Radiation-induced cardiopulmonary dysfunction
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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CHAPTER 03

PHYSIOLOGICAL INTERACTION OF HEART AND LUNG IN THORACIC IRRADIATION

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The risk of early radiation-induced lung toxicity (RILT) limits the dose and efficacy of radiotherapy of thoracic tumours. In addition to lung dose, co-irradiation of the heart is a known risk factor in the development of RILT. The aim of the present study is to identify the underlying physiology of the interaction between lung and heart in thoracic irradiation.

Methods: Rat hearts and/or lungs were irradiated to 20Gy using high-precision proton beams. Cardio-pulmonary performance was assessed using breathing rate measurements and 18F-FDG-PET scans biweekly, and left/right-sided cardiac hemodynamic measurements and histopathology analysis at 8 weeks post-irradiation.

Results: 2-12 weeks post-heart irradiation a pronounced defect in the uptake of 18F-FDG in the left ventricle (LV) was observed. At 8 weeks post-irradiation this coincided with LV perivascular fibrosis, an increase in LV end-diastolic pressure and pulmonary edema in the shielded lungs. Lung irradiation alone not only increased pulmonary artery pressure and perivascular edema, but also induced an increased LV relaxation time. Combined irradiation of lung and heart induced pronounced increases in LV end-diastolic pressure and relaxation time, in addition to an increase in right ventricle end-diastolic pressure, indicative of biventricular diastolic dysfunction. Moreover, enhanced pulmonary edema, inflammation and fibrosis were also observed.

Conclusions: Both lung and heart irradiation cause cardiac and pulmonary toxicity via different mechanisms. Thus when combined, the loss of cardio-pulmonary performance is even further intensified, explaining the deleterious effects of heart and lung co-irradiation. Our findings show for the first time the physiological mechanism underlying the development of a multi-organ complication RILT. Reduction of dose to either of these organs offers new opportunities to improve radiotherapy treatment of thoracic tumours, potentially facilitating increased treatment doses and tumour control.

Keywords: Heart-lung interaction, Thoracic irradiation.
Abstract

**Introduction:** The risk of early radiation-induced lung toxicity (RILT) limits the dose and efficacy of radiotherapy of thoracic tumours. In addition to lung dose, co-irradiation of the heart is a known risk factor in the development RILT. The aim of the present study is to identify the underlying physiology of the interaction between lung and heart in thoracic irradiation.

**Methods and Materials:** Rat hearts and/or lungs were irradiated to 20Gy using high-precision proton beams. Cardio-pulmonary performance was assessed using breathing rate measurements and $^{18}$F-FDG-PET scans biweekly, and left/right-sided cardiac hemodynamic measurements and histopathology analysis at 8 weeks post-irradiation.

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**Keywords:** Heart-lung interaction, Thoracic irradiation.
Introduction

For many thoracic tumours treated with radiotherapy, dose escalation is expected to improve local control. However, the dose that can be administered safely is limited by the risk of potentially lethal radiation-induced lung toxicity (RILT). In a clinical situation, RILT manifests as respiratory distress and changes on thorax X-ray/computed tomography \(^1\). Several treatment-related factors such as the radiation dose and amount of irradiated lung tissue correlate with the risk of developing RILT \(^1\). In addition to lung tissue, a part of the heart is often also irradiated \(^2\). Although the heart is generally considered not to be functionally affected early after irradiation, early radiation-induced effects have been reported in animal \(^3\) and clinical studies \(^4\). Co-irradiation of the heart was also shown to enhance the risk of RILT both in animal studies \(^5,6\) and in patients with non-small-cell lung cancer (NSCLC) \(^2\). Therefore the contribution of the heart as well as the lung in the development of early RILT should be taken into account for treatment optimization. Until now, however, the mechanism by which this interaction takes place is not known.

Recently, it was shown in rats that lung irradiation induces pulmonary hypertension and right ventricle hypertrophy in an irradiated-volume dependent manner \(^7\). Pulmonary hypertension can contribute to cardiac dysfunction \(^8\). The possible contribution of radiation-induced pulmonary hypertension in cardiac damage was suggested in the early 1990s in a canine model \(^9\), however, the underlying physiological changes, are still unknown.

Taken together, these observations indicate that lung irradiation can cause changes in the heart and that heart irradiation can cause changes in the lung, leading collectively to physiological changes in the cardio-pulmonary system. Therefore, in the present study we investigated whether physiological changes caused by irradiation of lung and/or heart can explain their interaction in the development of radiation-induced cardio-pulmonary dysfunction.

Materials and methods

Animals

Adult male Wistar rats were used. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III.1986).

