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ORIGINAL ARTICLE

Modulation of radiation-induced oral mucositis by thalidomide

Preclinical studies

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Abstract
Purpose Oral mucositis is a common, dose-limiting early side effect of radio(chemo)therapy for head-and-neck tumors. The epithelial radiation response is accompanied by changes in the inflammatory signaling cascades mediated by the transcription factor nuclear factor-kappa B (NF-κB). The present study was initiated to determine the effect of the NF-κB inhibitor thalidomide on the clinical manifestation of oral mucositis in the established mouse tongue model.
Materials and methods Treatment protocols comprised single dose irradiation and daily fractionated irradiation (5 fractions of 3 Gy/week) over 1 (days 0–4) or 2 weeks (days 0–4, 7–11), alone or in combination with daily thalidomide application (100 mg/kg intraperitoneally) over varying time intervals. Fractionation protocols were terminated by graded local radiation doses (day 7/14) to generate full dose-effect curves. Tongue epithelial ulcerations, corresponding to confluent mucositis, served as the clinically relevant endpoint.
Results Thalidomide application did not show a significant radioprotective potential when administered in combination with single dose irradiation. Thalidomide in combination with one week of fractionated irradiation significantly increased the isoeffective top-up doses. Similar results were observed during two weeks of fractionated irradiation in all but one experiment.
Conclusion Thalidomide treatment demonstrated a significant mucositis-ameliorating effect during fractionated irradiation, which is likely to result from NF-κB inhibition. However, further mechanistic studies are required to define the underlying mechanisms of the observed mucoprotective effect.

Keywords Radiotherapy · Oral mucositis · Thalidomide · NF-κB · Mouse model

Modulation der strahleninduzierten oralen Mukositis durch Thalidomid

Präklinische Studien

Zusammenfassung
Hintergrund Die orale Mukositis ist eine häufige, dosislimitierende frühe Nebenwirkung der Radio(chemo)therapie von Kopf-Hals-Tumoren. Die epitheliale Strahlenreaktion geht mit über den Transkriptionsfaktor Nuklearfaktor-kappa B (NF-κB) vermittelte Umstrukturierungen der Signalkaskaden der Entzündungsreaktion einher. Die vorliegende Studie soll den Effekt von Thalidomid, einem NF-κB-Inhibitor, auf die klinische Ausprägung der oralen Mukositis am etablierten Modell der Mäusezunge klären.
Material und Methoden Die Behandlungsprotokolle beinhalteten eine Einzeitbestrahlung und eine täglich fraktionierte Bestrahlung (5 × 3 Gy/Woche) über eine (Tage 0–4)

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oder 2 Wochen (Tage 0–4, 7–11), allein oder in Kombination mit täglicher Thalidomid-Gabe (100 mg/kg intraperitoneal) über verschiedene Zeitintervalle. Die fraktionierten Bestrahlungsprotokolle wurden von einer Aufsättigungsbestrahlung mit gestaffelten Dosen (Tag 7/14) zur Generierung kompletter Dosis-Effekt-Kurven abgeschlossen. Definiert als klinisch relevanter Endpunkt wurden Schleimhautulzerationen, entsprechend einer konfluenten Mukositis.

Ergebnisse Die Thalidomid-Gabe hatte bei Einzeitbestrahlung keinen radioprotektiven Effekt. Bei fraktionierter Bestrahlung über eine Woche führte Thalidomid zu einer signifikanten Erhöhung der isoeffektiven Aufsättigungsdosen. Während der 2-wöchigen fraktionierten Bestrahlung konnte, mit Ausnahme eines Experiments, ebenfalls ein signifikanter Effekt festgestellt werden.

Schlussfolgerung Die Thalidomid-Behandlung unter täglicher fraktionierter Bestrahlung zeigte eine signifikante Verminderung der oralen Mukositis, möglicherweise als Folge der NF-κB-Inhibition. Weitere mechanistische Studien sind jedoch notwendig, um die zugrundeliegenden Mechanismen dieses mukoprotektiven Effekts zu klären.

Schlüsselwörter Radiotherapie · Orale Mukositis · Thalidomid · NF-κB · Mausmodell

Introduction

Oral mucositis is the most frequent and often dose-limiting early side effect of radio(chemo)therapy for advanced head-and-neck malignancies, eventually resulting in ulcerative lesions in the oral cavity. Virtually all patients develop some grade of oral mucositis. The incidence of severe, confluent (grade 3) reactions in conventional radiotherapy is in general higher than 50 % [1, 2]. The epithelial radiation response has a significant impact on the patient's quality of life. Severe pain, swallowing difficulties, also associated with weight loss, often lead to unplanned treatment breaks, which result in a significant decrease in tumor control probability [3–5].

