Irradiation Induces a Biphasic Expression of Pro-Inflammatory Cytokines in the Lung

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Background and Purpose: The precise pathophysiological mechanisms of radiation-induced lung injury are poorly understood, but have been shown to correlate with dysregulation of different cytokines. The purpose of this study was to evaluate the time course of the pro-inflammatory cytokines tumor necrosis factor-(TNF-)α, interleukin-(IL-)1α and IL-6 after whole-lung irradiation.

Material and Methods: The thoraces of C57BL/6J mice were irradiated with 12 Gy. Treated and control mice were sacrificed at 0.5, 1, 3, 6, 12, 24, 48, 72 h, 1, 2, 4, 8, 16, and 24 weeks post irradiation (p.i.). Real-time multiplex RT-PCR (reverse transcriptase polmyerase chain reaction) was established to evaluate the expression of TNF-α, IL-1α and IL-6 in the lung tissue of the mice. For histological analysis, lung tissue sections were stained by hematoxylin and eosin.

Results: Multiplex RT-PCR analysis revealed a biphasic expression of these pro-inflammatory cytokines in the lung tissue after irradiation. After an initial increase at 1 h p.i. for TNF-α and at 6 h p.i. for IL-1α and IL-6, the mRNA expression of these pro-inflammatory cytokines returned to basal levels (48 h, 72 h, 1 week, 2 weeks p.i.). During the pneumonic phase, TNF-α, IL-1α and IL-6 were significantly elevated and revealed their maximum at 8 weeks p.i. Histopathologic evaluation of the lung sections obtained within 4 weeks p.i. revealed only minor lung damage in 5–30% of the lung tissue. By contrast, at 8, 16, and 24 weeks p.i., 70–90% of the lung tissue revealed histopathologically detectable organizing alveolitis.

Conclusion: Irradiation induces a biphasic expression of pro-inflammatory cytokines in the lung. The initial transitory cytokine response occurred within the first hours after lung irradiation with no detectable histopathologic alterations. The second, more persistent cytokine elevation coincided with the onset of histologically discernible organizing acute pneumonitis.

Key Words: Pneumonitis · Pro-inflammatory cytokines · Lung · Biphasic cytokine expression

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Biphasische Expression proinflammatorischer Zytokine im Rahmen der radiogenen Lungenreaktion

Hintergrund und Ziel: Die genaue Pathophysiologie der strahleninduzierten Lungenschädigung ist bislang nur unvollständig geklärt, scheint aber mit einer Dysregulation verschiedener Zytokine assoziiert zu sein. Das Ziel dieser experimentellen Studie war es, den zeitlichen Expressionsverlauf der proinflammatorischen Zytokine Tumor-Nekrose-Faktor-(TNF-)α, Interleukin-(IL-)1α und IL-6 nach Lungenbestrahlung zu untersuchen.

Material und Methodik: Bei C57BL/6J-Mäusen wurde eine Ganzlungenbestrahlung mit 12 Gy durchgeführt. Die Versuchstiere und unbestrahlte Kontrolltiere wurden zu unterschiedlichen Zeitpunkten (0,5, 1, 3, 6, 12, 24, 48, 72 h bzw. 1, 2, 4, 8, 16 und 24 Wochen) nach Bestrahlung getötet. Im Lungengewebe wurde die mRNA-Expression von TNF-α, IL-1α und IL-6 mit Hilfe der Real-Time-multiplex-RT-PCR (Reverse-Transkriptase-Polymerase-Kettenreaktion) quantifiziert. Für die histologische Beurteilung der Lungenpräparate wurde eine Hämatoxylin-Eosin-Färbung durchgeführt.

Ergebnisse: Im Rahmen der strahleninduzierten Lungenschädigung konnte mit Hilfe der Multiplex-RT-PCR eine biphasische Expression dieser proinflammatorischen Zytokine nachgewiesen werden. Nach einem initialen Anstieg – bereits 1 h nach Bestrahlung für TNF-α und nach 6 h für IL-1α und IL-6 – ging die Zytokinexpression auf Ausgangswerte zurück. Während der Pneumonitisphase war die mRNA-Expression von TNF-α, IL-1α und IL-6 signifikant erhöht und ereichte 8 Wochen nach Bestrahlung ihr Maximum. Während sich bis zu 4 Wochen nach Bestrahlung histopathologisch nur eine geringe Lungenschädigung in 5–30% des Gesamtlungengewebes beobachten ließ, waren nach 8, 16 bzw. 24 Wochen 70–90% des Gesamtlungengewebes im Sinne einer radiogenen Pneumonitis geschädigt.