Irradiation technique

Rat hearts and/or lungs were irradiated (single-fraction) using a high-precision proton beam (150MeV) as published previously \(^6\). Briefly, high-energy protons were employed leading to very uniform dose distributions in the longitudinal direction (1%) with sharp lateral field edges (20–80% isodose distance: 1mm). This steep penumbra (0.9-1.1mm) facilitates the separate irradiation of lung and heart required for dissecting the individual contributions of each on tissue damage/dysfunction. The rats were irradiated under anaesthesia (isoflurane) and suspended from their incisors. The isodose curves of the irradiation portals (Supplementary Table S1) are shown in Fig 1.
For many thoracic tumours treated with radiotherapy, dose escalation is expected to improve local control. However, the dose that can be administered safely is limited by the risk of potentially lethal radiation-induced lung toxicity (RILT). In a clinical situation, RILT manifests as respiratory distress and changes on thorax X-ray/computed tomography. Several treatment-related factors such as the radiation dose and amount of irradiated lung tissue correlate with the risk of developing RILT. In addition to lung tissue, a part of the heart is often also irradiated. Although the heart is generally considered not to be functionally affected early after irradiation, early radiation-induced effects have been reported in animal and clinical studies. Co-irradiation of the heart was also shown to enhance the risk of RILT both in animal studies and in patients with non-small-cell lung cancer (NSCLC). Therefore the contribution of the heart as well as the lung in the development of early RILT should be taken into account for treatment optimization. Until now, however, the mechanism by which this interaction takes place is not known.

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**Breathing rate assay**
In pre-clinical studies, BR is considered as a surrogate measurement for pulmonary function. The increase of the post-irradiation BR relative to the mean BR in weeks 0–2 after irradiation was used as an indicator of early pulmonary dysfunction. Current data (n=5/group) were combined with historical data.

**Electrocardiogram (ECG)-gated cardiac $^{18}$F-FDG (fluoride-deoxy-glucose) positron emission tomography (PET)**
ECG-gated cardiac $^{18}$F-FDG PET scans were used to investigate whether heart irradiation induces cardiac changes. Reduction in $^{18}$F-FDG uptake of the left ventricle (LV) was scored (Supplementary Table S2) (n=6/group) and was reported as LV $^{18}$F-FDG uptake defect.

**LV or right ventricle (RV) hemodynamic measurements**
LV or RV hemodynamic measurements were carried out to investigate cardiac physiological changes 8 weeks post-irradiation. For LV hemodynamic measurement (n=5/group), a pressure catheter was introduced via the carotid artery and guided through the aorta into the LV to obtain LV end-diastolic pressure (LVEDP) and Tau (LV relaxation constant). RV pressure (RVP) and pulmonary artery pressure (PAP) (n=4/group) were recorded from RV hemodynamic measurement. Wet lungs were weighed to check if they were oedematous.
The heart was divided into atria, ventricles and septum and weighed separately to assess RV hypertrophy (RVH)\(^7\).

**Morphological changes**

Pulmonary edema, inflammation and fibrosis, as well as cardiac perivascular fibrosis, were assessed in samples from the irradiated and the shielded lung and heart (n≥4/group). Pulmonary edema was assessed using Haematoxylin & Eosin (H&E) staining and semi-quantitative scoring of interstitial and perivascular edema. For interstitial edema, scores from 0-3 were given reflecting the severity of the damage to the interstitial tissue and the amount of affected tissue with/without interstitial infiltrate. For perivascular edema, scores from 0-2 were given to show the severity of the edema around vessels. At least 5 big vessels (>100μm) and 20 small vessels (<100μm) with/without perivascular infiltrate were scored. Using H&E staining, pulmonary parenchymal inflammation was scored from 0-3 based on the number of inflammatory cells in the lung parenchyma. For fibrosis, Masson Trichrome staining was used. Pulmonary parenchymal fibrosis was scored 0-3 based on the amount of deposited collagen. Cardiac perivascular fibrosis was quantified by calculating the ratio of the fibrosis area surrounding the vessel to the total vessel area using an image analysis system (CZ KS400;Imaging Associates, Bicester, UK). For this, at least 20 vessels were examined and depending on the amount of vessels, the average results per group were obtained from at least 4 rats’ LVs.

**QPCR**

Total RNA was extracted from the PBS-perfused rat LVs (n=4/group) and cDNA was generated subsequently according to the manufacturers’ protocols. Bio-Rad IQTM SYBR@ Green Supermix was used to perform amplifications in a Bio-Rad IQ iCycler machine as described by the manufacturer. The following primers were used to amplify fibronectin: 5’-agaccatactgcggaatgtag and gagaagcttctgtcctgtagag. The mRNA levels were normalized using 36B4 (ribosomal) primers with the sequences: 5’-gtttgctcagtcctcactc and 5’-gcagccgcaaatgcagatgg.