Various prophylactic and therapeutic approaches to reduce the severity of oral mucositis have been tested preclinically and also in initial clinical studies [6–10]. However, so far, no strategy has been established into clinical practice. Current measures to reduce oral radiation-induced mucositis are purely symptomatic, i. e., improvement of oral hygiene, mucosal coating agents, mouth washes, and administration of antibiotics and analgesics [11].

The pathobiology of oral mucositis includes activation of transcriptions factors, such as NF-κB, which leads to the up-regulation of pro-inflammatory signaling cascades [12–14]. NF-κB inhibition represents a promising strategy for prevention and/or mitigation of radiation-induced oral mucositis [15–17].

Thalidomide, a α-N-phthalmidoglutarimide, inhibits NF-κB activation [18] and has anti-inflammatory, anti-neoplastic, and anti-angiogenic properties [19]. Preclinical studies in a hamster model demonstrated a mucositis-ameliorating effect of this drug after chemotherapy [20].

The aim of the present study was to investigate the oral mucositis-ameliorating potential of thalidomide in the established mouse tongue model. Thalidomide administration was combined either with single dose or daily fractionated irradiation. Mucosal ulceration, corresponding to mucositis grade 3 of the classification of the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer (RTOG/EORTC), was analyzed as the clinically relevant endpoint. The time course parameters latency and duration of ulcerative lesions were analyzed as secondary endpoints.

Materials and methods

Animals

Eight to 12 weeks old mice of the inbred C3H/Neu strain from the breeding facility of the Department for Biomedical Research of the Medical University of Vienna were used for all experiments. Animals were housed under specific pathogen-free conditions with controlled humidity (55 ± 10 %), temperature (22 ± 2 °C), and a 12/12-h light–dark rhythm. Free access to standard mouse diet (ssniff Spezialdiäten GmbH, Soest, Germany) and fresh water from standard drinking bottles was provided ad libitum. A maximum of 5 animals were kept in Makrolon® cages (Techniplast GmbH, Hohenpeißenberg, Germany) on aspen wood bedding (ABEDD-LAB & VET Service GmbH, Vienna, Austria). All experiments were performed according to the current animal welfare legislation with approval of the respective authorities (file no. 66.009/0038-II/3b/2014).

Irradiation technique

Irradiation of the epithelium of the lower tongue surface was performed using two different techniques: Daily fractionated irradiation of the entire snout and local irradiation of a 3 × 3 mm^2 (test) area of the lower surface of the tongue. For both techniques an YXLON Maxishot device (YXLON International GmbH, Hamburg, Germany) was used. The beam was applied vertically.

Percutaneous irradiation of the entire snout was performed without anesthesia as described previously [21, 22]. In brief, animals were guided into perspex tubes (inner diameter 25 mm). Conical holes in a perspex block at the

Table 1 Experimental design

Groups	Fractionated irradiation (days)	Local irradiation (day)	Thalidomide administration (first–last day)	Doses for local top-up irradiation (Gy)
SD 0	–	0	–	7–10–12–14–17
SD 1	–	0	–3 to ulcus start	7–10–12–14–17
SD 2	–	0	–3 to ulcus healing	7–10–12–14–17
F 1.0	0–4	7	–	4–6–8–10–12
F 1.1	0–4	7	–3 to 4	3–6–8–10–13
F 2.0	0–4 and 7–11	14	–	4–6–8–10–12
F 2.1	0–4 and 7–11	14	–3 to 4	4–7–9–11–14
F 2.2	0–4 and 7–11	14	5 to 11	4–7–9–11–14
F 2.3	0–4 and 7–11	14	–3 to 11	4–7–9–11–14

Irradiation protocols comprised single dose irradiation (*SD*) or daily fractionated irradiation over one week (*F 1*) or two weeks (*F 2*), followed by graded local irradiation (5 dose groups, 10 animals each) on day 7 or 14, respectively. The first and the last day of thalidomide administration are represented in the second column from the right. The graded local top-up doses are listed in the rightmost column.

front end of the tubes served for positioning of the snouts. The back ends of the tubes were closed with a polystyrene plug to prevent withdrawal of the animals. Eight mice were irradiated simultaneously. The X-ray unit was operated at a tube voltage of 200 kV and a current of 20 mA. In addition to the inherent filtration by 3 mm Be, a 4 mm Al and 0.6 mm Cu beam filter was used. The dose rate at the focus-to-surface distance 45.5 cm was approximately 1 Gy/min. A 12-mm thick collimator plate, consisting of lead equivalent MCP-96, shielded the bodies of the animals caudally from a plane from the eyes to the throat, thus, including the entire tongue. The dose homogeneity between the individual snout positions was 3.2 ± 0.5 %.