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Schlussfolgerung: Im Rahmen der radiogenen Lungenreaktion konnte eine biphasische Expression der proinflammatorischen Zytokine nachgewiesen werden. Die initiale Zytokinerhöhung erfolgte in den ersten Stunden nach Lungenbestrahlung, ohne dass histopathologisch eine Lungenschädigung nachweisbar war. Die zweite, länger persistierende Zytokinerhöhung (einige Wochen nach Bestrahlung) korrelierte mit dem Beginn einer histologisch nachweisbaren radiogenen Pneumonitis.

Schlüsselwörter: Pneumonitis · Proinflammatorische Zytokine · Lunge · Biphasische Zytokinexpression

Introduction

The radiosensitivity of the lung limits the dose of radiation which can be delivered to tumors in the thoracic region. Moreover, radiation pneumonitis remains a major toxicity of total-body irradiation used in the preparation of patients for bone marrow transplantation. Radiation-induced lung damage may arise depending on the total dose of irradiation, the fractionation schedule, the volume of lung tissue irradiated, the existence of prior lung disease, and the use of chemotherapeutic drugs in the treatment of the disease [14, 15, 17, 25]. The pathophysiological tissue response after lung irradiation can be separated into an *early phase of acute pneumonitis* and a *later phase of fibrosis*. Acute pneumonitis is characterized by an enhanced vascular permeability with edema of the interstitium and exudation into air spaces and an influx and selective accumulation of different inflammatory and immune cells from the peripheral blood at the site of injury. A number of cytokines, known collectively as pro-inflammatory cytokines because they accelerate inflammation, regulate these inflammatory reactions and are released predominantly by recruited and resident inflammatory cells as well as pulmonary epithelial cells upon activation.

In the present study, we investigated the kinetics of radiation-induced upregulation of the pro-inflammatory cytokines tumor necrosis factor-(TNF-)α, interleukin-(IL-)1α and IL-6 in whole-lung-irradiated C57BL/6J mice. These pro-inflammatory cytokines are probably key mediators for the pathogenesis of radiation pneumonitis, because they show the following spectrum of biological activities: TNF-α enhances phagocytosis and cytotoxicity in neutrophilic granulocytes and modulates the expression of other cytokines including IL-1 and IL-6. TNF-α and IL-1 are strong chemoattractants for leukocytes and they also increase their adherence to the endothelium by enhancing the expression of molecules such as intercellular adhesion molecule-(ICAM-)1 and endothelial leukocyte adhesion molecule (ELAM) [9]. IL-1 and IL-6 influence antigen-specific immune responses by an activation of T-cells (induction of the differentiation of immature T-cells into cytotoxic T-cells, stimulation of T-helper cells) and B-cells (induction of the final maturation of B-cells into immunglobulin-secreting plasma cells and stimulation of the secretion of antibodies) [5]. In vivo, TNF-α and IL-1 are responsible for many alterations of the endothelium inhibiting anticoagulatory mechanisms and promoting thrombotic processes [13]. IL-1 is an endogenous pyrogen and induces a significant elevation of body temperature by causing the release of prostaglandins in the thermoregulatory center of the hypothalamus. IL-1 and IL-6 stimulate the synthesis of ACTH (corticotropin) in the pituitary. Glucocorticoids synthesized in response to ACTH inhibit the production of TNF-α, IL-1 and IL-6 in vivo, thus establishing a sort of negative feedback loop between the immune system and neuroendocrine functions [18].

There are two functionally almost equivalent forms of IL-1, IL-1α and IL-1β, that are encoded by two different genes but bind to the same receptor and therefore show similar if not identical biological activities. In the present study, we evaluated IL-1α, because it is the predominant form in mice, while IL-1β predominates in humans [8].

For the quantification of these pro-inflammatory cytokines we established and optimized a real-time multiplex RT-PCR (reverse transcriptase polymerase chain reaction). In the present paper, we describe the principle of the method employed and the validation of this technique for those pro-inflammatory cytokines playing a role in radiation-induced pneumonitis.

Material and Methods

Animals

C57BL/6J mice were purchased from Charles River Laboratories. Adult female mice, 8 weeks old and approximately 20 g in weight, were housed four to six per cage and allowed to acclimatize from shipping for 1 week prior to treatment.

