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Biology Contribution

Decreasing Irradiated Rat Lung Volume Changes Dose-Limiting Toxicity From Early to Late Effects

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Summary

Technological developments in radiation therapy have resulted in smaller irradiated volumes of normal tissue. Late radiation-induced rat lung dysfunction was observed to depend predominantly on dose and was mainly associated with inflammation and fibrosis, in contrast to the irradiated volume and vascular remodeling-dependent early dysfunction. Consequently, dose-limiting toxicity changed from early to late effects when the irradiated volume was reduced.

Purpose: Technological developments in radiation therapy result in smaller irradiated volumes of normal tissue. Because the risk of radiation therapy-induced toxicity generally depends on irradiated volume, changing volume could change the dose-limiting toxicity of a treatment. Recently, in our rat model, we found that early radiation-induced lung dysfunction (RILD) was closely related to irradiated volume dependent vascular remodeling besides inflammation. The exact relationship between early and late RILD is still unknown. Therefore, in this preclinical study we investigated the dose-volume relationship of late RILD, assessed its dependence on early and late pathologies and studied if decreasing irradiated volume changed the dose-limiting toxicity.

Methods and Materials: A volume of 25%, 32%, 50%, 63%, 88%, or 100% of the rat lung was irradiated using protons. Until 26 weeks after irradiation, respiratory rates were measured. Macrovascular remodeling, pulmonary inflammation, and fibrosis were assessed at 26 weeks after irradiation. For all endpoints dose-volume response curves were made. These results were compared to our previously published early lung effects.

Results: Early vascular remodeling and inflammation correlated significantly with early RILD. Late RILD correlated with inflammation and fibrosis, but not with vascular remodeling. In contrast to the early effects, late vascular remodeling, inflammation and fibrosis showed a primarily dose but not volume dependence. Comparison

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Therefore, pathologies with different dose-volume relationships may alter dose-limiting toxicity with changing irradiated volume.

of respiratory rate increases early and late after irradiation for the different dose-distributions indicated that with decreasing irradiated volumes, the dose-limiting toxicity changed from early to late RILD.

Conclusions: In our rat model, different pathologies underlie early and late RILD with different dose-volume dependencies. Consequently, the dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. In patients, early and late RILD are also due to different pathologies. As such, new radiation techniques reducing irradiated volume might change the dose-limiting toxicity of the radiation therapy treatment.

Introduction

Radiation therapy plays a pivotal role in the treatment of thoracic cancers. Unfortunately, radiation-induced lung dysfunction (RILD) is a potentially life-threatening and dose-limiting side effect of thoracic irradiation and thus the risk should be minimized (1).

Traditionally, RILD is divided into an early inflammatory phase known as "radiation pneumonitis" and a later fibroproductive phase referred to as "lung fibrosis." Clinically significant symptomatic early RILD occurs in approximately 5% to 50%, 5% to 10%, and 1% to 5% of patients irradiated for cancers of the lung, mediastinal lymphatics, and breast, respectively (2, 3).

Accurate prediction of the development of RILD is of great importance for treatment optimization. However, controversy exists about which dosimetric parameter(s) optimally predict RILD (4). Moreover, only models predicting early RILD are described. Because new advances in therapies will lead to a longer life expectancy of cancer patients, the occurrence of late radiation-induced normal tissue toxicity will become more relevant. Besides, technological developments in radiation therapy result in smaller irradiated volumes of normal tissue. Changing the irradiated volume could change the dose-limiting toxicity of a treatment.

In our rat model, early and late RILD occurs as a biphasic increase in breathing rates and histopathological changes (5). Morphologically, distinct types of lung injury can be observed: vascular remodeling, inflammation and fibrosis (6). Recently, we found that in concert with inflammation, vascular remodeling played a major role in the cause of early RILD (7, 8). It was shown that lung irradiation induced early vascular remodeling resulting in pulmonary hypertension and right ventricle hypertrophy eventually leading to cardiopulmonary dysfunction (7, 9).

The relationship between early RILD with its dependence on irradiation dose and volume and late RILD is still unknown. Therefore, in this preclinical study, we investigated the dose-volume relationship of late RILD, assessed its dependence on early and late pathologies and studied if decreasing irradiated volume could change the dose-limiting toxicity.

Methods and Materials

See Supplementary data online for complete material and methods (available online at www.redjournal.com).

Animals

Adult male albino Wistar rats (n = 3-7 per dose-volume group) were used in the experiments. The experiments were performed in agreement with The Netherlands Experiments on Animals Act (1977) and European Convention for the Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18.III. 1986).

Irradiation procedure

A volume of 25% (15-28 Gy), 32% (19-28 Gy), 50% (12-20 Gy), 63% (12-19 Gy), 88% (10-14 Gy) or 100% (10-13 Gy) of the rat lung was irradiated with protons. Figure 1 gives an overview of the irradiation ports.

