \(\alpha\)-tocopherol and cholecalciferol (vitamin D\(_3\)) level in lung tissue in the rats following BLM-induced fibrosis. Fourteen rats were randomly divided into two groups as a control and a BLM group. On the day of the experiment, the BLM group rats were instilled with BLM (7.5 mg/kg) and the control group with sterile saline intratracheally. Fourteen days after instillation, rats in each group were sacrificed and the lungs were prepared for histopathological examination and determination of the vitamin levels with a HPLC system. The levels of retinyl ester, \(\alpha\)-tocopherol and vitamin D\(_3\) in the lungs of the BLM group were determined to be lower than in the controls. There was statistically significant difference for the \(\alpha\)-tocopherol and vitamin D\(_3\) concentrations compared to the control group (\(p<0.01\), \(p<0.001\), respectively). According to these results in pulmonary fibrosis, vitamins were consumed by the lung tissue and their levels decreased.

Key Words bleomycin, pulmonary fibrosis, vitamins

Bleomycin (BLM) is mixture of glycopeptide antibiotics used in clinical practice against some carcinomas. The lung has an important susceptibility to a variety of chemotherapeutic agent damage. BLM causes a dose-dependent pulmonary fibrosis characterized by the extracellular proliferation matrix of transforming fibroblasts and the neutrophiles and eosinophiles in the alveolar structures (1). The induced damage is almost or completely irreversible and mechanism of the damage is unknown. But reactive oxygen species (ROS) like superoxide, hydrogen peroxide, and hydroxyl radicals produced by the molecular oxygen pathway (2) of ferrous ion and nitrogen species (RNS) including nitric oxide produced by infiltrating the inflammatory cells could be the changes involved (3). It is well-known that nitric oxide reacts with superoxide (produced by all aerobic cells) to form peroxynitrite, a potent oxidizing agent that can cause biological oxidative stress. BLM also promotes the depletion of endogenous antioxidant defenses, thus exacerbating oxidant mediated tissue injury (4).

The lung has strongly specialized and compartmentalized antioxidant defenses to protect from the ROS and the RNS (5). It was known that antioxidant defenses play a critical role in the respiratory tract where the exposure raised to the endogenous and environmental oxidants occurs, which can deteriorate the function of the ventilator by inducing the inflammation and the destruction of the alveolar walls (6). Infiltrating inflammatory cells, which accumulate in the lower airways, release harmful amounts of reactive oxygen species that result in lung injury and proliferation of fibroblasts in alveolar walls (7). Fibroproliferative changes via a concerted action of various cytokines lead to collagen accumulation in the lung (8).

Vitamin E plays crucial roles as a peroxyl-radical scavenger and chain-breaking antioxidant. Among the vitamin E family, \(\alpha\)-tocopherol is the most abundant and biologically most active form in plasma and tissues (9). Vitamin E affects oxidative changes which occur in other cell organelles and it prevents lipid peroxidation and cell destruction (10). The antioxidant activity of vitamin E has persuaded many groups to study its protective effect of tocopherol against BLM-induced lung injury (11, 12).

Vitamin A is an important factor in promoting the normal respiratory epithelial differentiation and growth (13). The positive effect of plasma retinol on lung function has been recognized in many studies (14, 15). During moderate vitamin A deficiency, the incidence for respiratory tract diseases is considerably increased and repeated respiratory infections can be influenced therapeutically by a moderate vitamin A supplementation (16).

Vitamin D is transported to the liver, where it is hydroxylated by vitamin D-25 hydroxylase, forming 25-hydroxyvitamin D\(_3\) which is the most abundant metabolite found in the circulation. The active hormone 1,25-dihydroxyvitamin D\(_3\), or calcitriol plays an important role in not only calcium metabolism but also in cell dif-
Vitamin Levels in Lung Tissue in Pulmonary Fibrosis

Materials and Methods

Materials. Fourteen male Wistar-Albino rats, at the age of 3.5 mo and weighing 180–200 g were selected for the study. The rats were housed in cages during the day time of 07:00–19:00 h at an ambient average temperature of 21±1°C. Their diet consisted of rat chow and tap water ad libitum. All the implied processes in the animals were approved by the institutional committee.

The rats were randomly divided into 2 groups having 7 animals in each. (1) BLM- group: the rats received an intratracheal BLM instillation (Nippon Kayaku, Tokyo, Japan), which was dissolved in 10 mL of saline solution; a single dose of 7.5 mg/kg BLM was given with chloroform under anesthesia. (2) Control group: the rats received an intratracheal injection of the same quantity of 0.9% normal saline sterile instead of BLM. The animals were gently shaken to help the distribution of the BLM and saline in the whole lung.