Radiation Schedule

A dose of 12 Gy to the midplane of the lungs was delivered in a single fraction via a posterior field using a linear accelerator. A plastic jig was used to restrain the mice without anesthesia, and lead strips were placed to shield the head and abdomen. The irradiation characteristics were as follows: beam energy, 10-MV photons; dose rate, 2.4 Gy/min; source–surface distance (SSD), 1 m; size of the radiation field, 18×10 cm. A film was taken to confirm that the entire lung was irradiated. Film dosimetry was used to deduce the relative dose distribution. Dosimetry was performed with a cylindrical ionization chamber. The depth of the maximum dose of the 10-MV photon beam was reduced by the tissue-equivalent plastic material in the restraining jig. 23 mm thickness displaces the build-up region of the 10-MV photon beam into the plastic material, thus achieving an acceptable dose uniformity throughout the thorax of individual mice.

Following irradiation the mice were maintained four to six per cage in laminar flow hoods in pathogen-free rooms to minimize pulmonary infections and supplied with standard laboratory diet and water ad libitum. Age-matched controls were maintained under identical conditions for the course of the experiment. Treated and control mice were sacrificed by cervical dislocation at 0.5, 1, 3, 6, 12, 24, 48, 72 h, 1, 2, 4, 8, 16, and 24 weeks post irradiation (p.i.). Three animals per assessment time point were used and untreated, sham-irradiated animals served as experimental controls. The experimental protocol was approved by the Medical Sciences Animal Care and Use Committee of the University of Münster, Germany.

Tissue Isolation

The complete lungs were removed immediately after death without being perfused. The left lobes were placed in fixative (4% neutral buffered formalin) for histological analysis and the right lobes were quickly frozen in liquid nitrogen for RNA isolation and subsequent PCR analyses. The lung tissue from three different mice for every time point was analyzed by a combination of light microscopy and PCR.

PCR Analysis

RNA Extraction. Total RNA preparations were performed using the RNeasy™ Total RNA Kit (Qiagen, Hilden, Germany). The lung tissue (a right lobe is equivalent to 75 mg tissue) was homogenized in 1.2 ml lysis buffer using a rotor-stator homogenizer (Ultra-Turrax, IKA, Staufen, Germany). Tissue lysates were kept at –85 °C prior to processing or were used directly in the procedure according to the manufacturer's protocol. RNA concentrations were determined by spectrophotometric absorption at 260 nm. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

First-Strand Complementary DNA (cDNA) Synthesis. Total RNA was converted to cDNA utilizing a first-strand cDNA synthesis kit according to the manufacturer's protocol (Pharmacia, Biotech, Freiburg, Germany). In this procedure, oligo dT primers were used for generating first-strand cDNA in a final reaction mix of 15 µl.

Real-Time Multiplex RT-PCR. The TNF-α, IL-1α and IL-6 mRNA expression in the lung tissue was quantified by real-time multiplex RT-PCR (ABI Prism 7700® sequence detection system). In this multiplex RT-PCR one fluorogenic probe labeled with VIC dye was used to detect the target amplicon (TNF-α or IL-1α or IL-6) and another fluorogenic probe, labeled with FAM dye, was used to detect the endogenous control gene (GAPDH).

Primer and Probe Design. PCR primers and fluorogenic probes for murine TNF-α, IL-1α and IL-6 and the reference gene GAPDH were designed using the computer program Primer Express® (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). Primers were located in two different exons and either one of the primers was located on an intron-exon junction to prevent co-amplification of genomic DNA. The melting temperature (T_m) of the primers was 58–60 °C and the probe T_m was approximately 69 °C, at least 10 °C higher than the primer T_m. The amplicon lengths were kept very short (Table 1). The fluorogenic probe contained a reporter dye (TNF-α/IL-1α/IL-6: VIC; GAPDH: FAM) covalently attached at the 5′ end and a quencher dye (TAMRA) covalently attached at the 3′ end.

Table 1. Primer and probe sequences for TNF-α, IL-1α, IL-6, and GAPDH. PCR primers and fluorogenic probes for murine TNF-α, IL-1α and IL-6 and the reference gene GAPDH were designed using the computer program Primer Express®. Forward (FW) and reverse (RV) primers were always located in different exons. Fluorogenic probes (FP) are VIC-labeled (TNF-α/IL-1α/IL-6) or FAM-labeled (GAPDH) at the 5′ end and TAMRA-labeled at the 3′ end.