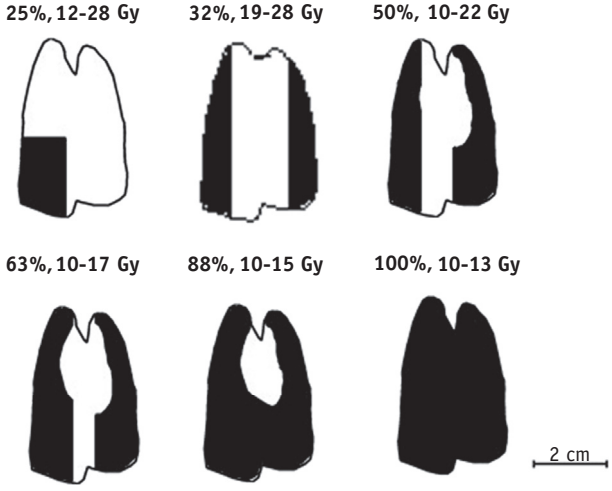


Fig. 1. Overview of the irradiation ports used.

Breathing rate assay

To assess response of pulmonary function to radiation, the breathing rate (BR) was measured up to week 26, as previously described (5, 10), and is shown in Figure 2. The mean increase in BR between 6 and 10 weeks after irradiation was used to assess the level of early RILD. For late RILD, the mean increase in BR between 16 and 26 weeks was measured.

Histologic examinations

Histologic examination was performed 26 weeks after radiation and compared with the histology at week 8 which we previously published (8). Details of the procedure and scoring have been published previously (5, 6).

Vascular remodeling was scored by assessing hypertrophy of the macrovasculature (Fig. 3). Both arterioles and venules were scored since these could not be distinguished in the lung tissue. No affected vessels received a score of 0; hypertrophic vascular walls received a score of 1; and heavily affected vessels, meaning smooth-muscle cells of the media layer were thickened and around the arterioles edema or fibrosis, received a score of 2.

Pulmonary inflammation was scored as the level of inflammatory cells in the lung tissue (Fig. 3). No cells = 0; only a few cells = 1; many nonclustered cells present = 2; and large amounts of clustered cells present and total

