RADIATION PNEUMONITIS: THE INFLUENCE OF PENTOXIFYLLINE AND DEXAMETHASONE ON THE ALVEOLAR SEPTAL THICKNESS AND THE AMOUNT OF NEUTROPHIL GRANULOCYTES

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Summary

We monitored the number of neutrophil granulocytes and the thickness of alveolar septa in (C57Bl/6xDBA/2)F_1 mice 168 days after γ - irradiation in the area of the lungs with a dose of 16 Gy (LD_5) and 180 days after irradiation with a dose of 18 Gy (LD_{50}). The laboratory animals were administered Pentoxifyline (3,5 and 35 mg/kg), Dexamethasone (1 and 10 mg/kg) and a combination of Pentoxifyline (35 mg/kg) and Dexamethasone (10 mg/kg) twice a week. In the animals examined 168 days after irradiation with a dose of 16 Gy, a significant decrease in the number of neutrophil granulocytes and a decrease in the thickness of alveolar septa were found in all the treated animals. The largest effect was seen in a combination of PTX and DXM. In the individuals examined 180 days after irradiation with a dose of 18 Gy, a similar trend was found. The largest effect was seen in DXM (10 mg/kg) and in a combination of DXM and PTX.

KEY WORDS: γ-Irradiation; Lung; Pentoxifylline; Dexamethasone (DXM); Image analysis.

Introduction

The radiation pneumonitis (RP) is defined as an exudative inflammation which occurs as a result of ionizing radiation by X-ray or gamma radiation (8) and it is regarded as alveolitis from the damage it causes to the pneumocytes and endothelial cells (11). This illness is usually manifested from the 4th to the 6th month after irradiation of the lungs with a single dose of 7 Gy and a higher dose (4). It involves the changes in the irradiated areas and so these are limited (23).

Pentoxifylline (PTX) is used in modern clinical practice mainly as rheologic and vasodilatans. It is indicated mainly in insufficiency of peripheral blood system, disorders of ischemic diseases of the extremities in all stages, chronic manifestations of cerebrovascular insufficiency, diabetic angiopathies and angiopathies of various aetiologies. Funk et al. (9) observed in vitro influence of cytokine production after the addition of PTX alone and PTX in a combination with DXM during experiments with human peripheral mononuclear cells, which were stimulated by Phytohaemagglutinin.

The authors state that PTX alone in a concentration of 50 μg/ml decreases significantly the production of TNF-α, IL-2 and IFN-γ. On the other hand, the production of IL-6 was not significantly influenced. During the application of a combination of PTX (50 and 100 μg/ml) and Dexamethasone (1 μmol of final concentration), production of the observed cytokines, with the exception of IL-6, was reduced statistically significantly in comparison with the cultivation with PTX alone. It follows from their results that PTX in vitro experiment inhibits the production of proinflammatory cytokines in a culture of human peripheral mononuclear cells. If the hypothesis that the cytokine cascade (TNF-α, IL-1 and others) indicates the expression of RP is right, then the causal therapeutic period influencing the expression of cytokines starts immediately after irradiation, because an increased production of proinflammatory cytokines was found immediately after irradiation (17).

In this study we wish to test the remedial effect of applying PTX and DXM (alone and in combination), administered from day 1 to day 180 after irradiation according to the degree of infiltration of the lungs irradiated with neutrophil granulocytes and for the measurement of the thickness of alveolar septa by means of computer image analysis.

Material and Methodology

Male (C57Bl/6xDBA/2)F_1 mice (Charles River, Salzfeld, Germany) aged 8-12 weeks and with a weight of 24-34 g were used for the experiment. The laboratory animals were irradiate locally in the area of the thorax using ^{60}Co unit (Chisotron Chirana) at a dose rate of 0.5 Gy/min (the target distance was 1 m). The animals were slightly aneasthesized before irradiation by means of a solution composed of one portion of Rometar (the Spofa Company, Prague) and 3 portions of Narkamon (the Léčiva Company, Prague) and 12 portions of physiological saline. This solution was applied intramuscularly in a 10 ml/kg dose. The local irradiation of the murine thoraxes was performed in a fixating box with a 10 cm thick layer of lead plates to prevent the irradiation of other parts of the body with a dose higher than 2-3% (20). The first application of substances was carried out 2 hours after irradiation.

The irradiated laboratory animals were divided into 6 groups according to the type of drugs applied subcutaneously twice a week. Each group of mice

included 6 individuals irradiated with 16 Gy and treated over 24 weeks after irradiation and 8 animals irradiated with a dose of 18 Gy with an application of drugs up to 180 days after irradiation. The first application was carried out 2 hours after irradiation and the last application two days before the animals were killed for the histologic examination.