**Statistical analysis**

Differences between groups were tested by comparing the functional and physiological parameters of the groups using one-way ANOVA with Bonferroni post-hoc analysis, to correct for multiple comparisons. For morphological assessments, non-parametric Kruskal-Wallis test was performed. P values < 0.05 were considered statistically significant. To determine to what extent the physiological changes can be explained by changes in the morphology, the Pearson’s product-moment correlation coefficient was used. A correlation was considered significant if the hypothesis of no correlation was rejected at p<0.05.

More details can be found in the Supplementary material.
Results

Radiation induces early cardiac damage
To confirm the interaction between lungs and heart on radiation-induced pulmonary dysfunction, we investigated the BR post-partial irradiation of the rat lung, with or without irradiation of the heart. Co-irradiation of the heart induced a pronounced BR-increase (Supplementary Fig. S1). This suggests that heart irradiation may induce early subclinical heart damage that manifests only in combination with lung irradiation resulting in an aggravated reduction in lung function. To determine whether heart irradiation induces early cardiac damage, cardiac metabolism was assessed by 18F-FDG-PET scans. Scans were taken biweekly for 12 weeks after irradiation of the heart and the adjacent lung tissue (25% of the lung) with a dose below and above 18 Gy; the threshold for lung/heart interaction. Indeed a significant defect in 18F-FDG uptake was observed in the LVs, at both dose levels (Fig. 2, P<0.01 H+25%L, 16 Gy vs. control and P<0.001 H+25%L, 25 Gy vs. control).

![Image](image.jpg)

**Figure 2: Cardiac metabolic changes after heart irradiation.** Scoring of reduced 18F-FDG uptake in irradiated hearts at diastolic phase (week2-12 post-irradiation) (a) shows 18F-FDG uptake defect in irradiated LVs to 16 and 25 Gy with more pronounced effect in 25Gy (b). The areas under the curves (week2-12) were compared. ***P<0.001 **P<0.01 *P<0.05 all when compared to control. Data are presented as mean ± SEM.

Heart irradiation induces cardiac fibrosis and myocardial damage
To investigate the origin of these metabolic changes, we investigated the morphology of the LVs. Immuno-histochemical staining (Fig. 3a) and morphological quantification (Fig. 3b, P<0.01) showed pronounced perivascular fibrosis in the irradiated LVs (20 Gy). Moreover,
in irradiated LVs a relative increase in fibronectin mRNA expression, a well-established profibrotic gene 10, was observed (Fig. 3c, P<0.05). Aside from perivascular fibrosis, contraction bands (Fig. 3d, yellow arrows) were observed in the myocardium of the irradiated LVs located predominately in endocardium and epicardium.

Figure 3: LV perivascular fibrosis and myocardial damage in irradiated hearts, observed by immuno-histochemical staining (a, d), morphological quantification of perivascular fibrosis (b) and relative fibronectin mRNA expression (c). Contraction bands (d, shown by yellow arrows) were observed in the epicardium and endocardium of the irradiated hearts. Scale bars on panel a, d and e represent 50μm, 1mm and 100μm respectively. Masson Trichrome staining was performed, red stain= muscle fiber, blue stain= collagen. ***P<0.001 **P<0.01 *P<0.05 all when compared to control. Data are presented as mean ± SEM.

Heart irradiation induces left ventricle diastolic dysfunction
Myocardial damage and perivascular fibrosis are known to be an important pathophysiological processes contributing to diastolic dysfunction by impairing cardiac relaxation 11,12. Therefore, we next investigated whether the observed histo-pathology and metabolic changes translate into changes in cardiac function. To this end, LV hemodynamics were assessed. Heart irradiation (20 Gy) induced a pronounced elevation of the LVEDP (Fig. 4a, P<0.05). Moreover, pericardial effusion was observed in 2 out of 9 irradiated hearts. Next we determined to what extent radiation-induced physiological changes can be explained by LV morphological changes, by correlating LV perivascular fibrosis with LVEDP. The perfect correlation (Supplementary Fig. S2a) indicated that LV diastolic dysfunction can be fully explained by changes in cardiac vasculature.
Heart irradiation enhances lung tissue damage
Since diastolic dysfunction is known to promote pulmonary edema, it may be the mechanism by which heart irradiation induces pulmonary dysfunction. If so, this would lead to pulmonary edema in shielded lung tissue. Morphological analysis showed significant pulmonary interstitial edema post-heart irradiation (Fig. 5a, P<0.001). Moreover, the wet weight of the lungs was higher when the hearts were irradiated (Supplementary Fig. S3).

