VOLUME-DEPENDENT EXPRESSION OF IN-FIELD AND OUT-OF-FIELD EFFECTS IN THE PROTON-IRRADIATED RAT LUNG

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Purpose: To investigate whether occurrence of early radiation effects in lung tissue depends on local dose only.

Methods and Materials: Twenty-five percent, 50%, 66%, 88%, or 100% of the rat lung was irradiated using single fractions of 150-MeV protons. For all volumes, in-field and out-of-field dose–response curves were obtained 8 weeks after irradiation. The pathohistology of parenchymal inflammation, infiltrates, fibrosis, and vascular damage and the relative expression of proinflammatory cytokines interleukin (IL)-1α, transforming growth factor-β, IL-6, and tumor necrosis factor-α were assessed.

Results: For all histologic endpoints, irradiated dose- and volume-dependent in-field and out-of-field effects were observed, albeit with different dynamics. Of note, the out-of-field effects for vascular damage were very similar to the in-field effects. Interestingly, only IL-6 showed a clear dose-dependent increase in expression both in-field and out-of-field, whereas the expression levels of IL-1α, transforming growth factor-β, and tumor necrosis factor-α were either very low or without a clear dose–volume relation. As such, none of the radiation effects studied depended only on local dose to the tissue.

Conclusion: The effects of radiation to lung tissue do not only depend on local dose to that tissue. Especially at high-volume irradiation, lung damage seems to present globally rather than locally. The accuracy of predictive modeling may be improved by including nonlocal effects. © 2011 Elsevier Inc.

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INTRODUCTION

Local progression after definitive (chemo-)radiation remains an important cause of treatment failure in inoperable non–small-cell lung cancer (1). The generally prescribed radiation dose ranging from 60 to 70 Gy is insufficient to achieve high rates of local tumor control (2). The dose required for achieving a significant probability of durable tumor control is estimated to be approximately 84 Gy (3). Therefore, tumor dose escalation is expected to result in higher cure rates (4). However, to date the radiosensitivity of normal tissues lying in the radiation field and the subsequent risk for severe complications, such as radiation pneumonitis, have frustrated attempts for dose escalation (5). Tumor dose escalation while preventing toxicity risks requires clinically applicable routine strategies that enable precise treatment tailoring. Currently used models to estimate the risk of lung toxicity are based on the mean lung dose or volumes irradiated to certain dose levels, such as the V20 (i.e., the percentage of the volume of the lung receiving 20 Gy or more) (6, 7). However, their predictive power is insufficient for clinical decision making (8, 9). In the recent QUANTEC review of current knowledge on radiation-induced dose–volume effects in the lungs, only V20 and mean lung dose are described, while acknowledging the many caveats in the reviewed literature (10). Improvement of prediction has been achieved by using additional information on the preradiotherapy overall lung function (8), inhomogeneous lung perfusion (8, 11), upper or lower lung tumor location (12, 13), and cytokine expression (14). However,
because of the limited variability in treatment plans, analysis of clinical data does not necessarily yield the parameter that best describes/predicts the biological process underlying the toxicity. Optimization of new treatment modalities, such as particle therapy, that allow new shapes of dose distributions using predictors derived exclusively from clinical data obtained with existing treatment techniques (e.g. three-dimensional and intensity modulated radiotherapy), may not lead to a full exploitation of the potential benefits.

By performing preclinical studies, more insight can be obtained in the mechanistic processes that cause the biological and clinical effects and the dosimetric predictors controlling them. Moreover, the effect of changing these predictors can be tested in a better-controlled manner and over a wider range than in clinical studies. Such studies have been performed in our laboratory and have shown dose–volume effects in the rat lung (15–18). Moreover, it was shown that concomitant irradiation of the heart severely reduces the radiation tolerance of the lung (19, 20) and that inclusion of this phenomenon in the critical-volume model was essential to be able to accurately predict the incidence of symptomatic radiation-induced loss of lung function (SRILF) (21). On the basis of this work, Huang et al. (22) recently showed that also in patients the most important variable predictive of radiation pneumonitis is heart related, and combining it with lung dose parameters in a multivariate logistic regression model results in a more accurate prediction than achieved with presently used models that are based on lung dose only. This demonstrates that predictors derived from preclinical studies may yield an alternative model that is more accurate than classic models exclusively derived from clinical data.

The impact of lung dose and other factors, such as spatial heterogeneity of the response of the lung (23–25), indicates that damage may not be best described by local dose to the lung. In the lung, two distinct forms of postirradiation morphologic damage have been shown: inflammatory vascular damage and parenchymal injury (16). Vascular damage already occurs at low doses but requires irradiation of large volumes to result in SRILF, whereas parenchymal damage occurs after irradiation of smaller volumes to higher doses, with minimal consequences for lung function. This indicates that “a lot to a little” is tolerated better than “a little to a lot” (26). Using a range of volumes and controlling for coirradiation of the heart, the relation between different mechanistic processes and dose–volume parameters may be dissected.