Tabelle 1. Sequenzen von Primern und Sonden für TNF-α, IL-1α, IL-6 und GAPDH. Die Primer und fluoreszenzmarkierten Sonden für murinen TNF-α, murines IL-1α und IL-6 sowie das Referenzgen GAPDH wurden mit der Software Primer Express® konstruiert. Die Sequenzen der „forward" (FW) und „reverse" (RV) Primer befanden sich immer in verschiedenen Exons. Die fluoreszierenden Sonden (FP) sind am 5′-Ende VIC- (TNF-α/IL-1α/IL-6) bzw. FAM-markiert (GAPDH), am 3′-Ende mit TAMRA.

Name	Sequence (5′→3′)	Amplicon length (bp)
TNF-α FW	CCA GGC GGT GCC TAT GTC T	
TNF-α RV	GGC CAT TTG GGA ACT TCT CAT	123
TNF-α FP	AGC CTC TTC TCA TTC CTG CTT GTG GCA	
IL-1α FW	GCC CGT GTT GCT GAA GGA	
IL-1α RV	ATA AGC AGC TGA TGT GAA GTA GTT CTT AG	118
IL-1α FP	TTG CCA GAA ACA CCA AAA CTC ATC ACA GGT	
IL-6 FW	ACA AGT CGG AGG CTT AAT TAC ACA T	
IL-6 RV	AAT CAG AAT TGC CAT TGC ACA A	80
IL-6 FP	TTC TCT GGG AAA TCG TGG AAA TGA GAA AAG A	
GAPDH FW	CAA CTC ACT CAA GAT TGT CAG CAA	
GAPDH RV	GGC ATG GAC TGT GGT CAT GA	118
GAPDH FP	CAT CCT GCA CCA CCA ACT GCT TAG CC	

PCR Amplification. PCR reactions were performed in the ABI-prism 7700® sequence detector, which includes a GeneAmp® PCR system 9600 (Perkin Elmer/Applied Biosystems).

PCR amplifications were performed in a total volume of 25 µl, containing 3 µl cDNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 60 nM Passive Reference 1 (Rox), 200 µM dATP, dCTP, dGTP and 400 µM dUTP, 5.5 mM $MgCl_2$, 0.05% gelatine, for each target gene (TNF-α/IL-1α/IL-6) 300 nM of each primer, for the reference gene (GAPDH) 80 nM (forward primer) and 60 nM (reverse primer), 1.25 U AmpliTaq Gold®, and 0.25 U AmpErase® Uracil N-Glycosylase (TaqMan® PCR core reagent kit; Perkin Elmer/Applied Biosystems). Each reaction also contained 200 nM of the corresponding detection probe (Table 1).

For all cytokines as well as for the reference gene GAPDH, identical thermal cycling conditions were used: 2 min at 50 °C

and 10 min at 95 °C, followed by a total of 40 two-temperature cycles: 15 s at 95 °C and 1 min at 60 °C.

Each PCR amplification was performed in triplicate wells. All values for the TNF-α, IL-1α and IL-6 mRNA expression were normalized to the constitutively expressed mRNA of the reference gene GAPDH.

Histology

For histological analysis, the left lobes were fixed in 4% neutral buffered formalin, paraffin-embedded, and sectioned at an average thickness of 5 µm. The mounted sections were stained by hematoxylin and eosin.

Statistical Methods

The 14 groups (corresponding to the 14 assessment time points) and the control group were tested for mean difference on a logarithmic scale of the five mediator parameters by simple analysis of variance followed by two-sided pairwise comparisons versus control according to Dunnett. Two outliers at 2 and 4 weeks p.i. in the IL-1α data with values $< 2 \times 10^{-4}$ were excluded prior to statistical testing. The statistical calculations were performed by SPSS statistical software. The criterion for statistical significance was $p < 0.05$.