![Graph showing LV Hemodynamics after heart and/or lung irradiation.](image)

Figure 4: LV Hemodynamics after heart and/or lung irradiation. Significant increases in LVEDP were observed in H+25%L and H+50%L groups (a) while elevation of Tau was observed in 50%L and H+50%L groups (b). ***P<0.001 **P<0.01 *P<0.05 all when compared to control. Data are presented as mean ± SEM.

Also in 3 out of 9 rats with irradiated hearts (20 Gy), pleural effusion was observed. A strong correlation between LVEDP and pulmonary interstitial edema (Supplementary Fig. S2a) suggests that LV diastolic dysfunction induces secondary lung damage. In addition to pulmonary edema, morphological evaluation revealed more pulmonary parenchymal inflammation and fibrosis (Fig. 5b, P<0.001 and P<0.01 respectively) when the heart was co-irradiated.

Combined lung and heart irradiation: Double trouble
If irradiation of heart and lung would both have a common consequence, this would explain the observed interaction between them. Importantly, irradiation of 50% lungs (20 Gy) has also been shown to induce not only pulmonary edema (Fig. 5a, Supplementary Fig. S3, P<0.05) and inflammation (Fig. 5b, P<0.05), but also affect the heart due to increased pulmonary tension, leading to RV systolic pressure overload and RVH (7, Supplementary Figure S4). Since all of these factors are known to cause LV dysfunction, we investigated LV function post-lung irradiation. Indeed, significant elevation of Tau (Fig. 4b, P<0.05) was observed. A strong correlation between pulmonary hypertension, perivascular edema and Tau (Supplementary Fig. S2b) indicates that secondary cardiac damage can be explained by radiation-induced lung damage.

We next investigated whether combining these effects by simultaneous irradiation of both organs results in enhanced response. To this end, cardiac performance was assessed after heart+50% lung irradiation (20 Gy). Significant elevation of both LVEDP (Fig. 4a, P<0.01) and Tau (Fig. 4b, P<0.05), in addition to perivascular fibrosis (Fig. 3b, P<0.001) were observed in this setting. Furthermore, enhanced wet lung weight increase (Supplementary Fig. S2, P<0.001), pulmonary interstitial edema (Fig. 5a, P<0.001), perivascular edema (Fig. 5a, P<0.05), parenchymal inflammation (Fig. 5b, P<0.001), fibrosis (Fig. 5b, P<0.001) and
pleural effusion (5 out of 9 rats) were observed in the group that received combined lung-heart irradiation. In addition to LV diastolic dysfunction, pulmonary edema may also develop from RV diastolic dysfunction, as it may be induced by pulmonary hypertension (Supplementary Fig. S4a,b). Consistent with this, we also observed a pronounced increase in RVDP (Fig. 6, P<0.001).

Figure 5: The morphology of the lung after heart and/or lung irradiation. a) Semiquantitative scoring of pulmonary interstitial (blue arrows) and perivascular edema (black arrows). The numbers in the pictures show the given scores (Interstitial edema: blue, Perivascular edema: black). The first, second and third rows show representative examples of the lung tissue taken from non-irradiated controls, shielded and irradiated parts respectively. From left to right, columns show increasing levels of the tissue damage. Significant increase of global pulmonary interstitial edema was observed when the heart was co-irradiated with the lungs (a). 50% lung irradiation with/without co-irradiation of the heart significantly increased pulmonary perivascular edema (a). b) The first and second rows show semiquantitative scoring of parenchymal inflammation in representative examples of the lung tissue taken from shielded and irradiated parts respectively (H&E staining). The third row shows semiquantitative scoring of parenchymal fibrosis in the lung tissue. The numbers on the left upper corner of the pictures show the given scores (Inflammation: black, fibrosis: blue). Co-irradiation of the heart significantly increased the number of inflammatory cells (b) and the amount of deposited collagen (b) in the lung tissue. Lung irradiation alone significantly increases the number of inflammatory cells. Scale bar shows 100μm. The purple bar shows increasing the level of tissue damage. ***P<0.001 **P<0.01 *P<0.05 all when compared to control. Data are presented as mean ± SEM.
Physiologic interaction of heart and lung