Therefore, in the present study we aimed to investigate to what extent a dose deposited in a subvolume of the lung determines lung toxicity inside as well as outside the irradiated volume at the time point when the peak in lung function loss occurs (20).

**METHODS AND MATERIALS**

**Animals**

Adult male albino Wistar rats of the Hsd/Cpb:WU strain weighing 300 ± 10 g at the start of the experiment were housed five to a cage under a 12-h light/12-h 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 procedure**

The rats were anesthetized with an i.p. injection of xylazine (Rompun; Bayer, Leverkusen, Germany) plus S-ketamine (Ketalar; Pfizer, Capelle aan de IJssel, The Netherlands) and placed in a holder hanging on a positioning rod by their upper incisors (19) for CT scanning or irradiation. The use of 150-MeV protons in a fixed beam line facilitated the irradiation of subvolumes of the lung with sharply demarcated (20–80% penumbra of approximately 1 mm) radiation fields (27, 28). Radiation portals were designed using planning CT images of 5 age-matched rats, as described previously (19, 20). Using protons, 100% (10–13 Gy), 88% (10–15 Gy), 63% (10–17 Gy), 50% (10–22 Gy), or 25% (12–28 Gy) of the lung was irradiated with a single fraction. The dose distributions were chosen such that the heart was either spared or irradiated only to a dose (<14 Gy) well below the threshold dose of 18 Gy where it starts influencing loss of pulmonary function (21). This ensured that the observed irradiation-induced injuries could be attributed to dose to the lung only. Control animals were anaesthetized and sham irradiated.

**Figure.** 1 gives an overview of irradiated volumes, the shape of the openings of the collimators used to achieve this, and the dose range used for each volume.

**Histologic examinations**

Histologic examination was performed 8 weeks after radiation at the peak in lung function loss (20). The numbers of animals used per dose/volume group are depicted in Table 1. Lung tissue samples were taken from both inside and outside the radiation field, with sufficient margins to ensure that the tissue was either irradiated (“in field”; i.e., tissue receives more than 97% of the prescribed dose) or shielded (“out-of-field”; tissue receives less than 7% of the prescribed dose) (27, 28). Details of the procedure and scoring have been published previously (16, 20).
Sections of 3 μm containing standardized samples of irradiated or nonirradiated lung tissue were stained with hematoxylin and eosin and examined by light microscopy. In the entire tissue cross-section on each slide, blinded scoring of vascular changes and parenchymal changes was carried out separately using four semiquantitative scoring scales independently by two observers.

Parenchymal inflammation. Parenchymal inflammation was scored 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 (Fig. 2A); only a few inflammatory cells in the lung = Score 1 (Fig. 2B and C); many nonclustered inflammatory cells present (200× magnification) = Score 2 (Fig. 2D); and large amounts of clustered inflammatory cells present (100× magnification) and total affected area volume of 50% or more of the total tissue cross-section = Score 3 (Fig. 2E).

Infiltrates. Clusters of inflammatory cells mostly closely connected to blood vessels were scored separately as focal lesions on each slide: no foci present = Score 0 (Fig. 2A); small to medium foci present (200× magnification field) = Score 1 (Fig. 2B and C); and large foci present (100× magnification field) and total affected area volume 50% of the total tissue cross-section = Score 2 (Fig. 2D and E).

Vascular damage. Mostly vascular hypertrophy, meaning that the smooth-muscle cells from the media layer are thickened, was scored as 0–2. No affected vessels = Score 0 (Fig. 2A); hypertrophic vascular walls (200× magnification field) = Score 1 (Fig. 2B and C); and heavily affected vessels, meaning smooth-muscle cells of the media layer are thickened and around the arterioles edema or fibrosis can be seen = Score 2 (Fig. 2E).

Early fibrosis. Early fibrosis was scored 0–2. No fibrosis = Score 0 (Fig. 2A); thickening of lung parenchyma, alveolar structures still visible = Score 1 (Fig. 2D); and complete breakdown of lung parenchyma, a clod of fibrotic tissue replaces alveolar tissue = Score 2 (Fig. 2F).