Lung tissue collection. Rats were sacrificed after 14 d of BLM treatment. All the lung tissues were collected and washed twice with cold saline solution, then placed into glass bottles and finally marked. The lung tissues were cut into small pieces with scissors and weighed as 500 mg and all the analyses were performed with them.

Determination of vitamin concentration in lung tissue. HPLC analysis was carried out with an agilent 1100 chromatograph (Germany). The process of extraction was modified from relevant literature (21–24). Tissues were homogenized with the 250 μL 10% ascorbic acid and then 250 μL of ethanol was added to each tube and vortexed for 1 min. Later, 2 mL of n-hexane was added and vortexed. The samples were then centrifuged at 2,000 rpm for 10 min. The hexane-containing upper layer was removed, evaporated and dried under a nitrogen stream. The residue was dissolved in 250 μL of methanol. Standard retinyl ester, tocopherol and vitamin D₃ (Sigma Co.) were prepared for analysis. One hundred microliters of tissue extract was injected into a HPLC column (C-18, 250×4.6 mm, Ace-Scotland) for the separation and quantitative determination of the vitamins. DAD (diode-array detector) was employed at a wavelength of 265 nm for the vitamin D₃, 290 nm for tocopherols and 325 nm for retinyl ester. As in the mobile phase, methanol-water (98 : 2) (Merck) was employed at a 1.5 mL/min flow rate (21, 23–25).

Results and Discussion

The levels of retinyl ester, α-tocopherol and vitamin D₃ levels in BLM and control groups are indicated in Table 1.

Histological studies. After the lung tissues were fixed with the 10% formalin buffer for 24 h, they were processed for routine paraffin embedding. Serial sections with a thickness of 7 μm were cut and the histological changes in the lungs were evaluated by hematoxylin-eosin stain (26).

Statistical analysis. Results were expressed as means±standard error of the mean (SD). Unpaired Student’s t test was also employed for the statistical analysis.

Table 1. The retinyl ester, α-tocopherol and vitamin D₃ levels in BLM and control group in lung tissue.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Control (S±SD)</th>
<th>BLM (S±SD)</th>
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<tr>
<td>Retinyl ester (μg/g)</td>
<td>7.6±0.014</td>
<td>12.9±6.03</td>
</tr>
<tr>
<td>α-Tocopherol (μg/g)</td>
<td>81.9±0.58</td>
<td>51.59±25.52**</td>
</tr>
<tr>
<td>Vitamin D₃ (μg/g)</td>
<td>7±0.01</td>
<td>1.11±0.58***</td>
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**p<0.01, ***p<0.001.

The histological changes were completely obvious in the BLM-treated animals. Large fibrous areas in the interalveolar septa tissue, peribronchial and perivascular regions were seen with apparent infiltration of lymphocytes. None of these changes was observed in the healthy controls (Fig. 1).

In this study, the retinyl ester, α-tocopherol and vitamin D₃ levels in the lung tissue in rats according to the fibrosis induced by BLM were measured. The average retinyl ester in the lung tissue of the BLM group (12.93 μg/g) was found to be lower than that of the control group (16.25 μg/g). No statistically significant difference was found (p>0.05) compared to the control group.

Vitamin A and its active metabolite credits are important factors for supporting epithelial differentiation and normal respiratory growth (13). Retinoic acid (RA) stimulates the cell proliferation (27) and effects the polyamine transport and synthesis in cultured type II pneumocytes. Polyamine may be important in the lung cell repair process (28). Habib et al. (29) reported that the rats pretreated with vitamin A showed a statistically significant reduction of total bronchoalveolar lavage fluid cell count and alveolar production of superoxide anions of macrophage after 7 d the BLM competed with the control. It was recently discovered that retinoic acid could induce the regeneration of lung alveoli in the experimentally damaged adult rat lung (30, 31).

Retinoids also have anti-fibrotic and anti-inflammatory properties (32–34). Tabata et al. (32) checked the preventive effect of All-trans-RA (ATRA) on the progression of the fibrosis of lung in irradiated and BLM-treated rats. They found that ATRA will improve the fibrosis
vitamin E could not protect from BLM-induced pulmonary fibrosis, and a dietetic deficiency of vitamin E worsens BLM lung damage that was produced by emphysema in the hamster. Kato et al. (12) noted that the concentration of vitamin E in the lung tissue was increased significantly after intratracheal administration of BLM. Vitamin E deficiency produces a great number of free radicals at the early stage after the BLM treatment and could induce the imbalance of protease-antiprotease in the lung which probably causes an emphysematous change in the BLM-treated lung at a late stage after administration of BLM.