Discussion

The present results document the physiology of how cardiac and pulmonary damage can mutually influence cardio-pulmonary function. We observed that heart irradiation directly affects cardiac vasculature and myocardium and therefore increases end-diastolic pressure, leading to LV diastolic dysfunction, which promotes pulmonary interstitial edema. Lung irradiation indirectly impairs LV diastolic function through radiation-induced pulmonary hypertension, pulmonary perivascular edema and the resultant effect on LV relaxation time. Combined lung-heart irradiation enhances cardiac diastolic dysfunction via both mechanisms, which may result in bi-ventricle dysfunction, explaining the observed aggravated cardio-pulmonary dysfunction. We propose that cardiac dysfunction, either originating directly from heart irradiation or indirectly caused by lung irradiation, may be an important factor causing and/or enhancing cardio-pulmonary toxicity after thoracic radiotherapy.

Consistent with our observations, changes in cardiac $^{18}$F-FDG uptake were observed in patients with oesophageal cancer 3-9 months post-radiotherapy. Moreover, these changes correlated with elevated plasma levels of brain natriuretic peptide, a known marker of cardiac diastolic dysfunction in patients with acute dyspnoea. In pre-clinical studies on RILT, BR increase is often considered a surrogate measurement of radiation pneumonitis. Indeed, these BR changes may reflect a reduced diffusion capacity due to parenchymal damage such as alveolitis. In addition, it may reflect decreased ventilation efficiency as a consequence of reduced perfusion, a symptom frequently observed in patients with pulmonary hypertension and edema. Radiation-induced pulmonary hypertension was shown to contribute to BR changes not only in single irradiation dose preclinical studies but also in fractionation study in a canine model. Clinical symptoms of cardiac diastolic dysfunction are also predominantly respiratory in nature, due to backward failure of the LV causing congestion of the pulmonary vasculature and pulmonary edema. Consistent with this, cardiac dysfunction was shown to play key
role in the development of BR increase in the present study. Taken together, this suggests that dyspnoea may originate from physiological cardio-pulmonary changes such as cardiac diastolic dysfunction and pulmonary hypertension rather than parenchymal damage alone. However to substantiate our findings, studies with fractionated dose in larger animal models and/or in patients should be performed.

Current practice in the optimization of the treatment of NSCLC is mainly based on the dose distribution in the lungs and the dose to spinal cord. The local control of the lung tumours could be improved to ~80-90% by increasing the tumour dose by ~20% \(^{16}\). At present however, this dose escalation is not possible in the majority of patients due to the associated risk of RILT. Importantly, in our pre-clinical studies differences in tolerance dose between co-irradiating and sparing of the heart were typically in the same range \(^{5,6}\). Currently, conforming to the dose constraints of the lung often results in an increase of dose to the heart. Our present work shows that for a given lung dose, increasing dose to the heart may in fact increase the risk of RILT, thus hampering efforts for dose escalation. Therefore, adding dose constraints for the heart in the treatment optimization for NSCLC may allow dose escalation and consequently increase the local control. Recently, pre-clinical and clinical studies have suggested that models of RILT can be improved by accounting for the impact of dose to the heart on the risk of complications \(^{2,6}\). However, the approach to optimally distribute dose over the lung and heart is still unclear. Since we now show that symptomatic RILT results from physiological cardio-pulmonary changes, dose-response relationships of these changes such as LVEDP and PAP in patients incorporating both lung and heart dose-volume variables are needed to build more accurate predictive models.

In addition to dosimetric factors, pre-treatment clinical factors could be incorporated into the predictive models. Pre-existing lung diseases \(^{17}\) and cardiac and pulmonary comorbidity \(^{18}\) are known to predict adverse effects of a treatment. More accurate models will help to identify patients with a higher risk of RILT. Notably, dose limits for the entire population is currently based on these patients, accurately identifying them increases the tolerance of the residual population, thereby allowing tumour dose escalation. The patients for whom tumour location and size prevents radiotherapy with conventional modalities are suitable candidates for dose escalation by more focalized radiation treatment modalities such as particle radiotherapy \(^{19}\). Here, the dose is more confined to the target and co-irradiation of the heart and large volumes of healthy lung tissue can be more effectively spared \(^{19}\).

Symptoms of RILT were shown not to be limited to patients with radiologic changes, nor were these changes exclusively associated with symptomatic patients \(^{20}\). Radiographically occult factors such as vascular damage \(^{7}\) and cardiac damage (present results) may contribute to the development of RILT and the associated symptoms. Therefore, in addition to diagnostic tools of lung damage, (sub-clinical) cardiac damage should be assessed for a better assessment of RILT.