Reverse transcription–polymerase chain reaction analysis

For quantitative polymerase chain reaction (qPCR) analysis, a separate set of experiments (50%: 13, 17, and 20 Gy; 63%; 13 and 17 Gy; and 100%: 13 Gy) were performed, of which rat lungs (n = 3 per group) were removed 8 weeks after irradiation and divided in irradiated and shielded parts as described above. Messenger RNA was isolated from cubic tissue sections (snap-frozen in liquid nitrogen) of no more than 1 mm³ each using the RNeasy Plus Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s protocol. Complementary DNA was synthesized from the messenger RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Oligo-(dt) 12–18 primers were used for generating first-strand complementary DNA in a final reaction mix of 20 μL. For qPCR, Absolute qPCR ROX Mix from Thermo Scientific Rockford, IL was used as master mix. Expression levels of interleukin (IL)-1α, IL–6, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β were quantified using Taqman Gene expression assays from Applied Biosystems (Foster City, CA) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Beta-actin levels were used for normalization because its expression levels were not altered by radiation, as examined by measuring the difference in expression levels after irradiation of three housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, β-2-microglobulin, and β-actin) in random test samples of nonirradiated and irradiated rat lungs.

Statistical analysis

Dose or volume dependence of responses was tested by linear regression analysis. Dose or volume dependence was considered significant if the regression coefficient significantly differed from zero. The nominal level of statistical significance was 5%.

RESULTS

First the level of inflammation (Fig. 2) in the lungs was assessed 8 weeks after partial and 100% lung volume irradiation. Fig. 3A shows the dose–response curve of the score of parenchymal inflammation after irradiation of 50% lung as an example. As expected, a clear dose-dependent (p < 0.001) increase in the in-field number of parenchymal inflammatory cells was observed. Interestingly, the number of inflammatory cells in the parenchyma also clearly increased nonlocally out of field, and this increase was dependent on the delivered in-field dose (p < 0.001; Fig. 3A). 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 (identical at 13 Gy; Fig. 3B and Fig. 4). Moreover, at a fixed dose level the number of inflammatory cells increased with irradiated volume (p < 0.01 for in field and out of field after 15 Gy irradiation and in field after 13 Gy irradiation).

Similar effects were seen for infiltrates and fibrosis, albeit with higher in-field threshold doses of approximately 12 Gy and approximately 14 Gy, respectively, for 100% lung volume irradiation (all data are summarized in Fig. 4).

Vascular damage developed rather independently from the parenchymal inflammatory response. At irradiated lung volumes exceeding 50%, vascular hypertrophy was already observed at 10 Gy (Fig. 3C), albeit at a low level. Interestingly, the out-of-field effects were virtually as severe as the in-field effects (Fig. 3D and Fig. 4). Similar to parenchymal inflammation, vascular damage also occurred in an irradiated volume–dependent manner both within and outside the radiation field (p < 0.01).
These results indicate that even in nonirradiated areas, depending on the irradiated volume, severe inflammatory and vascular changes can be induced.

**Cytokines**

To assess whether the inflammatory and vascular changes were dose/volume-dependently associated with cytokines known to be involved in radiation-induced lung damage (24, 29, 30), we assessed the expression of IL-1α, TGF-β, IL-6, and TNF-α in irradiated and shielded parts of the lung 8 weeks after irradiation. As expected on the basis of these previous published works, increases in the relative expression of all the cytokines were observed after irradiation (Fig. 5). However, the expression of IL-1α never increased more than twofold (Fig. 5A and B), making its biological relevance in our experiment questionable. Although TGF-β substantially increased, no clear dose- or volume-dependent response could be observed (Fig. 5C and D). Both in field and out of field, IL-6 did show a clear dose-dependent ($p < 0.05$) increase after 50% lung volume irradiation (Fig. 5E), albeit without a clear volume dependency (Fig. 5F). Interestingly, the reverse seems true for the relative expression of TNF-α, which was always enhanced independent of dose (Fig. 5G) or irradiated volume.

Fig. 2. Quantification of lung morphology 8 weeks after irradiation. (A) Normal lung, sporadic inflammatory cells, thin vascular wall (see Inset). Score 0 for parenchymal inflammation, infiltrates, vascular damage, and fibrosis. (B, C) Small or medium infiltrates present (*), moderate increase inflammatory cells, normal septa, hypertrophic vascular walls (see Inset in B). Score 1 for parenchymal inflammation, infiltrates, and vascular damage and Score 0 for fibrosis. (D) Medium-size foci, a lot of nonclustered inflammatory cells, thickened septa (arrowheads), hypertrophic vascular walls. Score 1 for vascular damage and fibrosis, Score 2 for parenchymal inflammation and infiltrates. (E) Large foci of inflammatory cells, edema (open arrowheads), thickened septa, degradation alveoli, extreme hypertrophic vascular wall (large arrow), perivascular infiltrates. Score 3 for parenchymal inflammation, Score 2 for infiltrates and vascular damage, Score 1 for fibrosis. (F) Big clod of fibrotic tissue (star), surrounded by normal-looking lung parenchyma. Score 2 for fibrosis. Scale bars: 200 μm.
The out-of-field relative expression of cytokines mimicked the in-field expression, indicating that the expression of cytokines is not directly induced by the irradiation dose directly but rather by other related factors, such as the inflammatory cells.