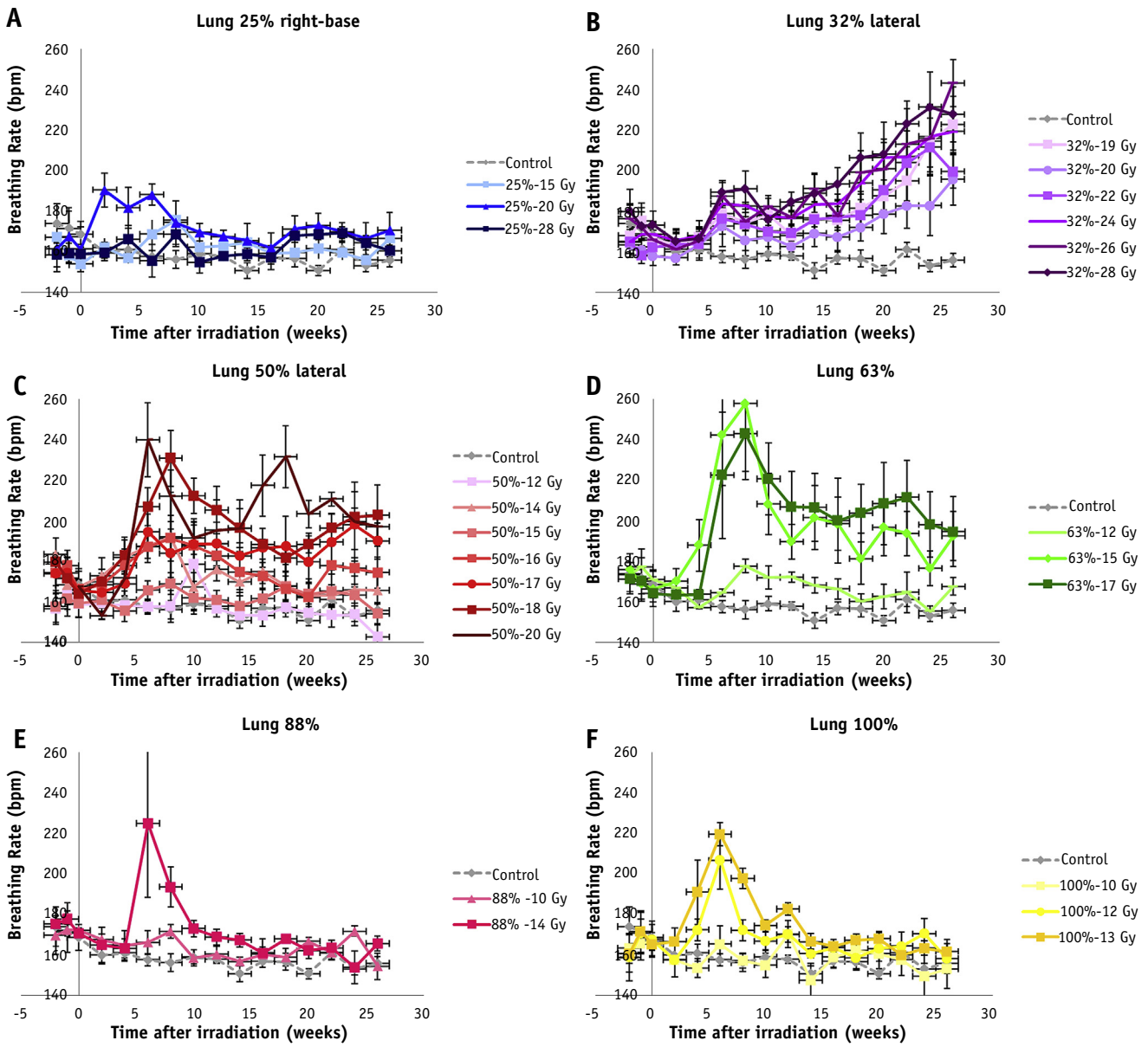


Fig. 2. Respiratory rate of lung-irradiated rats. Respiratory rate of rats irradiated to a lung volume of 25% (A), 32% (B), 50% (C), 63% (D), 88% (E), and 100% (F) with doses ranging from 10 to 28 Gy. The gray dotted line in all panels indicates the breathing rate of unirradiated animals. N = 3-7 per dose-volume group. Error bars indicate SEM.

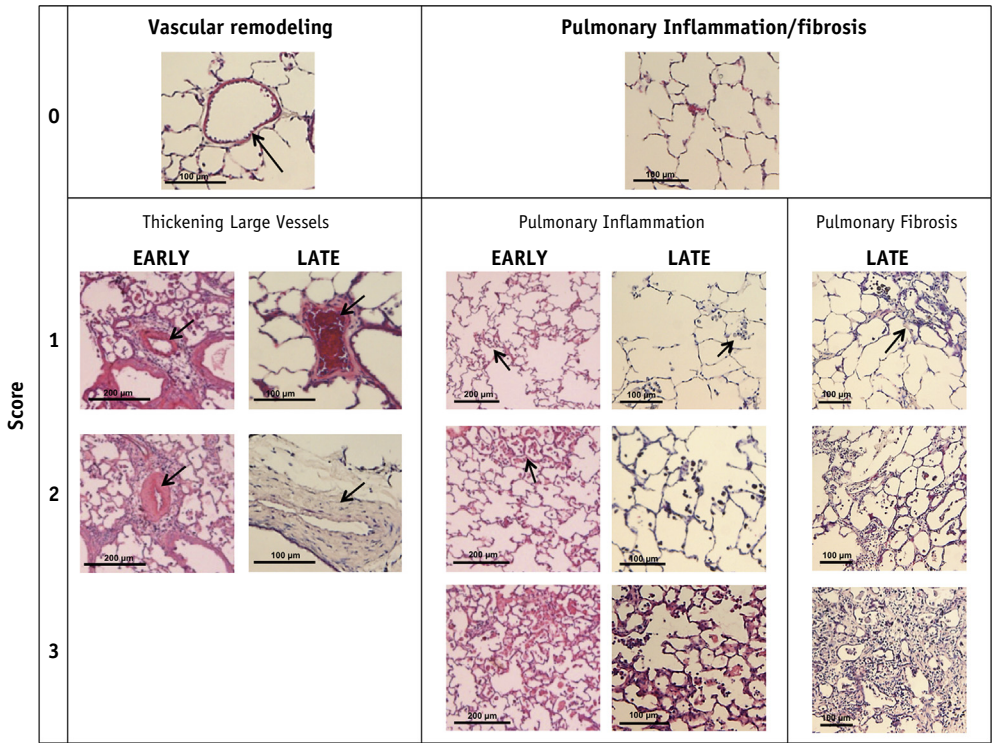


Fig. 3. Quantification of rat lung morphology early and late after irradiation. A score of 0 for macrovascular remodeling indicates thin vascular walls; a score of 1 hypertrophic indicates vascular walls; and a score of 2 indicates extreme hypertrophic vascular walls. A score of 0 for pulmonary inflammation represents normal lung tissue with sporadic inflammatory cells. A score of 1 indicates a moderate increase of inflammatory cells; a score of 2 indicates a lot of nonclustered inflammatory cells; and 3 indicates large foci of inflammatory cells. A score of 0 for fibrosis shows normal lung tissue without fibrosis; a score of 1 is small foci of fibrosis. A score of 2 indicates medium foci of fibrosis, and a score of 3 indicates large foci of fibrosis.

affected area volume of 50% or more of the total tissue cross-section = 3.