Taken together, our findings show for the first time that reducing the risk of the multi-organ complication RILT, requires specific optimization of the treatment with respect to cardiac and pulmonary dose. This may lead to the development of more accurate prediction and more effective treatment of thoracic cancers, thereby facilitating individualized dose escalation, increased local control and quality of life of patients undergoing thoracic radiotherapy.
the predictive models. Pre-existing lung diseases and cardiac and pulmonary comorbidity are significant factors in predicting and more effectively treating thoracic cancers, thereby facilitating individualized dose escalation, increased local control and quality of life of patients. Organ complication RILT, requiring specific optimization of the treatment with respect to dose-volume effects in the rat lung. Int J Radiat Oncol Biol Phys 2007 10/01;69(2):552-559.

Taken together, our findings show for the first time that reducing the risk of the multi-organ complication RILT, requires specific optimization of the treatment with respect to dose-volume effects in the rat lung. Int J Radiat Oncol Biol Phys 2007 10/01;69(2):552-559.

To substantiate our findings, studies with fractionated dose in larger animal models and/or in patients should be performed. However, this dose escalation is not possible in the majority of patients due to the distribution in the lungs and the dose to spinal cord. The local control of the lung tumours may allow dose escalation and consequently increase the local control. Recently, pre-treatment clinical factors could be incorporated into accurate predictive models.

In addition to dosimetric factors, the role of pre-treatment clinical factors could be incorporated into accurate predictive models. Incorporating both lung and heart dose-volume variables are needed to build more dose-response relationships of these changes such as LVEDP and PAP in patients.

Symptoms of RILT were shown not to be limited to patients with radiologic changes, nor to diagnostic tools of lung damage, (sub-clinical) cardiac damage should be assessed for a more complete understanding of the development of RILT and the associated symptoms. Therefore, in addition to dosimetric factors, pre-treatment clinical factors could be incorporated into accurate predictive models.

Contribution of the heart to radiation-induced lung injury has been studied extensively, but the precise mechanisms remain unclear. In the present study, we aimed to evaluate the role of the heart in the development of bronchiolitis obliterans with organizing pneumonia (BOOP) and the toxic pneumonitis following thoracic irradiation in rats. The impact of heart irradiation on dose-volume effects in the rat lung was investigated. Int J Radiat Oncol Biol Phys 2007 10/01;69(2):552-559.

Physiologic interaction of heart and lung

References

Supplement material

Animals
Adult male albino Wistar rats (n=50, 270gr-320gr) of the Hsd/Cpb:WU strain bred in a specific pathogen free colony (Harlan-CPB, Rijswijk, The Netherlands) were used. They were housed five to a cage under a 12h light-12h dark cycle and fed rodent chow (RMH-B, Hope Farms, Woerden, The Netherlands) and water ad libitum. The experiments were performed in agreement with the Netherlands Experiments on Animals Act (1977) and the European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg,18.III.1986).

Irradiation technique
Rats’ heart and/or lung were irradiated with 150 MeV protons (single fraction) from the cyclotron at the Kernfysisch Versneller Instituut, Groningen, using the shoot-through technique as previously published 1,2. In short, the shoot-through technique only employs high-energy protons (the plateau of the Bragg curve). In the plateau the stopping power (and consequently dose) increases by approximately 1% within the rat, along the beam axis. A homogeneous dose distribution of scattered proton beams was created, using a double scatter foil system. This resulted in a very uniform dose distribution in the longitudinal direction (1%) and sharp lateral field edges (20–80% isodose distance: 1 mm). The steep penumbra (0.9-1.1 mm) of the proton beam with this irradiation technique facilitates the separate irradiation of lung and heart required for dissecting the individual contributions of each on tissue damage and cardio-pulmonary dysfunction. The rats were irradiated while they were under anaesthesia (isoflurane) and hung from their incisors. The irradiation ports were designed using computed tomography scans of 5 rats of the same age and weight in the same set up as previously published 1,2. The irradiation ports and their fields of irradiation are described in table S1. Overview of isodose curves of applied irradiation ports is shown in Fig. 1.