This particular scheme of application of drugs was selected as appropriate to the technical resources of the working place.

- The 1st control group was dosed with physiological saline (S).
- The 2nd group of mice was administered Pentoxifyline (Pentillin, Krka - 1 ampulla 100 mg/5 ml) which was diluted using physiological saline administered in a dose of 3,5 mg/kg (PTX 3,5).
- The 3rd group of laboratory animals was dosed with 35 mg/kg of Pentoxifyline (PTX 35).
- The 4th group of individuals was dosed with Dexamethasone (Dexona, Cadila Laboratories Ltd. - 1 ampulla 4 mg/1 ml) which was diluted using physiological saline in a 1mg/kg dose (DXM 1).
- The 5th group was administered Dexamethasone in a dose of 10 mg/kg (DXM 10).
- The 6th group was administered a combination of Pentoxifyline in a dose of 35 mg/kg and Dexamethasone in a dose of 10 mg/kg (COMB).
- The 7th group contained nonirradiated animals and nontreated animals (C).

The animals irradiated with 16 Gy (6 mice in each group) were killed and histologically examined 24 weeks after irradiation (LD_5 for this strain) (27). The animals irradiated with 18 Gy (8 mice in each group) were killed 180 days after irradiation (LD_{50} for this strain) (27) and their lungs were histologically examined. The values of the observed markers of 6 mice from the 7th group were used as referrence values.

Histologic Examination

When the given time intervals had passed, the mice were killed using cervical dislocation. During the dissection, the removed lungs were fixed in 10% neutral buffered formalin, tissue particles were then embedded into paraffin, and histologic preparations were stained using chloracetatesterase stain (to determine neutrophil granulocytes), combined trichrom (to measure the thickness of septa) and Gram's stain (to evaluate bacterial infection).

The Calculation of Neutrophil Granulocytes

In histologic preparations with chloracetasterase stain, neutrophil granulocytes were indentified using the Amplival light microscope (Carl Zeiss, Jena). 15 viewing fields were observed in each preparation at a 640 fold magnification (282 743,3 μm^2 per field).

The Measurement of the Thickness of Alveolar Septa

Histologic preparations stained with combined trichrom were evaluated using the BX 40 light microscope (the Olympus Company, Prague) and a computer image analysis - LUCIA M (the Laboratory Imaging, Prague). 6 randomly selected viewing fields by size of 71447.8 μm^2 were evaluated in each preparation at a 400 fold magnification. In each viewing field, 10 randomly selected alveolar septa were measured in the narrowest point of their length.

Data Processing

In the data obtained the diameter ± SEM was calculated and a Mann-Whitney's test was used for statistical analysis.

Results

Within a time interval of 24 weeks after irradiation with a dose of 16 Gy, in total only 3 mice died in all the groups. One mouse from the 2nd group died on day 77 and 2 mice from the 6th group, one on day 105, another on day 116. From mice surviving 180 days after irradiation with a dose of 18 Gy, in total 6 mice died. Two mice died on day 20, 1 mouse from the 5th group and 1 mouse from the 6th group. On day 166, 3 mice died, 2 mice from the 3rd group and 1 mouse from the 4th group. On day 177, 1 animal from the 1st group died.

During histologic examination of the lungs of the laboratory animals, no infectious agents were found.

The Infiltration of the Lungs with Neutrophil Granulocytes

168 days after irradiation with 16 Gy, a statistically significant increase in the amount of neutrophil granulocytes was noted in the viewing field in the individuals of the control group in comparison with the animals of the nonirradiated group ($p<0.0001$). In other groups a statistically significantly lower number of neutrophil granulocytes was noted in comparison with the group with the application of physiological saline ($p<0.005$). By observing the number of neutrophil granulocytes, a significant difference between nonirradiated laboratory animals and animals treated with DXM (1 mg/kg) and a combination of PTX and DXM was not found.

In other groups we found a significantly higher number of neutrophils in comparison with nonirradiated mice ($p<0.05$). After administration of Pentoxifyline alone (3.5 and 35 mg/kg), no correlation between the number of neutrophil granulocytes in the lungs and the concentration of the substance was observed. Nor did we note after applications of DXM alone (1 and 10 mg/kg), any significant difference in the number of neutrophils in correlation to concentration of DXM. The animals given repeated applications of Dexamethasone in a dose of 1 mg/kg showed a significant difference in the number of neutrophil granulocytes in comparison with the groups given an application of PTX ($p<0.005$).

