Asian Journal of Chemistry Vol. 21, No. 8 (2009), 6167-6175

COX-2 as Inhibitors to Protect Radiation Induced Pneumonia

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The aim of this study is to assess whether the celecoxib has a radioprotective effect in the rat model of radiation-induced lung injury. Rats were given a single dose of 20 Gy to the right hemithorax and mediasten region from 80 cm distance and 1 cm depth by a ^{60}Co teletherapy unit. Histopathological evaluation was fulfilled using light microscope and the biochemical results were evaluated by considering membrane lipid peroxidation. Biochemical and histopathological results of 20 Gy irradiated lungs revealed significant tissue damage. In only radiation applied group (Group-3); macrophage clusters in alveolar structures, diffuse alveolar septum thickness, bronchial lymphocytes in groups and diffuse peribronchial edema were determined (radiation pneumonitis). It was seen that plasma MDA level was fairly elevated in irradiated lungs. But four dose of celecoxib precluded the radiotherapy induced lung damage significantly when compared to the control group ($p < 0.05$). It was thought that two dose of celecoxib alleviated the radiotherapy dependent lung damage. Biochemical and pathological evaluation of the animals in Group-4 showed that four dose of celecoxib lowered the radiotherapy induced lung damage significantly. In only celecoxib applied group (Group-5), biochemical and pathological results showed that celecoxib didn't cause any change in normal lung tissue.

Key Words: Radiotherapy, Radiation pneumonia, Celecoxib, MDA, Radioprotector agents.

INTRODUCTION

Radiation therapy is an important therapeutic modality in the treatment of several thoracic tumors including lung, breast and lymphoma[1]. The purpose of radiation therapy is to kill or damage cancer cells. Because lung is one of the important dose-limiting organs for radiation therapy of tumours in the thoracic region, the ability of radiation therapy to treat tumors involving the thoracic region is significantly limited by tolerance of lung to radiation[2,3]. Also the dose-response relation for normal tissue injury is the limiting factor in the amount of irradiation that can be given.

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Therefore, it is important to consider the dose limiting factors like tissue tolerance[4,5]. As the size of the tumor increases and the dose needed for local control likewise increases, the risk of injury to normal tissue becomes greater[6].

Irradiation of the thorax is not without harmful side effects. Radiation pneumonitis (RP) is one of the most important and serious complications of this treatment[7]. In normal tissues radiotherapy induced side effects are acute, subacute and late radiation complications. In lungs, acute and subacute radiation damage is known as radiation pneumonitis. Late radiation damage is known as radiation fibrosis, chronic radiation pneumonitis, korpulmonole and heart insufficiency[8]. Radiation pneumonitis can lead to severe respiratory dysfunction and even death and some patients may develop chronic respiratory insufficiency as a result of pulmonary fibrosis. Progressive irreversible fibrosis is one of the most clinically significant consequence of ionizing radiation in normal tissues. In the case of the lung, the dose limitations imposed by normal-tissue tolerance presently precludes successful radiotherapeutic treatment for most malignancies, because radiation-induced pulmonary fibrosis is mostly refractory to treatment. Therefore many methods of prevention, or at least moderation of chronic radiation injury, have been attempted in view of these clinical considerations[9].

Although the mechanism of radiation induced lung damage is not known clearly, it is believed that lung injury from ionizing radiation is a consequence of a cascade of cytokine activity, which ultimately begins with oxidative stress from radiolytic hydrolysis and formation of reactive oxygen species (ROS)[10]. These ROS include superoxide (O_2P^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), with superoxide dismutase (SOD) playing a key role in cellular defenses against oxidative damage[1]. These ROS eventually causes toxic peroxidation products like malondialdehyde (MDA) and various changes in membrane lipids. Radioprotectors have been studied for decades to minimise the oxidative damage[11].