Cardiac gated \(^{18}\text{F-FDG-PET scans}\)
Electrocardiogram (ECG)-gated cardiac\(^{18}\text{F-FDG} \) (fluoride-deoxy-glucose) positron emission tomography (PET) scans were used to investigate whether heart irradiation induces cardiac changes). Rats (n=6) were scanned under isoflurane anaesthesia biweekly till week 12 after heart irradiation (+25% inevitably lung irradiation) to 16 or 25 Gy. Each cardiac cycle, delimited by peaks in the ECG, was divided to 8 gates. Because of its high workload, the left ventricle (LV) uses more glucose compared to the surrounding tissues and is therefore clearly visible on an \(^{18}\text{F-FDG-PET scan}\). Prior to the scan 1ml of saline containing 60 MBq of \(^{18}\text{F-FDG} \) was injected in the superficial penile vein. Emission scans were made and then reconstructed for all the gates. \(^{18}\text{F-FDG} \) uptake defects were scored blindly by two observers. Each image was given a score for the estimated part of the myocardium that had a reduced uptake of \(^{18}\text{F-FDG} \) (Table S2). Subsequently the average of the scores in diastolic phase was calculated for each group and was reported as LV \(^{18}\text{F-FDG} \) uptake defect. To compare the difference between the groups the area under the curves from week 2 till 12 were calculated.
Cardiac Hemodynamic measurements
To investigate early cardiac physiological changes LV or right ventricle (RV) hemodynamic measurements were carried out 8 weeks after irradiation. For LV hemodynamic measurement, a pressure catheter was introduced via the carotid artery and guided through the aorta into the LV to obtain hemodynamic parameters (n=5 per group) and LV end-diastolic pressure (LVEDP) and Tau (LV relaxation constant) were recorded. Right ventricle pressure (RVP) and pulmonary artery pressure (PAP) (n=4 per group) were recorded from RV hemodynamic measurement as previously published. All procedures were carried out while the rats were anesthetized (isoflurane) and ventilated with room air. Subsequently the thorax was opened and heart and lungs were examined in situ for overt abnormalities such as pericardial and pleural effusion and then excised for further histological analysis. Wet lungs were weighted to check if they are oedematous. The heart was divided into atria, ventricles and septum and weighted separately to assess RV hypertrophy (RVH) as published previously.

Histopathology
Morphological changes in the heart and lung tissue were assessed in samples from heart, irradiated and shielded (distance >3 mm from the irradiated field-edge) parts of the lung. Tissue sections (5-μm thick) were stained with Haematoxylin & Eosin and Masson’s trichrome and analysed morphologically for signs of pulmonary edema, parenchymal inflammation and fibrosis as well as myocardial damage. Pulmonary edema was assessed on Haematoxylin & Eosin stained lung tissue by semi-quantitative scoring of interstitial and perivascular edema. For interstitial edema: score 0: no edema in the interstitial tissue (thin septa); score 1: Mild-moderate edema without interstitial infiltrate, affected area (a) <50% of total tissue section; score 2: moderate-sever edema with interstitial infiltrate, a <50%; score 3: sever edema with interstitial infiltrate, a >50%. For perivascular edema: score 0: no edema around vessels; score 1: mild-moderate edema with/without perivascular infiltrate; score 2: moderate-severe edema with/ without perivascular infiltrate. All vessels in the tissue section (at least 5 big vessels >100μm and 20 small vessels <100μm) were scored blindly by two observers separately and then averaged for the representative score of that section. Pulmonary parenchymal inflammation was scored on Haematoxylin & Eosin stained lung tissue as the level of inflammatory cells in the lung parenchyma on each slide. No distinction was made between the different cell types. No inflammatory cells = Score 0; only a few inflammatory cells in the lung = Score 1; many nonclustered inflammatory cells present (200x magnification) = Score 2; and large amounts of clustered inflammatory cells present (100x magnification) and total affected area volume of 50% or more of the total tissue cross-section = Score 3. Pulmonary parenchymal fibrosis was scored on Masson Trichrome stained lung tissue. No fibrosis = Score 0; thickening of lung parenchyma, alveolar structures still visible = Score 1; complete breakdown of lung parenchyma, a clot of fibrotic tissue replaces alveolar tissue (total affected area < 50% of the total tissue section) = Score 2; complete breakdown of lung parenchyma, a clot of fibrotic tissue replaces alveolar tissue (total affected area volume of 50% or more of the total tissue cross-section) = Score 3.

Cardiac perivascular fibrosis was quantified on Masson Trichrome stained heart tissue by calculating the ratio of the fibrosis area surrounding the vessel to the total vessel area using an image analysis system (CZ KS400; Imaging Associates, Bicester, UK). For this at
least 20 vessels were examined and depending on the amount of vessels the average results per group were obtained from at least 4 rats’ LVs.

**QPCR**
Total RNA was extracted from the PBS-perfused rat LVs using the Stratagene Absolutely RNA miniprep-kit and cDNA was generated subsequently using the M-MLV Reverse Transcriptase kit (Invitrogen) according to the manufacturers’ protocols. Bio-Rad IQTM SYBR® Green Supermix was used to perform amplifications in a Bio-Rad IQ iCycler machine as described by the manufacturer. The following primers were used to amplify fibronectin: 5’-agaccatacctgccgaatgtag and gagagcttcttgtcctgtagag. The mRNA levels were normalized using 36B4 (ribosomal) primers with the sequences: 5’-gttgcctcagtgcctcactc and 5’-gcagccgcaaatgcagatgg.