Table 1

The influence PTX/DXM on amount of neutrophil granulocytes in the lung after irradiation of the thorax

Dosis of irradiation	S.	PTX 3,5	PTX 35	DXM 1	DXM 10	COMB.
168 days after 16 Gy	3,63 ± 0,39	1,95 ± 0,75	1,76 ± 0,79	0,58 ± 0,16	0,67 ± 0,20	0,50 ± 0,13
180 days after 18 Gy	2,31 ± 0,46	1,57 ± 0,79	0,78 ± 0,35	1,36 ± 0,43	0,42 ± 0,11	0,23 ± 0,12

Average amount of neutrophil granulocytes of non-irradiated and non-treated mice (C.) - 0,42 ± 0,33 S.E.M. (standard error of mean)

Table 2

The influence treatment on the thickness of alveolar septa after irradiation of the thorax

Dosis of irradiation	S.	PTX 3,5	PTX 35	DXM 1	DXM 10	COMB.
168 days after 16 Gy	7,18 ± 0,23	6,02 ± 0,26	5,91 ± 0,34	5,83 ± 0,29	5,92 ± 0,38	5,47 ± 0,10
180 days after 18 Gy	6,60 ± 0,19	5,77 ± 0,15	6,12 ± 0,23	5,44 ± 0,07	5,13 ± 0,26	5,38 ± 0,23

The alveolar septal thickness of non-irradiated and non-treated mice (C.) - 4,56 μm ± 0,12 S.E.M.

When the dose of DXM was increased to 10 mg/kg, a significant difference was noted in comparison with the groups of individuals given an application of PTX alone ($p<0.05$). The number of neutrophil granulocytes after applying a combination of Pentoxifyline in a dose of 35 mg/kg and Dexamethasone in a dose of 10 mg/kg was statistically significantly lower than after application of PTX alone ($p<0.01$). No significant difference in the observed marker was noted between the groups given an application of a combination of PTX and DXM and DXM alone.

Dose of 18 Gy, 180 days after irradiation together with applications of physiological saline, led to a statistically significantly higher number of neutrophils in the viewing field in comparison with both nonirradiated and treated mice ($p<0.01$), with the exception of the groups given PTX (3.5 mg/kg) and DXM (1 mg/kg). By observing the number of neutrophil granulocytes a significant difference between the nonirradiated laboratory animals and animals treated with DMX (10 mg/kg), PTX (35 mg/kg) and a combination of PTX and DXM was not found.

In other groups, we found a significantly higher number of neutrophils in comparison with the nonirradiated mice ($p<0.005$). In laboratory animals given Pentoxiphyline, a significant correlation to the concentration of the drug ($p<0.05$) was noted. After applying Dexamethasone, we also noted a correlation with the concentration of the substance ($p<0.001$). The number of neutrophils in repeated applications of lower concentrations of PTX/DXM alone, was significantly higher in comparison with the higher concentrations of DXM/PTX ($p<0.05$) alone. A combination of higher concentrations of Pentoxifyline (35 mg/kg) and Dexamethasone (10 mg/kg) significantly restricted infiltration of the pulmonary tissue with neutrophil granulocytes in comparison with both the groups that received an application of PTX ($p<0.05$) and the animals given an administration of DXM ($p<0.001$).

The Measurement of the Thickness of Alveolar Septa

In the control group (given physiological saline) significantly higher thicknesses of alveolar septa were measured 168 days after irradiation with a dose of 16 Gy in comparison with the nonirradiated and treated animals ($p<0.001$). By measuring the thickness of alveolar septa, we found a significant difference between irradiated and nonirradiated animals ($p<0.005$). The values of the thickness of alveolar septa did not show any statistically significant difference between the groups given an application of PTX and DXM, and between individual concentrations not only of PTX but also DXM. The size of the observed indicator after applications of a combination of Pentoxifyline in a dose of 35 mg/kg and Dexamethasone in a dose of 10 mg/kg was significantly lower than after both the administration of PTX alone ($p<0.005$), and DXM alone ($p<0.01$).

After a dose of 18 Gy, 180 days after irradiation and with applications of physiological saline, the measured thickness of alveolar septa was significantly lower ($p<0.001$) than in nonirradiated and treated animals, with the exception of mice treated with PTX (35 mg/kg) ($p<0.1$). By measuring the thickness of alveolar septa, we found a significant difference in nonirradiated animals in comparison with

irradiated animals ($p<0.005$). In comparison with the animals given repeated applications of DXM (10 mg/kg), the significant difference was lower ($p<0.02$). In laboratory animals given applications of Pentoxifyline in a concentration of 35 mg/kg, the average thickness of alveolar septa was significantly higher ($p<0.005$) than in the concentration of PTX 3,5 mg/kg.