The main problem that limits the success rate of radiotherapy (RT) in lung cancer is the radiosensitivity of the normal tissue into which the tumor infiltrates and which, therefore, has to be given very high radiation doses[12]. When all standart fractions and doses (1.5/2 Gy/fraction) are applied to whole lung as 18-20 Gy, in less than 5 % of radiation pneumonitis patients observed[13]. Complications of radiation pneumonitis can range from relatively mild to life threatening, depending on a number of factors including the total dose of radiation given, the fractionation schedule, the volume of lung irradiated, the existence of prior lung disease and the use of chemotherapeutic drugs together with radiosensitizers in the management of the disease[14]. The use of radiotherapy together with chemotherapy increases the risk incidense. In the case of determining the risk factors, the ratio of symptomatic radiation pneumonitis can increase to 10-20 %[13]. Radiation pneumonitis can have a considerable impact on patient morbidity and infrequently mortality. Clinically significant radiation pneumonitis usually develops in 13-37 % of patients receiving radical dose of radiation therapy for lung cancer[7]. Acute radiation pneumonitis

occuring 2-3 months after radiation treatment can be significant cause of morbidity or mortality, while fibrosis occuring after 6 months can result in chronic pulmonary distress. Thus the lung is a major dose-limiting organ in radiotherapy[15].

Non-steroidal antiinflammatory drugs (NSAIDs) are included in the present study as radioprotectors. Fruta *et al.*[10] showed that indomethacin has a radioprotective effect on lung tissue. But its success was considerably limited. Many researchers attracted attention to the increase of various eicosanoids including prostaglandin (PG) in response to ionized radiation[16]. Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), also known as prostaglandin-H synthethase 1 and 2, inhibit the endogen PG synthesis[17]. COX-1 occurs in normal tissues, including that of the stomach lining, intestine, kidney, platelets and it helps regulate basal level of prostaglandin necessary for normal function and may play a role in preventing mucosal damage[16]. COX-2, on the other hand, is not found in normal tissues in more than minute quantities. However COX-2, the enzyme that converts arachidonic acid to prostaglandin, is over expressed in a variety of different tumors, including colon, pancreatic, prostate, lung and head and neck cancers[18]. It has been shown that when the selective COX-2 inhibitors are used in combination with radiation and anti-cancer drugs, efficacy of treatments increased and also side affects of the treatment decreased[17]. The reason for prefering COX-2 inhibitors is that their side effects in gastrointestinal system (GIS) mucosa are slightly. Two specific COX-2 inhibitors, namely, rofecoxib (Vioxx, Rahway, NJ, USA) and celecoxib (Celebrex, Roche Pharmaceuticals) are extensively studied and the results were hopeful[16,17].

In this study we determined whether or not celecoxib (COX-2 inhibitor) has preventive role on acute and subacute effects in rat lung after irradiation. We evaluated this criteria by determining the tissue damage through monitoring malondialdehyde content in plasma and by histopathological evaluatin of the tissues under light microscope.

EXPERIMENTAL

Female Wistar Albino rats, aged 8 weeks (150-190 g), obtained from Erciyes University Medical School Hakan Çetinsaya Experimental and Clinical Research Center (DEKAM) were housed in a controlled environment and provided with standard laboratory nourishment (rodent chow) and water.

Animal preperation: Specific pathogen free 25 Wistar female rats were distributed randomly and equally into the following five groups: 1. Shame control, 2. Two doses of COX-2 inhibitor + radiation therapy, 3. Radiation therapy alone, 4. Four doses of COX-2 inhibitor + radiation therapy and 5. Four doses of COX-2 inhibitor.

Rats in the "shame control" group provided with serum physiologic and gavage during 4 days and rats in the third group provided only with serum physiologic during 4 days. Rats in all groups were sacrificed 6 weeks after irradiation.

Irradiation: The animals were anesthesized prior to irradiation with ketamine hydrochloride (67.5 mg/kg) and xylazine (4.5 mg/kg). For the application of radiation to the right hemithorax and mediasten region, rats were put in prone position. Total irradiation area for every individual rat was 2 × 4 cm. Rats were given a single dose of 20 Gy to the right hemithorax and mediasten region from 80 cm distance and 2 cm depth. Radiation is delivered at a dose rate of 143.3 cGy/min by a ^{60}Co teletherapy unit (Theratron 780C). The unirradiated areas were shielded using lead blocks.

Drug application: COX-2 inhibitor (Celecoxib, Celebrex®, Roche Pharmaceuticals) was administered two times orally 6 h before and 12 h after irradiation. Celecoxib at the dose of 20 mg/kg body weight diluted with serum physiologic to prepare 0.3 mL solution and orally gavaged. Celebrex was given once daily. Previous studies are taken into consideration in determining and preparing the application dose of celebrex[19].