Late fibrosis was scored from 0 to 3 (Fig. 3), where no fibrosis = 0; small foci present = 1; medium foci present = 2; and large foci present and total affected area ≥50% of the total tissue cross-section = 3.

Statistical analysis

Pearson's linear correlation coefficient r was calculated to test for associations between respiratory changes after irradiation with vascular remodeling and inflammation. To evaluate dose- or volume-dependencies, a multivariate logistic regression analysis was performed in Matlab using the GLMFIT algorithm (http://www.mathworks.com/products/matlab/). A P value of $<.05$ was considered significant.

Results

Relationship of vascular remodeling and inflammation with respiratory rate

To investigate early and late lung function changes, we assessed respiratory rate up to 26 weeks after lung irradiation (Fig. 2). In general we see a dose dependent biphasic increase in respiratory rate as reported before (5, 6).

Next, we assessed the level of known radiation-induced lung pathologies, vascular remodeling, pulmonary inflammation, and fibrosis (3, 6, 7, 9, 11-16) by scoring the histology of lung slides (Fig. 3). Finally, to investigate the role of these different pathologies in the development of late RILD, we correlated the level of early and late lung effects with respiratory rate changes.

In contrast to early vascular remodeling, which is closely related to respiratory rate (Fig. 4A) as reported earlier (7), no clear correlation was found for late vascular remodeling with respiratory rate (r=0.79; 95% confidence interval [CI]: 0.53-0.91; respectively. 0.53; 95% CI: 0.07-0.81) (Fig. 4B). Consequently, the role of vascular remodeling in late RILD seems less important than in early RILD.

Next, we assessed the relationship between early and late pulmonary inflammation and respiratory rate. Typically, fibrosis is used as a late marker of RILD (17). However, since in this study relatively low radiation doses were used not leading to early fibrosis, inflammation was assessed to compare pathologies underlying early and late RILD. This parameter is present in both phases. Contrary to early RILD, where vascular remodeling and inflammation correlated strongly with respiratory rate