Local irradiation was applied to a 3 × 3 mm² treatment field in the center of the lower tongue surface as described previously [7, 23]. Briefly, mice were immobilized by pentobarbital sodium, 60 mg/kg intraperitoneally (Release®, WDT, Garbsen, Germany), and placed in a supine position in the central bore (diameter 25 mm) of an aluminum block. The tongue was pulled gently through a hole (diameter 3 mm) in the roof of the block and the upper surface was fixed to the block with adhesive tape. In order to prevent tension at the base of the tongue, a polystyrene wedge supported the head of the mice. A 1-mm thick aluminum plate with a 3 × 3 mm² window was placed centrally over the tongue to define the treatment field. The X-ray unit was operated at a tube voltage of 25 kV with a tube current of 20 mA. In addition to the inherent filtration with 3 mm Be, a 0.3 mm Al beam filter was used. The dose rate at the focus-to-surface distance of 15 cm was approximately 4 Gy/min.

Thalidomide

Thalidomide powder ((±)-thalidomide T44, Sigma–Aldrich®, St. Louis, MO, USA) was dissolved in DMSO (dimethyl sulfoxide, Sigma–Aldrich®, St. Louis, MO, USA) at a concentration of 40 mg/ml and injected intraperitoneally at a daily dose of 100 mg/kg (injection volume 0.05–0.075 ml).

Experimental design

A summary of the experimental protocols is given in Table 1.

Single dose irradiation was performed on day 0 with graded doses of 7, 10, 12, 14, or 17 Gy (experiment SD 0). Thalidomide was administered from three days prior irradiation until diagnosis (experiment SD 1) or complete healing (experiment SD 2) of tongue ulcerations. At the day of irradiation, the drug was administered 2 hours postradiotherapy. In animals that did not develop ulceration, thalidomide treatment was stopped when the ulcerations of the responders had healed (Table 1).

Fractionation (experiment F 1, F 2) comprised daily 3 Gy fractions over one (days 0–4, F 1.0) or two weeks (days 0–4, 7–11, F 2.0), followed by graded local top-up doses on day 7 or day 14, respectively (Table 1). During one week of fractionated irradiation, thalidomide was administered from day –3 until day 4 (F 1.1). Thalidomide in combination with two weeks of fractionation was applied from day –3 until day 4 (F 2.1), day 5 until day 11 (F 2.2) or day –3 until day 11 (F 2.3).

Follow-up and endpoints

Scoring of the tongues was done daily from the onset of first symptoms of mucositis until complete re-epithelialization. For this, the mice were immobilized with approximately 40 mg/kg pentobarbital sodium (Release®, WDT, Garbsen, Germany) intraperitoneally. Mucosal ulceration was documented as a quantal endpoint. The incidence of

Table 2 The effect of thalidomide on radiation-induced oral mucositis in mouse tongue

Groups	ED_{50} ± SD (Gy)[a]	p_{dose}[b]	$p_{vs.\ control}$[c]	Latency time ± SD (days)[d]	Ulcer duration ± SD (days)
SD 0	11.9 ± 1.2	0.0004	–	11.8 ± 0.9	3.1 ± 0.4
SD 1	12.1 ± 1.0	0.0005	0.6340	11.3 ± 1.2	3.2 ± 1.0
SD 2	12.2 ± 1.1	0.0008	0.7089	12.1 ± 1.2	2.9 ± 1.0
F 1.0	6.9 ± 3.2	0.0061	–	8.2 ± 0.8	3.3 ± 0.6
F 1.1	12.8 ± 0.1	< 0.0001[e]	0.0001	8.3 ± 0.5	2.7 ± 0.9
F 2.0	8.4 ± 2.1	0.0005	–	8.1 ± 0.9	3.1 ± 1.0
F 2.1	10.2 ± 1.5	0.0004	0.0692	8.9 ± 1.1	2.7 ± 0.9
F 2.2	13.5 ± 1.0	0.0068	< 0.0001	7.7 ± 0.8	2.9 ± 0.4
F 2.3	13.9 ± 0.1	< 0.0001[e]	< 0.0001	9.1 ± 0.4	2.8 ± 0.7

[a]Standard deviation of ED_{50} value, resulting from logit analyses
[b]*p*-values for the radiation dose dependence of ulcer incidence, resulting from logit-analyses
[c]*p*-values for the difference between dose-effect curves, resulting from maximum-likelihood analysis
[d]Relative to the day of local irradiation; i. e., in fractionation protocols relative to the day of top-up irradiation
[e]Alternative test (see statistical analysis)
SD standard deviation

ulceration was analyzed as the primary endpoint. Latency (time between irradiation and first ulcer diagnosis) and ulcer duration (from first diagnosis to macroscopic healing) served as secondary endpoints.