Drug applications were performed in the following way:

Group-1: 4 dose of serum physiologic
Group-2: 2 dose of COX-2 inhibitor
Group-3: 4 dose of serum physiologic
Group-4: 4 dose of COX-2 inhibitor
Group-5: 4 dose of COX-2 inhibitor

While the rats were under ketamine anesthesy, lung tissue and blood samples were obtained. Afterwards all rats were sacrificed 6 months after irradiation. Blood samples were kept at (-) 70 ºC until the time of observation. Lung tissue was homogenized in a solution containing 10 % formalin.

Determination of plasma malondialdehyde (MDA) content: The extent of lipid peroxidation was determined by measurement of MDA levels according to the method of Stocks and Dormandy, which is modified by Jain[20]. MDA, an end product of fatty acid peroxidation reactions, can react with thiobarbituric acid (TBA) to form a coloured complex that has maximum absorbance at 532 nm.

One volume of plasma sample (0.6 mL) was mixed with 4 volume of phosphate buffered saline and diluted at a rate of 1/5. Then 30 % of thiobarbituric acid was added to these test tubes (1/2 volume) and vortexed. To the blank tube, 0.6mL distilled water added instead of plasma sample and the same procedures are applied as in the above. Sample and blank tubes are incubated in a boiling water bath for 2 h. After cooling, the precipitate was removed by centrifugation at 2000 rpm for 10 min and 3 mL of supernatant was transferred to test tubes. After that, 0.225 mL of 0.1 M EDTA and 0.75 mL of freshly prepared 1 % of thiobarbituric acid was added to these test tubes.

Histopathological examination: After the rats were killed, their lungs were removed and fixed in 10 % formalin solution for 24 h[14]. Tissues are dehydrated by graded alcohol and embedded in paraffin blocks. Cross-sections (5-8 µ thick) were cut along the longitudinal axis of the lung using a tissue slicer. Tissue sections were

deparaffinized with xylene and stained with hematoxylin-eozin. Then slides were examined under light microscope (BH2-Olympus)[19]. Right and left lung were evaluated histopathologically at the same time and comparisons made between the groups.

Statistical analysis: All data are expressed as mean ± SD. Data from the experiments were subjected to analysis of variance (ANOVA and post-ANOVA-Scheffe) using SPSS for Windows[21]. A p value < 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

Biochemical and histopathological results from lungs irradiated by 20 Gy RT revealed significant tissue damage. Histopathological evaluation was fulfilled using light microscope and biochemical results were evaluated by considering membrane lipid peroxidation.

Biochemical findings: Biochemical results of plasma MDA levels are summarized in Table-1. In the control group of animals (shame control) average MDA content was found to be 1.55 µmol/L. Minimum and maximum plasma MDA contents were 1.55 µmol/L and 1.56 µmol/L, respectively. These were normal metabolic values. Group-3 (radiotherapy alone) and Group-4 (4 dose of COX-2 inhibitor + radiotherapy) showed significant difference when compared with the control.

TABLE-1
PLASMA MDA CONTENTS OF THE GROUPS (µmol/L)

Groups	Number of test subject	Mean MDA values (± SD)
Shame control	5	1.554 ± 0.005
2 Dose of COX-2 + Radiation	5	1.546 ± 0.069
Radiation alone	5	1.740 ± 0.015
4 Dose of COX-2 + Radiation	5	1.358 ± 0.008
4 Dose of COX-2	5	1.530 ± 0.018

In Group-2 (2 dose of COX-2 inhibitor + radiotherapy) average MDA content was 1.54 µmol/L. Minimum and maximum plasma MDA contents were 1.46 µmol/L and 1.63 µmol/L, respectively. These values were considerably close to that of control. The results of Group-2 (2 dose of COX-2 inhibitor + radiotherapy) were significantly different from those of Group-3 (radiotherapy alone) and Group-4 (4 dose of COX-2 inhibitor + radiotherapy).

It was seen that in Group-3 (radiotherapy alone) the plasma MDA content was fairly high; average, minimum and maximum values were 1.74, 1.72 and 1.76 µmol/L, respectively. These results were significant when compared with other four group. Although radiation was applied in Group-2, Group-3 and Group-5, it was evident that oxidative damage was higher in radiotherapy-alone group.

In Group-4 (4 dose of COX-2 inhibitor + radiotherapy) average, minimum and maximum plasma MDA values were 1.35, 1.35 and 1.37 µmol/L, respectively. These values were significantly lower than those of other four group.