After applying Dexamethasone, we observed a correlation with the concentration of the substance ($p<0.005$). It was found that after the administration of DXM the measured values are significantly lower than after the applications of PTX ($p<0.005$).

A combination of higher concentrations of Pentoxifyline (35 mg/kg) and Dexamethasone (10 mg/kg) led to a significantly lower thickness of alveolar septa than in laboratory animals after the application of PTX alone ($p<0.001$) and the administration of DXM alone in the concentration of 1 mg/kg ($p<0.05$). By comparing the results of measurements with the administration of a combination of PTX and DXM and DXM in the concentration of 10 mg/kg, no significant difference was found.

Discussion

At present, PTX is considered to be a preparation with various immunomodulatory effects which were found in vivo in plasmatic concentrations as low as 10 μg/ml (9). The main effect of PTX in immune reactions is a decrease in the production of TNF-α (9, 15) at the mRNA level (6). Because of this effect, we used Pentoxifyline for the treatment of RP. It also causes a significant decrease in the expression of GM-CSF and IFN-γ in the peripheral mononuclear cells (24). The influence of PTX on IL-1 is more complicated. The influencing of IL-1α after the application of this drug has not been found so far. In IL-1β, the influencing of mRNA production (19) was not found, but the alteration of the target protein was noted (2, 16, 28).

The suppression of IL-10 after the administration of PTX (26) is an interesting fact. This fact proves a negative influence of PTX on the production of both proinflammatory and antiinflammatory cytokines. It was also noted that the amount of TNF-α increased by endotoxin in irradiated tissue after the transplantation of bone-marrow is actively involved in the induction of apoptosis of endothelial cells in the pulmonary vessels (7).

In our study, we tried to reduce the intensity of observed indicators in RP by applications of PTX and DXM. By investigating the effects of PTX at the cellular level, it was found that this drug inhibits the chemotaxis, degranulation, and adherence of neutrophils to endothelial cells, which serve for the infiltration of target tissue, and it also reduces the production of superoxide in neutrophil granulocytes (13, 25, 18). In addition has found on the model of the Paraquat affected tissue culture formed by isolated pulmonary cells (22) that PTX not only reduces the production of oxygen radicals but also functions as the scavenger of free radicals.

As far as the influence of PTX at the tissue and organ level is concerned, it was found that prevention of over-production of TNF-α using PTX leads to a visible improvement of damage to pulmonary tissue (3). Dezube et al. (5) noted an inhibition of TNF dependent cachexia when PTX was used, which is important in all the irradiated individuals both in experimental and clinical practice.

The proinflammatory effects of DXM are a well-known fact and the protective effect of corticosteroids was evaluated according to the survival of the irradiated animals and the dose reduction factor was determined as equal to 1,2 (12). The suppressive influence of DXM on the production of IL-2 (10), which is observable at mRNA level (1), is known. In vitro, an inhibition of IL-1 (21) was also found.

Our study tried to answer the question whether PTX and DXM alone and a combination of PTX and DXM will have a some effect in the treatment of RP in animals. The application of PTX seemed to be more effective here than the application of DXM alone. We also noted the synergistic effect of DXM and PTX but according to the observed markers DXM had larger effect than PTX.

It follows from our results that after the repeated applications of PTX, DXM and a combination of both substances, there is a significantly lower amount of neutrophil granulocytes and lower values of the thickness of alveolar septa in the irradiated pulmonary tissue in comparison with the individuals of the control group. The effect of a long-term administration of corticoids has a larger influence than repeated applications of PTX. This, however, does not reduce the role of repeated applications of PTX, when we take into account that PTX has incomparably fewer side-effects. We found that administrations of higher concentrations of PTX alone and DXM alone 168 days after irradiation with a dose of 16 Gy, do not have a significantly higher effect on the observed markers. We even noted a venostasis of alveolar septa after the administration of higher concentrations of PTX (35 mg/kg) 180 days after irradiation with a dose of 18 Gy. We assume that it is an intensification of vasodilatation, the main effect of this drug. From the therapeutic point of view, the best effect of a combination of PTX and DXM was found and we may therefore assume that corticoids play an important role here.

Further investigation of the effects of the above mentioned drugs on the development of an inflammatory cytokine cascade at the protein level in the irradiated animals will be the objective of our further work.

Conclusion

Reduction of the expression of TNF-α and IL-1 in the irradiated lungs becomes more important. Especially as we know that these cytokines step up their mutual effect. The use of PTX in medium and lower concentrations may be perspective. It is, however, necessary to investigate the mechanisms of PTX, which prevent the inception and development of RP, in detail, in order to be able to prevent RP after irradiation of the lungs with high doses of ionizing radiation on the basis of tested knowledge.

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