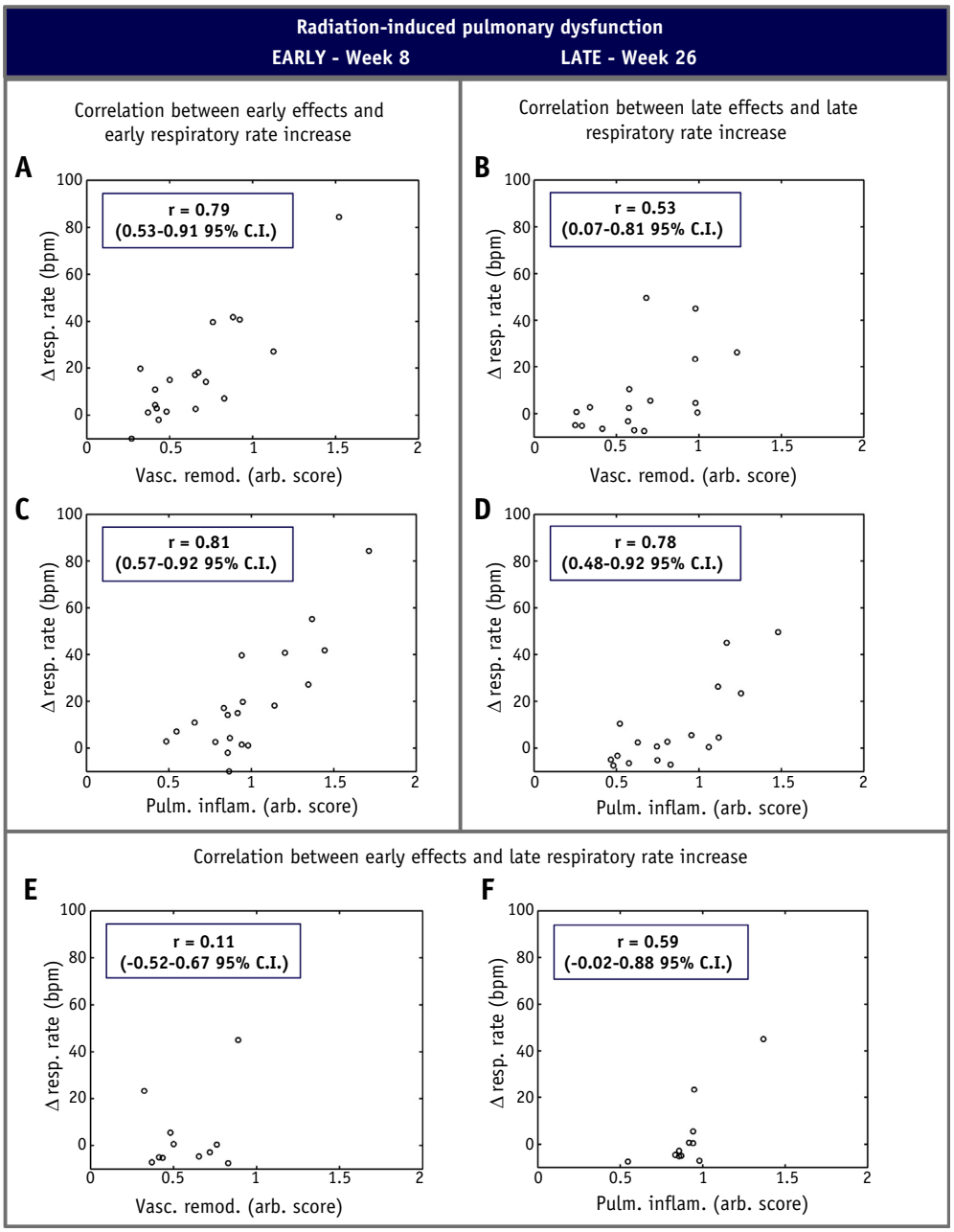


Fig. 4. Correlation of respiratory rate changes with vascular remodeling and pulmonary inflammation. Correlation between macrovascular remodeling and respiratory rate early (A) and late (B) after irradiation. Correlation of inflammation and respiratory rate early (C) and late (D) after irradiation. Correlation between early macrovascular remodeling and late respiratory rate (E) and of early inflammation and late respiratory rate (F). Pearson's linear coefficient r and 95% confidence intervals are shown in the graphs.

(respiratory r=0.79 [95% CI: 0.53-0.91] and r=0.81 [95% CI: 0.57-0.92]) (Fig. 4A and 4C), late after irradiation a similarly strong correlation was observed only for inflammation (r=0.78 [95% CI: 0.48-0.92]) (Fig. 4D). To assess the possible influence of early vascular remodeling and early inflammation on late RILD, we correlated early effects with late respiratory rate increases. Figure 4E shows that there is no correlation between early vascular remodeling and late respiratory rate increase (r=0.11 [95% CI: −0.52 to 0.67]). No correlation was found between early inflammation and late respiratory increase either (r=0.59 [95% CI: −0.02 to 0.88]) (Fig. 4F). Therefore, vascular remodeling and inflammation play a role in the development of early RILD, whereas the role of vascular remodeling seems to be reduced in late RILD. Furthermore, neither early vascular remodeling nor early inflammation seems to influence late respiratory difficulties.

Dose-volume dependency of vascular remodeling, inflammation and fibrosis

Next, we investigated the dose-volume dependency of late RILD and compared it with early effects. Figure 5A shows the dose-response curve of the score of vascular remodeling after irradiation of 50% as an example. Consistent with early RILD (7, 8), at 26 weeks after irradiation vascular remodeling was already observed at low doses (12 Gy) (Fig. 5A), albeit at a low level. Similar to early vascular remodeling, the out-of-field effects were virtually as severe as the in-field effects (Fig. 5A). However, in contrast to early vascular remodeling (7, 8), which was both dose- and volume-dependent (Fig. 5G), late vascular remodeling was associated only with dose- (Fig. 5A and 5G) and not with volume (Fig. 5B and 5G).

Next, we investigated the dose-volume dependency of late inflammation and fibrosis. Figure 5C shows the dose-response curve of the score of inflammation after irradiation of 50% lung as an example. Consistent with early inflammation, a dose-dependent increase in the in- and out-of-field number of inflammatory cells was observed at 26 weeks (Fig. 5G). This phenomenon was observed for all irradiated volumes, but with increasing volumes, the number of out-of-field inflammatory cells became more similar to the in-field score (Fig. 5D). Early after irradiation, the number of inflammatory cells increased with irradiated volume at a fixed dose level (8) (Fig. 5G). This volume-dependency was not observed at 26 weeks (Fig. 5D and 5G). The dependence on dose and irradiated volume of late inflammation and fibrosis are similar (Fig. 5C-F). Comparable to inflammation, no volume-dependency was shown for the level of fibrosis late after irradiation (Fig. 5F and 5G).

Thus, the development of late RILD in our model is mainly dependent on radiation dose whereas early RILD is dependent on irradiated dose as well as volume.