In Group-5 (4 dose of COX-2 inhibitor) average, minimum and maximum plasma MDA values were 1.54, 1.35 and 1.76 µmol/L, respectively. These values were significantly higher than those of Group-3. However these same values were significantly lower than those of Group-4.

Histopathological findings: Under microscopic examination the parameters were; erythrocyte in alveoli, peribronchial edema, lymphocyte at peribronches and septa, macrophages in alveoli and interalveolar septal thickness. Histopathological results of tissue analysis are summarized in Table-2. Acording to the ratio of infiltration, results were grouped as 1, 2 and 3.

TABLE-2
HISTOPATHOLOGICAL EVALUATION IN RAT GROUPS

Group	Erytrocyte in alveol	Inflammatory cells	Periadventitial edema	Thickness of alveolar septa	Macrophage in alveol
Control (right lung)	1	1 Peribrochial	+	Few number of focal thickness	-
Control (left lung)	1	1 Peribrochial	+	Few number of focal thickness	-
2 dose of drug +RT (right lung	1	2 In groups at septa	+	Thickness at septum	+ in a few foci
2 dose of drug +RT (left lung	1	1 Peribrochial	+	Few number of focal thickness	-
RT right lung	1	2 In groups at septa	+	Thickness at septum	+(macrophage clusters)
RT left lung	1	1 Peribrochial	+	Few number of focal thickness	-
4 dose of drug + RT (right lung	2	1 Peribrochial	+	Thickness at septum	-
4 dose of drug +RT (left lung)	1	1 Peribrochial	+	Few number of focal thickness	-
Drug (right lung)	1	1 Peribrochial	+	Few number of focal thickness	-
Drug (left lung)	1	1 Peribrochial	+	Few number of focal thickness	-

RT = Radiotherapy.

For erythrocytes: (1) determination of several erythrocyte (2) focal erythrocyte infiltration (3) erythrocyte infiltration that fills alveoli.

For lymphocyte: (1) scarce in a few foci (2) in groups (3) diffuse.

Thickness of alveolar septa: (1) available (present) (2) non-existent

Macrophage: (1) available (present) (2) nonexistent.

Periadventitial edema: (1) available (present) (2) non-existent.

When the right lung of the rats in control group were examined, a few erythrocyte in alveoli, 1 or 2 lymphocyte in a few peribronchial foci, peribronchial edema and few number of focal thickness were observed. Similar findings were observed in the left lung of experimental animals. These findings, observed under microscope, were evaluated as normal lung values.

In group-2 (2 dose of COX-2 inhibitor + radiotherapy) in irradiated right lung a few erythrocyte in alveoli, groups of lymphocyte in a few peribronchial foci, macrophage clusters in alveolar structures, peribronchial edema and thickness of the alveolar septa were determined. In the left lungs few number of erythrocyte in alveoli, several lymphocyte, peribronchial edema and few number of alveolar septal thickness were determined. No macrophage were determined in alveoli.

In group-3 (radiotherapy-alone) in the right lungs of the rats several erythrocytes in alveoli, groups of lymphocyte around the bronchiols, macrophage clusters in alveolar structures, peribronchial edema and diffuse alveolar septal thickness were determined. In left lungs the same microscopic findings were observed as in the control.

In group-4 (4 dose of COX-2 inhibitor + radiotherapy) in the right lungs of the rats diffuse erythrocytes in alveoli, a few number of lymphocytes around the bronchiols, few number of periadventitial edema and thickness of alveolar septa were determined. No macrophage were seen in alveoli. In the left lungs a few erythrocytes in alveoli, rare lymphocytes in a few foci around the bronchiols and periadventitial edema were determined. Focal septal thickness were seen in alveoli, but no machrophage were detected.

In group-5 (4 dose of COX-2 inhibitor), in the left and right lungs the same findings were determined as in the control.

It has long been suggested that prostoglandins, the products of cyclooxigenase (*i.e.*, COX-1, COX-2) enzyme activity on arachidonic acid, play a role in cell survival after ionizing radiation[22]. The addition of prostoglandins or their analogs has been shown to be radioprotective in a number of cell types, whereas others have suggested that prostoglandins have differential effects depending of the cell type[23].