Statistical analysis

The Statistical Analysis System, SAS, version 9.3 (SAS Institute Inc., Cary, NC, USA) was used for all statistical procedures. Probit analyses were performed to establish dose–effect relationships, assuming a log-normal distribution (logit analysis), without a threshold dose. Dose-effect curves were characterized by the ED_{50} values (doses, where ulcerations are expected in 50 % of the animals) and their standard deviation σ. P_{dose}-values for the effect of dose on ulcer incidence were calculated, based on the slope of the regression line of the probit curve. In cases where *p*-values for dose-dependence of the response could not be calculated by probit analysis, a Cochrane–Armitage trend test (labelled as "Alternative test") was applied to analyze for a monotonic trend of the incidence (SAS PROC FREQ). Dose–effect relationships from different experiments were compared with a likelihood ratio test, based on the logit model, without assumption of a threshold dose. Time course parameters were compared by two sided t-tests.

Results

The results of the present study are summarized in Table 2. Irradiation and thalidomide treatment were well tolerated. No treatment-associated adverse effects, such as a reduction in body weight or food consumption, or changes in appearance or behavior of the animals, other than the mucosal radiation response were observed.

Thalidomide and single dose irradiation

The ED_{50} value for single dose irradiation alone was 11.9 ± 1.2 Gy. Ulcer incidence was highly dose-dependent (p_{dose} = 0.004). As shown in Fig. 1, neither thalidomide administration from day –3 until first ulcer diagnosis nor from day –3 until ulcer healing had a significant influence on the dose–effect curves (ED_{50} = 12.2 ± 1.0 Gy and 12.2 ± 1.1 Gy, respectively).

The mean latent time for SD alone was 11.8 ± 0.9 days, the mean ulcer duration was 3.1 ± 0.4 days. Thalidomide treatment from day –3 until first ulcer diagnosis and from day –3 until ulcer healing did not significantly change these time course parameters.

Thalidomide and one week of fractionated irradiation

One week of fractionated irradiation followed by graded top-up doses resulted in a top-up ED_{50} of 6.9 ± 3.2 Gy. A significant increase in isoeffective doses, with an ED_{50} of 12.8 ± 0.1 ($p_{vs.\ control}$ 0.0001) was observed after thalidomide treatment from day –3 until day 4 (Fig. 2). The mean latent time after one week of fractionation alone was 8.2 ± 0.8 days, the mean ulcer duration was 3.3 ± 0.6 days (Table 2). Thalidomide application had neither a major impact on the mean latent time (8.3 ± 0.5 days) nor on the mean ulcer duration (2.7 ± 0.9 days; Fig. 2).