<table>
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<tr>
<th>Fields</th>
<th>Irradiation details</th>
<th>Corresponding panel</th>
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</thead>
<tbody>
<tr>
<td>25%L</td>
<td>Irradiation of 25% of the right base of the lung</td>
<td>Figure 1a</td>
</tr>
<tr>
<td>H + 25%L</td>
<td>Irradiation of the heart plus unavoidable 25% of the surrounding lung tissue</td>
<td>Figure 1b</td>
</tr>
<tr>
<td>H + 50%L</td>
<td>Irradiation of the 25% of the lateral part of the lung in addition to H+25%L</td>
<td>Figure 1c</td>
</tr>
<tr>
<td>50%L</td>
<td>Irradiation of the 50% of the lateral part of the lung</td>
<td>Figure 1d</td>
</tr>
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</table>

*Table S1: Detailed description of the irradiation fields.*

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</thead>
<tbody>
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<td>No reduction</td>
</tr>
<tr>
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<td>x ≤ 25%</td>
</tr>
<tr>
<td>2</td>
<td>25% &lt; x ≤ 50%</td>
</tr>
<tr>
<td>3</td>
<td>x &gt; 50%</td>
</tr>
</tbody>
</table>

*Table S2: Scoring system for left-ventricle FDG uptake reductions.*
Figure S1: Co-irradiation of the heart enhances early radiation-induced pulmonary dysfunction. Small lung irradiation (25%) does not translate to BR-increase whereas larger irradiated lung volume (50%) clearly induces pulmonary dysfunction shown by significant BR-increase (***P<0.001, 50%L vs. Control). Co-irradiation of the heart adversely enhances this response (†P<0.05, 50%L vs. H+50%L). The graph shows the area under the curve of BR increase (week 0-8 post-irradiation, containing the peak response (1,2)). Current data supplemented with data from (1,2). Data are presented as mean ± SEM.
Chapter 03 | Physiologic interaction of heart and lung

Figure S2: Correlation between different morphological and physiological parameters indicates that irradiation of heart and lung can independently cause both cardiac and pulmonary damage/dysfunction via different mechanisms. a) An almost perfect correlation between an increase in left ventricle end-diastolic pressure (LVEDP) and left ventricle (LV) perivascular fibrosis (r=0.99) suggests that heart irradiation induces LV perivascular fibrosis leading to an increase of LVEDP and LV diastolic dysfunction. This cardiac damage in turn may cause secondary lung toxicity by promoting pulmonary interstitial edema both in irradiated (close symbols, r=0.98) and shielded lung tissue (open symbols, r=0.95). b) Lung irradiation induces pulmonary hypertension (4) which promotes pulmonary perivascular edema both in irradiated (close symbols, r=0.91) and shielded lung tissue (open symbols, r=0.83). A strong correlation between Tau and pulmonary perivascular edema both in irradiated (close symbols, r=0.99) and shielded lung tissue (open symbols, r=0.98) suggests that this global lung damage indirectly leads to impairment of LV diastolic function through its resultant effect on LV relaxation time (Tau). Data are presented as mean ± SEM.
Figure S3: Wet lung weight is increased after lung irradiation (\( ^*P<0.05 \) 50%L vs Control) which further elevated when co-irradiated with the heart (\( ^{†}P<0.05 \) 50%L vs H+50%L; \( ^{***}P<0.001 \) H+50%L vs Control). Data are presented as mean ± SEM.

Figure S4: 50% lung with or without heart irradiation (20Gy) induces pulmonary hypertension and right ventricle hypertrophy. This was shown by a) significant elevation of mean pulmonary artery pressure (mPAP), \( ^{*}P<0.05 \) (50%L vs. Control) and b) right ventricle hypertrophy (RVH), \( ^{*}P<0.05 \) 50%L vs. Control and \( ^{**}P<0.01 \) (H+50%L vs. Control). RVH was assessed indirectly by \(^{18}\)F-FDG scans and directly by measuring the ratio of the weights of RV to the combination of IVS and LV. \(^{18}\)F-FDG scans show the top view of the hearts. \(^{18}\)F-FDG uptake of the RV increased dramatically after lung irradiation indicative of increased work load of the RV. Data are presented as mean ± SEM.
Supplementary references