Irradiated volume determines which toxicity is dose limiting

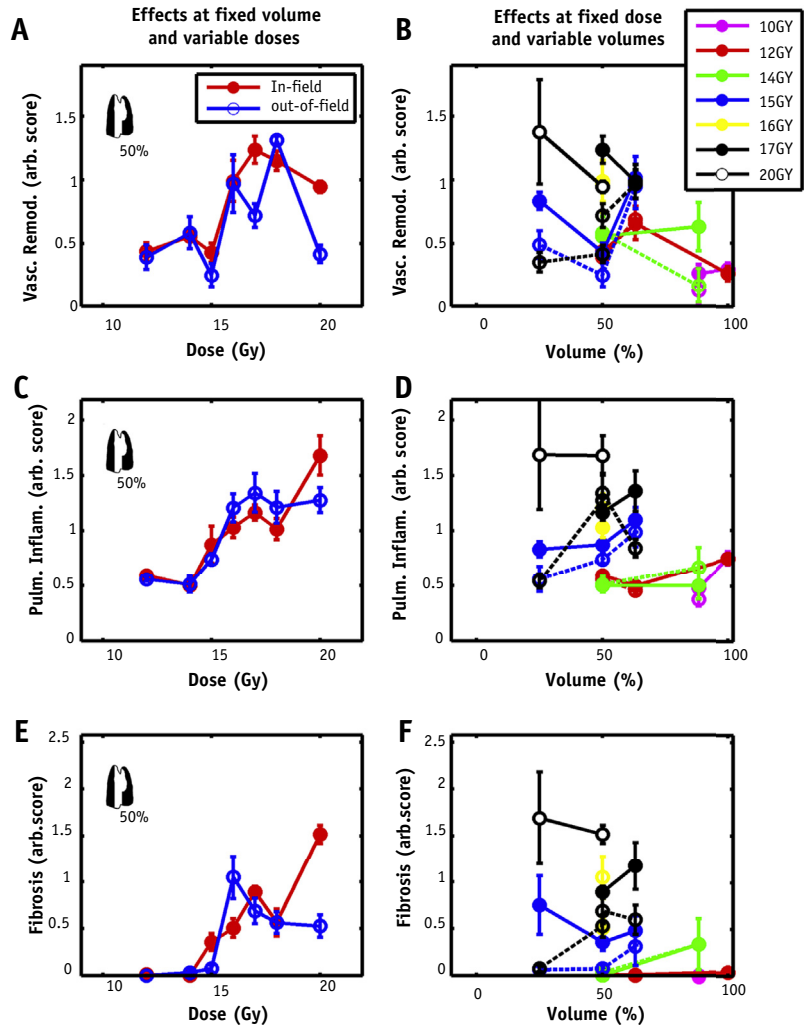
So far we have shown that early RILD is associated with vascular remodeling and inflammation in a dose- as well as a volume-dependent manner. Late RILD on the other hand was mainly associated with inflammation and fibrosis in a dose-dependent manner. As such, this model shows that different prediction models may be required for early and late RILD.

To investigate whether dose-limiting toxicity varies with irradiated volume, we assessed early and late respiratory rate after irradiation for various volumes (Fig. 6A and 6B). These early and late respiratory changes were compared in Figure 6C to assess which toxicity would be more severe and therefore dose-limiting. With decreasing irradiated volumes the dose-limiting toxicity changed from early RILD towards late. At higher irradiated volumes (100%, 88%) early function loss was dose-limiting due to its dependence on irradiated volume-dependent vascular remodeling (Figs. 3D and 5G) (7), although no significant late RILD was observed. With decreasing irradiated volume, the tolerance dose for early RILD increased, whereas dose-dependent late inflammation and fibrosis increased (Fig. 5C-5G), leading to late RILD (eg, 63%, 50%, respectively). At an irradiated volume of 32% the tolerance dose for early RILD even exceeded that for late RILD. Therefore, respecting dose-volume limits for early RILD might not always prevent late RILD.

Discussion

In our rat model, we show that depending on time after lung irradiation, different pathologies determine functional outcome. In addition, we observed that these pathologies differ in their dependence on irradiated dose and volume. Late RILD was associated with inflammation and fibrosis, mainly depending on dose. In contrast, early RILD was associated with vascular remodeling besides inflammation and mainly depended on irradiated volume. These observations were described before by us and others (3, 5-8, 11-13). Interestingly, we observed a new phenomenon. We found that the dose-limiting toxicity can change depending on irradiated volume. With decreasing irradiated volume the dose-limiting toxicity changed from early to late RILD. This finding may be very relevant in an era of technical developments in radiation therapy leading to smaller irradiated volumes of normal tissue.