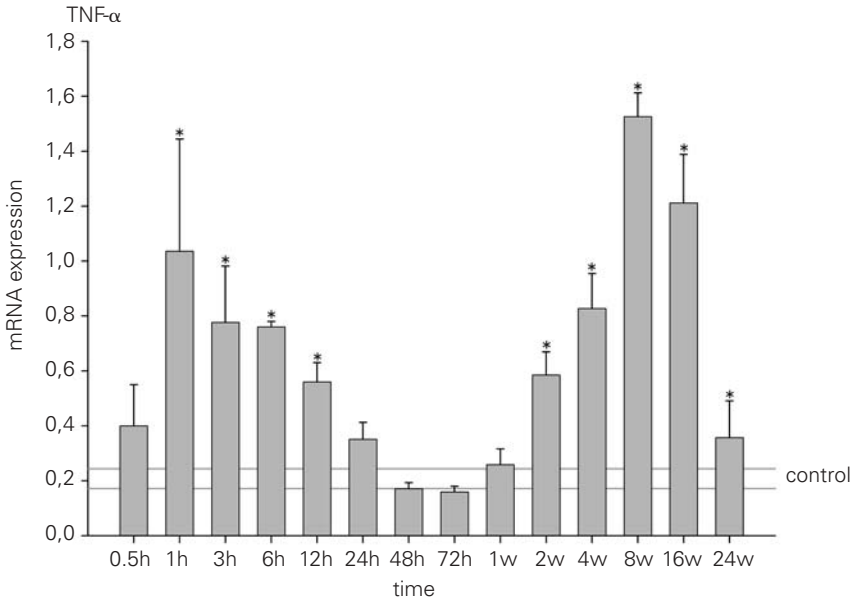

Figure 1. Time course of the TNF-α mRNA expression in the lung tissue of mice that underwent thoracic irradiation with 12 Gy (data are mean ± SD of triplicate determinations from three different mice; ∗: statistically significant). Control: TNF-α mRNA expression in nonirradiated lung tissue of control animals.

Abbildung 1. Zeitlicher Verlauf der TNF-α-mRNA-Expression im Lungengewebe von Mäusen nach Ganzlungenbestrahlung mit 12 Gy (Mittelwert ± Standardabweichung von Dreifachbestimmungen an drei verschiedenen Mäusen; ∗: statistisch signifikant). Kontrolle: TNF-α-mRNA-Expression im Lungengewebe unbestrahlter Kontrolltiere.

Results

Histology

The qualitative and quantitative histopathologic alterations of the radiation-induced lung injury conformed to those described previously [1, 22, 23]. In brief, the histopathologic alterations that contributed to the pleomorphic picture of radiation-induced lung injury included macrophage infiltration in air spaces, edema in the alveolar walls and/or air spaces, desquamation of epithelial cells from the alveolar walls, thickening of the alveolar septa by infiltration of inflammatory cells, collagen deposition, progressive fibrosis of alveolar septa, and obliteration of the alveoli. Histopathologic evaluation of the lung sections obtained within 4 weeks after thoracic irradiation revealed only minor lung damage in 5–30% of the lung tissue. A significant increase in the percent of lung containing histopathologically detectable organizing alveolitis was observed at 8, 16, and 24 weeks p.i. At these time points, 70–90% of the lung tissue was found to be seriously affected.

PCR Analysis

The results of the quantitative assessment of TNF-α, IL-1α and IL-6 mRNA expression in lung tissue after thoracic irradiation are demonstrated in Figures 1, 2, and 3, respectively. Nonirradiated lung tissue of control animals exhibited low TNF-α, IL-1α and IL-6 expression levels (TNF-α: 0.207 ± 0.036, IL-1α: 0.048 ± 0.015, and IL-6: 0.059 ± 0.022). Following thoracic irradiation with a single dose of 12 Gy, radiation-induced TNF-α mRNA expression in lung tissue revealed a distinct increase as early as 1 h p.i. (relative mRNA expression: 1.04 ± 0.41) and was elevated during the 1st day after irradiation (1, 3, 6, and 12 h p.i.; Figure 1). In addition, we observed a marked increase in IL-1α (relative mRNA expression: 2.12 ± 0.7) and IL-6 mRNA levels (relative mRNA expression: 1.41 ± 0.32) at 6 h p.i. (Figures 2 and 3). Subsequently, the radiation-induced mRNA levels for TNF-α, IL-1α and IL-6 returned to baseline levels (24 h, 48 h, 72 h, 1 week p.i.; Figures 1 to 3).

At the beginning of the pneumonic phase, radiation-induced TNF-α mRNA expression increased at 2 and 4 weeks and reached maximal values at 8 and 16 weeks p.i. (relative mRNA expression: 1.52 ± 0.09 and 1.21 ± 0.18, respectively; Figure 1). Irradiation-mediated IL-1α and IL-6 mRNA expression had risen at 4 weeks and demonstrated a pronounced increase at 8 weeks p.i. (relative mRNA expression of IL-1α: 0.6 ± 0.05; of IL-6: 1.36 ± 0.16) followed by a decrease to moderate levels at 16 and 24 weeks p.i. (Figures 2 and 3).