COX-2 has been reported to be present or induced in inflammatory conditions such as rheumatoid arthritis, osteoarthritis and cancer. A recent report suggests that elevated COX-2 expression correlates with reduced patient survival after radiation therapy. These observations raised the possibility that COX-2 may play a role in survival after ionizing radiation therapy[24]. This hypothesis has now become testable with the discovery of compounds that selectively inhibit COX-2 (such as celecoxib) and has generated some renewed interest in prostaglandin modulation as an approach to radioprotection.

It is hypothesized that inhibition of COX-2 will have an inhibitory effect on the radiation induced inflammatory process of the lung tissue cells and will preclude the radiation pneumonia, a concept first proposed by present authors.

In this article, the authors have combined radiation therapy with COX-2 inhibitor to observe whether it will protect the lungs from the ionizing radiation harmful effect or not.

Biochemical and histopathological end points were analyzed in rats, after single dose irradiation with 20 Gy to the right lung. In the present study, we have tested the radioprotective effect of celecoxib, a selective COX-2 inhibitor, in a rat model

of radiation-induced lung injury. Present results clearly showed that celecoxib can both significantly delay and reduce the extent of radiation induced pulmonary injury in rats. A significant changes in plasma MDA levels were also found to occur 6 weeks after 20 Gy hemithoracic irradiation. These data suggest that, in rats, plasma MDA level might be a potential marker of normal tissue injury after irradiaiton. Significant decrease in plasma MDA content after the application of celecoxib made us think that this drug caused antioxidant effect. However to obtain more reliable data about the effect of this drug, more detailed determinations have to be performed regarding the dose, application duration and effect of other antioxidant enzymes. Histopathological analysis showed that in irradiated groups, alveolar macrophages, clusters of lymphocytes around the bronchiols, diffuse thickness of alveolar septa and peribronchial edema were evident. But in celecoxib treated groups, histopathological evaluation suggest that celecoxib may help to delay radiotherapy dependent radiation pneumonitis. In "2 dose of celecoxib + radiotherapy" group, near complete decrease in the number of macrophages and marked decrease in the number of lymphocytes were observed. It was thought that 2 dose of celecoxib fairly relieved the radiaiton pneumonitis. In "4 dose of celecoxib + radiotherapy" group, a few lymphocyte were seen, however, macrophages were completely disappeared. In this group, histopatological results also showed that lung structure was normal (Fig. 1).

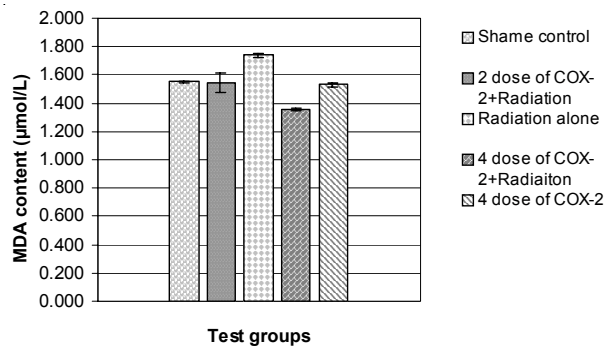

Fig. 1. Plasma MDA contents of the groups (µmol/L)

Conclusion

Radiation pneumopathy represents one of the greatest challenges to radiotherapy, because radiation induced lung disease is the limiting factor for improvements of the cure of the most common and most aggressive cancer in humans. The results of those examinations on the distribution of lung cancer deposits within the thorax recently demonstrated that to sterilize those and the subclinical deposits between them, large volumes of lung tissue need to be given doses that would be too high to be tolerated by the lung tissue locally, causing long-term, progressive fibrotic lung disease with crippling or even lethal consequences[25]. However, the results of research into the pathogenesis have raised hopes that there may be a fair chance for therapeutic intervention into the progressive nature of radiation-induced lung disease,

because all the available evidence rules out a straight deterministic radiation effect in favour of a regulated, but complex, progression scenario. No clinically exploitable procedure of therapeutic intervention has been developed yet, but this hope is a strong stimulus for further research[26].

In conclusion, present results indicate that, 4 dose of celecoxib has potent antagonistic activity against the radiation pneumonitis. Therefore, inhibitors of COX-2, in combination with radiation treatment, may be an alternative strategy to protect the radiation pneumonitis.

ACKNOWLEDGEMENTS

The authors would like to thank to Dr. O.Galip Yildiz and Kadir Yaray for his excellent technical help and design of data of this manuscript.

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(*Received*: 3 November 2008; *Accepted*: 23 May 2009) AJC-7594