Radiation-induced lung pathologies, such as vascular damage, inflammation, and fibrosis, assessed in this study have been recognized previously in animals as well as in patients (3, 6-8, 11, 13-16). However, the impact on RILD and exact dose-volume relationships of these different pathologies have not been investigated before. Besides, so far, studies aimed at investigating the dose-volume relationship of the development of "radiation pneumonitis" and not "late fibrosis" (4, 18). As reported in our previous studies (6-8), we showed that in addition to early inflammation and early fibrosis, vascular remodeling may play an important role in the development of early RILD. Late RILD on the other hand was associated with inflammation and fibrosis whereas the role of vascular remodeling seemed to be reduced. This was supported by the finding that the pulmonary pressure and right ventricle hypertrophy did not further increase and even decreased at 26 weeks after irradiation (Fig. E1; available online at www.redjournal.com). This might indicate that vascular remodeling in our model stabilizes, is repaired or compensated for over time, while inflammation and fibrosis remain or even further progress. As such, early and late RILD have different dose-volume dependencies due to the different underlying pathologies. Therefore, different prediction models may be required.



G Dose-volume dependencies assessed by multivariate logistic regression analysis:

Time	Variable	Dose		Volume	
		β-Coefficient*	p-Value	β-Coefficient#	p-Value
EARLY - Week 8	Vasular remodeling	0,03	0,01	0,76	< 0,001
	Pulmonary inflammation	0,06	< 0,00001	0,55	< 0,01
	Early Fibrosis	0,02	< 0,0001	< 0,001	0,57
LATE - Week 26	Vascular remodeling	0,04	< 0,01	0,03	0,90
	Pulmonary inflammation	0,06	< 0,0001	0,16	0,48
	Late Fibrosis	0,08	< 0,00001	< 0,001	0,74

* Increase in histopathology score per Gy
\# Increase in histopathology score per % irradiated volume

Fig. 5. Quantification of the level of late vascular remodeling, pulmonary inflammation, and fibrosis after irradiation of rat lung. Relationships are shown between (A) the level of macrovascular remodeling and dose after 50% lung irradiation; (B) level of macrovascular remodeling and irradiated volume at fixed dose levels; (C) number of inflammatory cells and dose after 50% lung irradiation; (D) number of inflammatory cells and irradiated volume at fixed dose levels; (E) dose and level of fibrosis after 50% lung irradiation; and (F) level of fibrosis and irradiated volume at fixed dose levels. (G) Table shows the β coefficients and *P* values of multiple linear regression analysis predicting dose and volume dependencies of vascular remodeling and inflammation and fibrosis early and late after irradiation. (B, D, F) Solid lines indicate in-field levels of lung damage; dotted lines indicate out-of-field damage. (N = 3-7 per dose-volume group.)