These data establish that pulmonary TNF-α, IL-1α and IL-6 mRNA levels were increased initially in the first hours

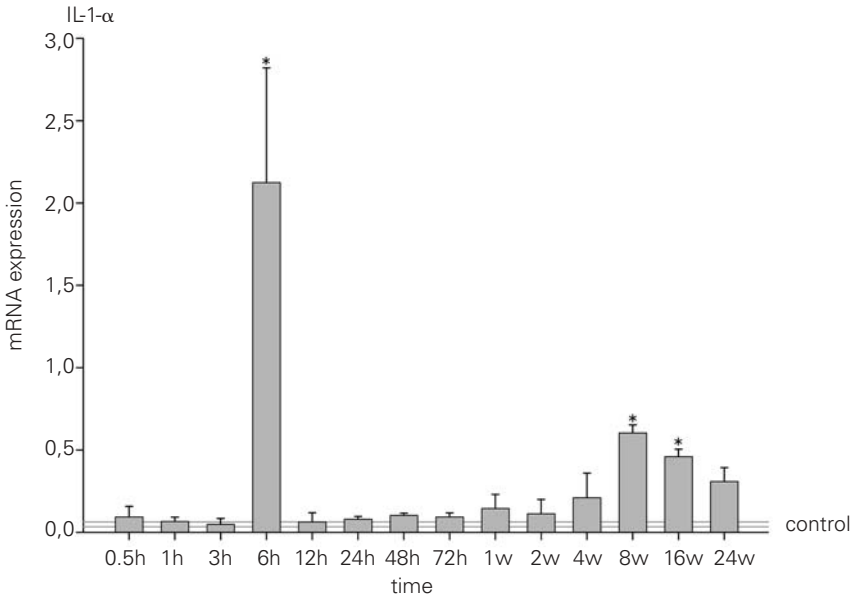

Figure 2. Time course of the IL-1α mRNA expression in the lung tissue of mice that underwent thoracic irradiation with 12 Gy (data are mean ± SD of triplicate determinations from three different mice; ∗: statistically significant). Control: IL-1α mRNA expression in nonirradiated lung tissue of control animals.

Abbildung 2. Zeitlicher Verlauf der IL-1α-mRNA-Expression im Lungengewebe von Mäusen nach Ganzlungenbestrahlung mit 12 Gy (Mittelwert ± Standardabweichung von Dreifachbestimmungen an drei verschiedenen Mäusen; ∗: statistisch signifikant). Kontrolle: IL-1α-mRNA-Expression im Lungengewebe unbestrahlter Kontrolltiere.

after whole-lung irradiation followed by a drop to control levels (24 h, 48 h, 72 h, 1 week p.i.). The second peak increase in TNF-α, IL-1α and IL-6 mRNA levels was associated with the onset of acute pneumonitis visible in the histological changes associated with organizing alveolitis.

Discussion

For the present study, we designed and optimized an accurate multiplex RT-PCR for the relative quantification of inflammatory cytokines playing a role in radiation-induced pneumonitis. To compensate for variations in input RNA amounts and efficiency of reverse transcription, an endogenous control gene (GAPDH) was quantified, and results were normalized to its values. In this multiplex RT-PCR, one fluorogenic probe, labeled with VIC dye, was used to detect the target amplicon (TNF-α/IL-1α/IL-6), and another fluorogenic probe, labeled with FAM dye, was used to detect the endogenous control gene (GAPDH). Running both assays in a single tube reduces both the running costs and the dependence on accurate pipetting when splitting a sample into two separate tubes. As this method combines PCR amplification and product detection in one single step, the technique is very fast and easy to perform, compared to classic RT-PCR techniques. This method provides accurate measurements over a wide range of starting template quantities. The use of internal fluorogenic probes guarantees the specificity of the PCR product to be measured.