Our previous studies performed with Deger et al. (41) and Dede et al. (42) proved that the pulmonary formation of fibrosis was prevented by the supplementation of vitamin E. Deger et al. (41) noted that rats given vitamin E as α-tocopherol at a dose of 10 mg/kg body weight twice a week starting from one week before the induction of fibrosis to the end of experiment, had less severe fibrotic lesions. Dede et al. (42) had also proven that rat lungs treated with BLM were seriously damaged and vitamin E seemed to prevent certain damage as indicated by the difference of the concentrations in serum of major elements. The Fe and Mg concentration in the group of BLM were found to be lower than the controls and Cu, Ca, and K levels were not appreciably changed among the three groups.

This research was made to support our previous studies that the obtained results explained cellular damage in lung tissue such as catalase, glutathione, malondialdehyde and Fe, Mg changes in the serum of BLM-treated rats. Thus by these two articles and research presented, the effect BLM had on the formation of the lung fibrosis had been supported by diagnosing the biochemical changes of the serum and tissue. In the present study, the average α-tocopherol concentration in the lung tissue of the BLM-treated group decreased appreciably more than in the control group (p<0.01).

The active metabolite of vitamin D₃, 1,25-dihydroxyvitamin D₃, is a member of the lipophile family of ligands that exerts its actions by a nuclear receptor, the 1,25-dihydroxyvitamin D₃ receptor (VDR) (43). The presence of 1α,25(OH)₂D₃ receptors in the alveolar type II cells of the lung and the participation of 1α,25(OH)₂D₃ in maturation and differentiation of these cells and synthesis of surfactant released from these cells indicated that the alveolar type II cells may be principal targets for 1α,25(OH)₂D₃ (44). 1,25 dihydroxyvitamin D₃ inhibits the production of monocyte macrophage-derived cytokines such as interleukin-1α, interleukin-6, and TNF-α on the post-transcriptional level. Phan and Kunkel (45) reported that increased production of TNF-α and TGF-β are important components of the fibrotic process. There is no research on the relationship between pulmonary fibrosis induced by BLM and vitamin D₃ in the literature.

In this study, the vitamin D₃ levels in the lung tissue of the control and BLM groups were found to be 2.25±0.01 µg/g and 1.11±0.58 µg/g, respectively. The observed decreases of the average vitamin D₃ levels

induced by BLM in the lung tissues of rats and their data may provide a reasoning to explore the clinical use of ATRA for the prevention of fibrosis induced by pathologic radiation of the lung implying pulmonary fibrosis.

Tumor necrosis factor (TNF)-α, a proinflammatory cytokine, was shown to play an early and fundamental role in the pathogenesis of pulmonary inflammation and the fibrosis induced by BLM (4). ATRA was shown to antagonize the effects of TNF-α on target cells (35). Segel et al. (36) reported that their study did not support the use of ATRA at either dose (0.5 mg/kg per day and 2 mg/kg per day) to prevent or ameliorate BLM-induced lung fibrosis.

BLM induces pulmonary fibrosis by the production of oxidants. However, current data prove that BLM can generate ROS such as superoxide and hydroxyl radicals (37). Vitamin E serves as a natural antioxidant that reduces the oxidized cellular components, breaks up the ROS and detoxifies toxic oxidation products (38). Suntries and Shek (39) suggested that the pretreatment of the rats with α-tocopherol liposomes can ensure a significant protection against BLM-induced lung damages. Kilinc et al. (37) reported that a high amount of the vitamin E (15 mg/animal) considerably reduces the fibrotic effect of BLM on lung tissue in mice.

Takahashi (40) reported that a dietetic supplement of

Fig. 1. Histologic analysis of lung after 14 days administration of drugs (HEX80). (A) Control group: Normal lung parenchyma. (B) BLM group: Large fibrosis areas (F) in the interalveolar septa, peribronchial regions. Lymphocyte infiltration (arrows) is also evident.
in the lung tissue were statistically important (p<0.001).

According to these results in pulmonary fibrosis, it was observed that vitamins were consumed by the lung tissue and their levels decreased.

REFERENCES

30) Tabata C, Kadokawa Y, Tabata R, Takahashi M, Okoshi K, Sakai Y, Mishima M, Kubo H. 2006. All-trans-retinoic acid prevents radiation- or bleomycin-induced pulmo-