**DISCUSSION**

To develop predictive models for the occurrence of normal tissue radiation damage with the highest possible accuracy, preclinical studies are needed to specifically test potentially relevant variables that can be modulated in current and anticipated clinical practice.

In the present study a clear volume effect was seen for all parameters investigated, except cytokine expression. Moreover, strong volume-dependent out-of-field effects were observed, indicating that radiation-induced lung damage manifests itself as a tissue systemic disease rather than as local disease due to local dose. With proton irradiation, scattered dose from the shield is too low to provoke a pathohistologic response (e.g., Fig. 2). Interestingly, the out-of-field effects were distinct for the different parameters. In contrast to the parenchymal inflammatory response, the vascular damage out-of-field effects were almost as severe as the in-field effects, indicating strong cross-talk between the irradiated and nonirradiated parts of the lung.

Dose–volume effects for the lung have been recognized for a long time in both animal and human studies (10, 31). Regional differences (18, 23), out-of-field effects (25), and interaction with other organs, such as the heart (19, 20), have demonstrated that the response of a lung to irradiation cannot simply be predicted from the irradiated volume and the local dose. In this study we confirmed previous observations (16) that parenchymal and vascular damage are separate phenomena occurring at different dose levels/irradiated volumes and therefore may have a different impact on clinical outcome with various irradiation techniques.

These two types of damage occur in the early phase of pulmonary radiation injury (32). Moreover, recent work showed that, indeed, vascular injury after whole-thorax irradiation had already occurred at relatively low doses (33, 34). In humans this could translate into reduction of lung perfusion, for which even regional differences have been observed (13, 35). Reduced perfusion may lead to oxidative stress, tissue hypoxia, and perpetuation of radiation injury (36). Reactive oxygen species (ROS) induced by radiation may damage the endothelium, inducing...
hypoxia, which again results in a further formation of ROS (36). This is, however, contradicted by the observation that vascular damage can resolve after lower doses with little or no remaining damage (16, 34). This suggests that vascular damage precedes parenchymal damage and possibly plays a primary role in the development of SRILF, as suggested as early as 1968 by Rubin and Casarett (37). Interestingly, vascular hypertrophy was also found outside the radiation field to virtually the same level as inside the radiation field. This points to a more systemic effect in the lung, which is not likely induced by short-lived ROS. Especially for the out-of-field effect, the induction of vascular damage differs from parenchymal inflammation because it is more severe and closely follows the in-field damage. Further knowledge on the mechanism may yield opportunities for the development of improved normal tissue complication probability (NTCP) models and possibilities to intervene.

Inflammatory cytokines have been implicated in the development of radiation pneumonitis (1, 29, 30) and may be involved in the further regulation of ROS (24, 38, 39). Also in our study, changes in the expression of IL-1α, TGF-β, IL-6, and TNFα, which are considered the most relevant cytokines induced after irradiation in the lung (24), were observed. However, except for IL-6 we did not observe any
significant dose–volume relation for the expression of these cytokines. Although TNF-α expression increased, no significant correlation with dose or volume could be detected. Expression of these cytokines has the tendency to fluctuate in time (24, 29, 30), so we may have missed the peak expression levels by probing at one specific point in time only. For IL-6 a dose–effect relationship of potential interest was found. Interleukin-6, a pleiotropic cytokine that plays an important role in the immune response and inflammation, is produced by a variety of cells, including monocytes, macrophages, and endothelial cells (40). However, injection with monoclonal IL-6 receptor antibodies could not reduce radiation-induced lung injury in mice (41). These results indicate that single-parameter cytokine expression is not a good read-out of normal tissue complications. Interestingly, out-of-field IL-1α, TGF-β, and TNF-α were modulated in a similar manner to parallel the in-field response, albeit to a bit lower level. Indirect effects of the inflammatory response has been suggested to trigger the generation of ROS inducing out-of-field DNA damage in rats (42). Further investigation of this effect should shed light on the mechanism of out-of-field effects in the lungs and whether it can be related to a specific type of damage, to use it as a read-out.

CONCLUSION

The effects of radiation to lung tissue do not only depend on local dose to that tissue but also generally depend on irradiated volume. This shows that radiation-induced lung damage should be considered as a systemic lung disease rather than as a phenomenon limited to the irradiated area. Inclusion of these nonlocal effects in NTCP models, which until now have been based on the assumption that the damage is limited to the irradiated volume, could improve the predictive power of these models.

REFERENCES