The mechanism of pulmonary irradiation acute effects and its correlation with late effects is the subject of controversy. Current concepts of the pathogenesis of pulmonary fibrosis propose an initial inflammatory stage involving an influx of inflammatory cells into the interstitium, which together with activated resident cells are thought to release a variety of cytokines [2–4, 6, 10, 11, 19–21, 26] and other polypeptide mediators such as chemokines [12] that stimulate fibroblast proliferatsion and collagen secretion. While pulmonary fibroblasts are probably the most central cell populations in this process, because of their ability to produce collagen, it is obvious that the regulation of the fibroblast responses in injury is influenced by specific interactions among multiple cell types. Cytokine expression by recruited and resident inflammatory cells, as well as by pulmonary epithelial and endothelial cells, has been implicated in the development of radiation-induced lung injury [4, 19, 20].

The present work is a detailed study on the temporal release of the pro-inflammatory cytokines TNF-α, IL-1α and IL-6 in the lung tissue of C57BL/6J mice after thoracic irradiation. Inflammatory cytokines are of key interest for modulating and ameliorating the effects of inflammatory reactions after lung irradiation (acute radiation pneumonitis) and their sequelae (late radiation fibrosis). Our results demonstrate a complex pattern of elevation of cytokine mRNA levels following total-lung irradiation but with a clear indication of a two-phase mechanism in the molecular pathology. We observed an immediate release of pro-inflammatory cytokines in the first hours after lung irradiation with no detectable appearance of histological changes and a second long-lasting increase in pro-inflammatory cytokine mRNA levels correlating with the onset of organizing alveolitis. The radiation-induced TNF-α mRNA expression revealed a significant increase as early as 1 h after irradiation (Figure 1). This early and prominent TNF-α increase precedes the peak of IL-1α and IL-6 at 6 h p.i. Since TNF-α enhances the production of different cytokines, including IL-1 and IL-6, which are thought to further promote the inflammatory process, the authors presume that TNF-α may induce the expression of IL-1 and IL-6 in irradiated lung tissue, therefore having a prominent role in the initiation of the cytokine cascade. A slight upregulation of the TNF-α and IL-6 expression was already observed 4 weeks after lung irradiation. The histological evaluation of the corresponding lung sections revealed

only minor histopathologic alterations in 10–20% of the lung tissue. Therefore, the upregulation of TNF-α and IL-6 at 4 weeks p.i. pre- cedes histologically discernible organizing alveolitis, suggesting a pathogenetic role of these cytokines in initiating radiation-induced pneumonitis. During the phase of acute pneumonitis, significant-ly increased TNF-α mRNA expression was observed at 8, 16, and 24 weeks p.i. These results confirm and extend those of our previous study on radiation-induced TNF-α expression in lung tissue [20]. In the previous study, the cellular source of the TNF-α protein as well as the association between TNF-α immunoreactivity and specific histopathologic alterations could be demonstrated by immunohistochemistry. In the first hours after thoracic irradiation, the bronchiolar epithelium revealed an intense and homogeneous staining for TNF-α; in lung parenchyma, resting alveolar macrophages stained positive. During the stage of acute pneumonitis, the lung tissue revealed an accumulation of positive inflammatory cells particularly in perivascular and peribronchial areas, as well as in subpleural regions.

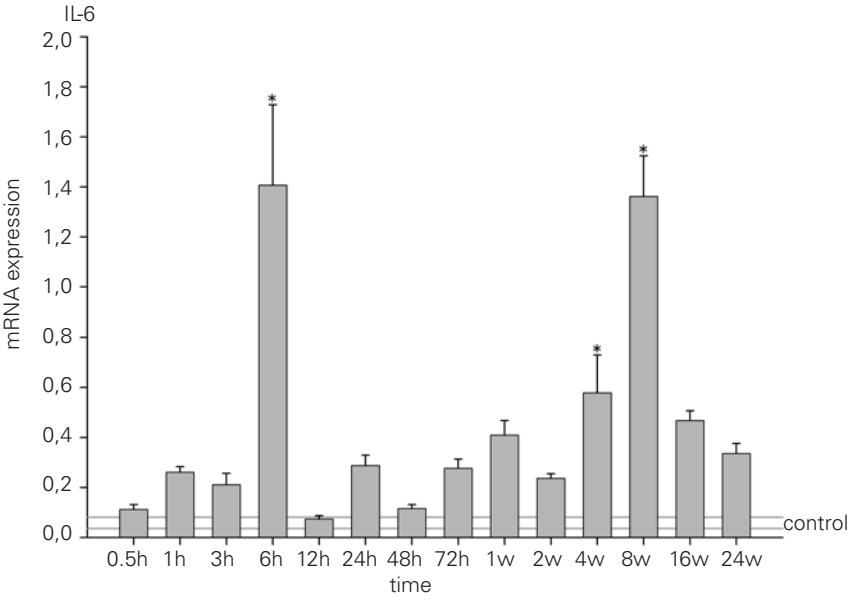

Figure 3. Time course of the IL-6 mRNA expression in the lung tissue of mice that underwent thoracic irradiation with 12 Gy (data are mean ± SD of triplicate determinations from three different mice; ∗: statistically significant). Control: IL-6 mRNA expression in nonirradiated lung tissue of control animals.