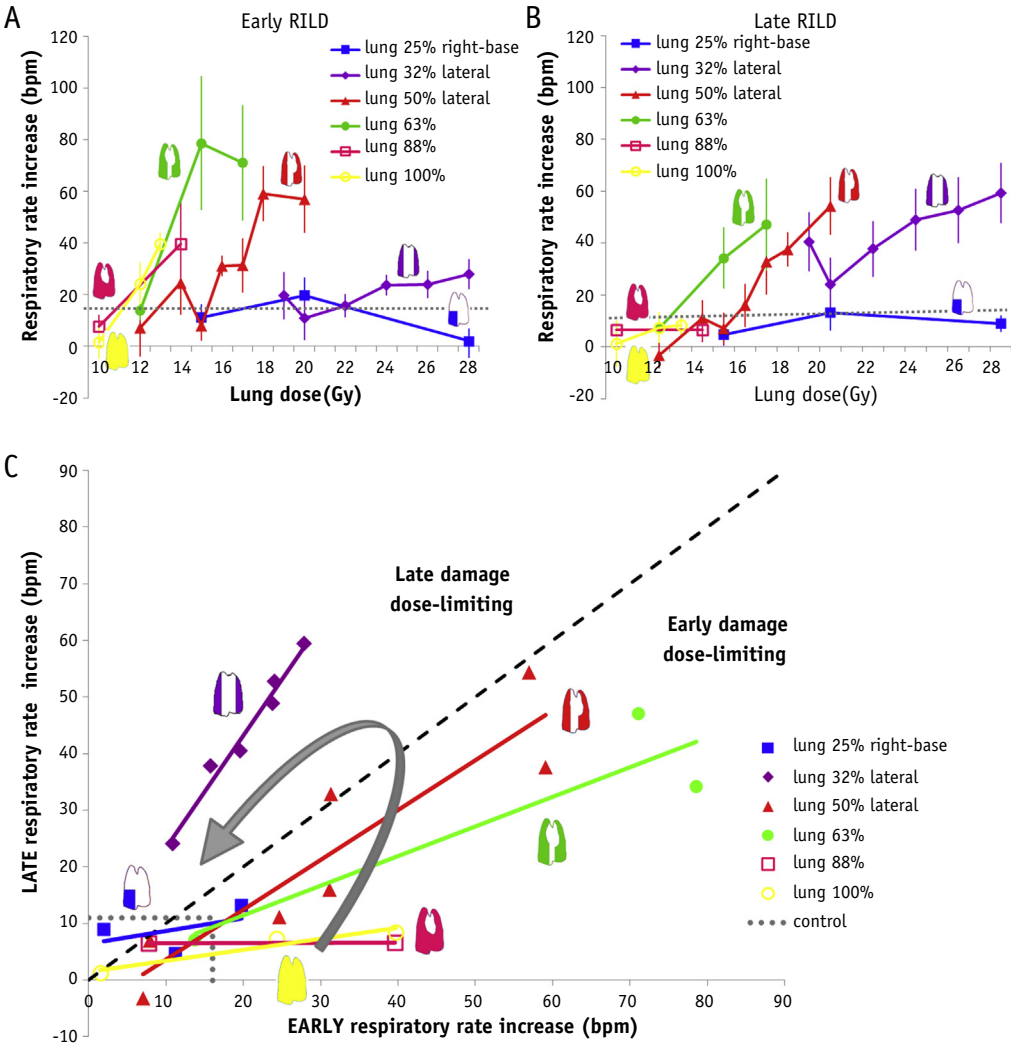


Fig. 6. Variations in the increase in respiratory rate as a function of dose for various irradiated lung volumes. Respiratory rate increases early (A) and late (B) after irradiation of different dose distributions. (C) Comparison of the panel A and B depicted respiratory rate increases early and late after irradiation. The arrow indicates that with decreasing irradiated volumes, the dose-limiting toxicity changes from early RILD toward late. The gray dotted lines in all 3 panels indicate respiratory rate increase of unirradiated animals (early phase: 19 bpm, late phase: 11 bpm). Error bars indicate the standard error of the mean. N = 3-7 per dose-volume group. *Abbreviations:* bpm = beats per minute; RILD = radiation-induced lung dysfunction.

Interestingly, we found that late RILD is not always preceded by early dysfunction. Irradiating small rat lung volumes (25%) up to a single dose of at least 28 Gy does not lead to functional changes either early or late after irradiation. However, irradiating a somewhat larger volume, 32%, leads to severe late RILD with, in contrast, a minor loss of function early after irradiation. Larger irradiated volumes (50%-63%) lead to both early and late RILD with more severe dysfunction in the early than in the late phase. Irradiated volumes of 88% and 100% showed early RILD but no late RILD. However, the doses of these irradiated lung volumes could not exceed 14 Gy, whereas the threshold dose of inflammation/fibrosis may be somewhere in the range of 14 to 15 Gy. As such, early and late RILD is limited by different dose-volume constraints. Consistent with our findings, Fröhlich et al (19) reported the presentation of symptomatic fibrosis without preceding pneumonitis in patients. Thus, late symptomatic RILD is not always a consequence of the clinical occurrence of early RILD. As such, prediction models of early RILD might not necessarily predict late RILD.

In patients also different pathologies underlie early and late RILD (3, 15). Therefore, different prediction models might also be required for early and late RILD in patients. Clinically, however, this is only relevant if respecting the dose-volume limitation of early RILD does not also prevent late RILD. Because this study shows that late RILD is not always proceeded by early RILD, late prediction models may be relevant. Unlike our rat model, in patients, for example, genetic differences and pre-existing pulmonary disease exist which may influence the development of the different pathologies (2) and consequently the development

of RILD (2). As such, to establish these models clinical studies should be performed including the aforementioned factors.

Development of new models predicting early as well as late RILD may be of importance since new advances in therapies, like stereotactic radiation technique (20-22), irradiation with protons (23) or specific molecular targeted therapies (24) lead to a longer life expectancy of cancer patients. Moreover, new radiation techniques lead to a reduced irradiated volume of normal tissue. This preclinical study showed that the dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. This could be explained by different pathologies underlying early and late RILD with different dose-volume dependencies. In patients, also different pathologies underlie early and late RILD. As such, to optimize radiation therapy treatment, models predicting both early and late toxicity may have to be used. To establish these models clinical modeling studies should be performed.

Conclusions

In contrast to early RILD in rats, late RILD predominantly depends on dose and is associated with inflammation and fibrosis, rather than irradiated volume and vascular remodeling. Consequently, dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. In patients, early and late RILD are also due to different pathologies. As such, new radiation techniques reducing irradiated volume might change the dose-limiting toxicity of the radiation therapy treatment.

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