Abbildung 3. Zeitlicher Verlauf der IL-6-mRNA-Expression im Lungengewebe von Mäusen nach Ganzlungenbestrahlung mit 12 Gy (Mittelwert ± Standardabweichung von Dreifachbestimmungen an drei verschiedenen Mäusen; ∗: statistisch signifikant). Kontrolle: IL-6-mRNA-Expression im Lungengewebe unbestrahlter Kontrolltiere.

During the phase of acute pneumonitis, IL-1α and IL-6 mRNA levels were elevated at 8 and 16 weeks p.i. (Figures 2 and 3). Human alveolar macrophages have been shown to release IL-1, predominantly IL-1β, within hours after irradiation in vitro [16]. Low-dose irradiation induces IL-1 and IL-6 expression in mouse macrophages in vitro [7]. Tavakoli et al. [24] described an increased IL-6 production in airway epithelial cells after stimulation with prostaglandin E_2 in vitro. No published data are available evaluating the cellular source of increased IL-1 and IL-6 expression after lung irradiation in vivo. Therefore, immunohistochemical detection methods were established in our laboratory to evaluate the protein expression of IL-1 and IL-6 in the lung tissue after thoracic irradiation. Our preliminary results seem to confirm the presented mRNA expression data, with the bronchiolar epithelium becoming a significant source of these pro-inflammatory cytokines especially in the first hours after irradiation; at the onset of radiation pneumonitis, the bronchiolar epithelium as well as inflammatory cells in the lung parenchyma produce high amounts of these inflammatory cytokines. Further investigations including the quantification of protein production in the lung sections by automated image analysis are under way to validate these data.

In a previous study by Johnston et al. [11], fibrosis-sensitive (C57BL/6) and fibrosis-resistant (C3H/HeJ) mice received thoracic irradiation of 5 and 12.5 Gy. The animals were killed at day 1 and 1, 2, 8, 16, and 26 weeks after irradiation. The lung tissue of these animals was evaluated for TNF-α, IL-1α and IL-1β mRNA expression by slot-blot analysis. While IL-1β mRNA levels were unaltered after irradiation, IL-1α mRNA levels in the lung tissue of C57BL/6J mice were increased at 8, 16, and 26 weeks after thoracic irradiation with 12.5 Gy. In the C57BL/6J mice, TNF-α mRNA levels were increased at 2 weeks p.i., but decreased during the pneumonic phase. These results are in contrast to our observation of a long-lasting increase of TNF-α on mRNA and protein level during the acute phase of pneumonitis, predominantly secreted by inflammatory cells in the lung parenchyma [20].

Previous reports of cytokine expression following lung irradiation focused on the remodeling phase but not the initial response within the first hours after irradiation [3, 11, 21]. In the present study, we can clearly demonstrate the immediate release of pro-inflammatory cytokines in the first hours after lung irradiation. Our results are consistent with the observation of Hong et al. [6] that TNF-α and IL-1 mRNA levels (measured by RNase protection assay) were induced in the lung tissue of C57BL/6J mice within 24 h after irradiation. The immediate release of pro-inflammatory cytokines demonstrates the absence of a so-called latent period.

Conclusion

This study demonstrates that irradiation induces a biphasic expression of pro-inflammatory cytokines in the lung: an initial transitory cytokine response within the first hours after lung irradiation and a second, more persistent cytokine elevation which coincides with the onset of acute pneumonitis. Insights into the molecular mechanisms of radiation-induced lung injury may provide new strategies for the treatment of radiation pneumonitis. Inhibition of appropriate cytokines at an early stage may provide a new tool for effective treatment of radiation-induced lung injury. Pharmacological regulation of the pro-inflammatory cytokine production in the first hours after irradiation may provide protection against radiation-induced, cytokine-mediated damage.

Acknowledgments

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