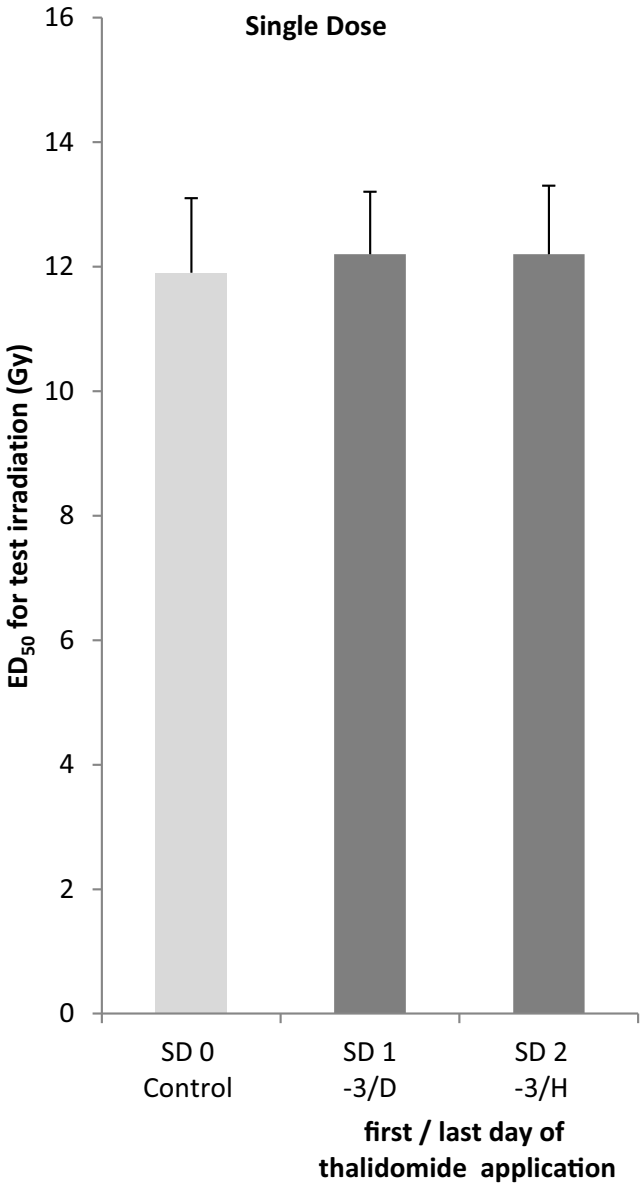

Fig. 1 Effect of thalidomide in combination with single dose irradiation. The bars represent the ED_{50} values based on experiments with 5 graded dose groups with 10 animals each. ED_{50} values and their standard deviation σ (error bars) were calculated by logit analyses. Thalidomide was applied daily from day –3 until the day of first diagnosis of ulcerations (SD 1) or until the day of healing of ulcerations (SD 2). The *light grey bar* (SD 0) indicates the control group, which received irradiation alone

Thalidomide and two weeks of fractionated irradiation

The ED_{50} for top-up irradiation after two weeks of fractionation was 8.4 ± 2.1 Gy. Thalidomide administration from day –3 until day 4 increased the ED_{50} value to 10.2 ± 1.5 Gy (Fig. 3), but this effect was not significant ($p_{vs.control}$ 0.0692). However, thalidomide given during the second week of irradiation (day 5–11) significantly increased the top-up ED_{50} value to 13.5 ± 1.0 Gy. The most significant change in isoeffective doses was observed when thalidomide was administered both weeks (day –3 until day 11) with an ED_{50} value of 13.9 ± 0.1 Gy (Fig. 3).

After two weeks of fractionated irradiation the mean ulcer manifestation was on day 8.1 ± 0.9 and lasted for 3.1 ± 1.0 days on average. Thalidomide administration over the above mentioned time intervals did not significantly or systematically influence these time course parameters.

Discussion

Oral mucositis is a frequent and dose-limiting side effect of radio(chemo)therapy of head and neck cancer. It is likely linked to NF-κB activation and the consequent upregulation of pro-inflammatory cytokines [12, 14, 24]. Therefore, the present study assessed the effect of NF-κB inhibition by thalidomide on the clinical manifestation of oral mucositis in the established mouse tongue model. Thalidomide administration prior to and during one week of fractionated irradiation resulted in a highly significant increase in isoeffective doses. No change was observed for latency and ulcer duration. Thalidomide combined with two weeks of fractionated irradiation was most effective when applied during both weeks. A significant effect was also observed when the drug was administered in the second week of irradiation only. When thalidomide was given only in the first week, the increase of the ED_{50} value did not reach significance.

The biological mechanisms underlying the mucoprotective effect of thalidomide are still unclear. In the initial phase of mucositis, DNA damage and reactive oxygen species result in activation of NF-κB and up-regulation of pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6, and interleukin-1ß in the epithelium. Several preclinical and clinical studies revealed that increased levels of these cytokines correlate with the development and also the severity of oral mucositis [13, 25]. A positive feedback loop between TNF-α and NF-κB may further amplify the inflammatory signal. In a hamster model for chemotherapy-induced oral mucositis, thalidomide reduced mucositis incidence [20]. However, selective inhibition of TNF α in the mouse tongue model had no effect on ulcer incidence [8]. The mucoprotective potential of thalidomide may hence be based on the direct inhibition of NF-κB and down-stream inflammatory signaling cascades in the crucial phase of mucositis development. However, once the ulcerative phase is initiated, thalidomide does not accelerate healing of the oral mucosal epithelium.

Irradiation also stimulates the expression of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) in endothelial cells and fibroblasts in the submucosa. However, this is only seen during the maximum ulcerative mucosal re-

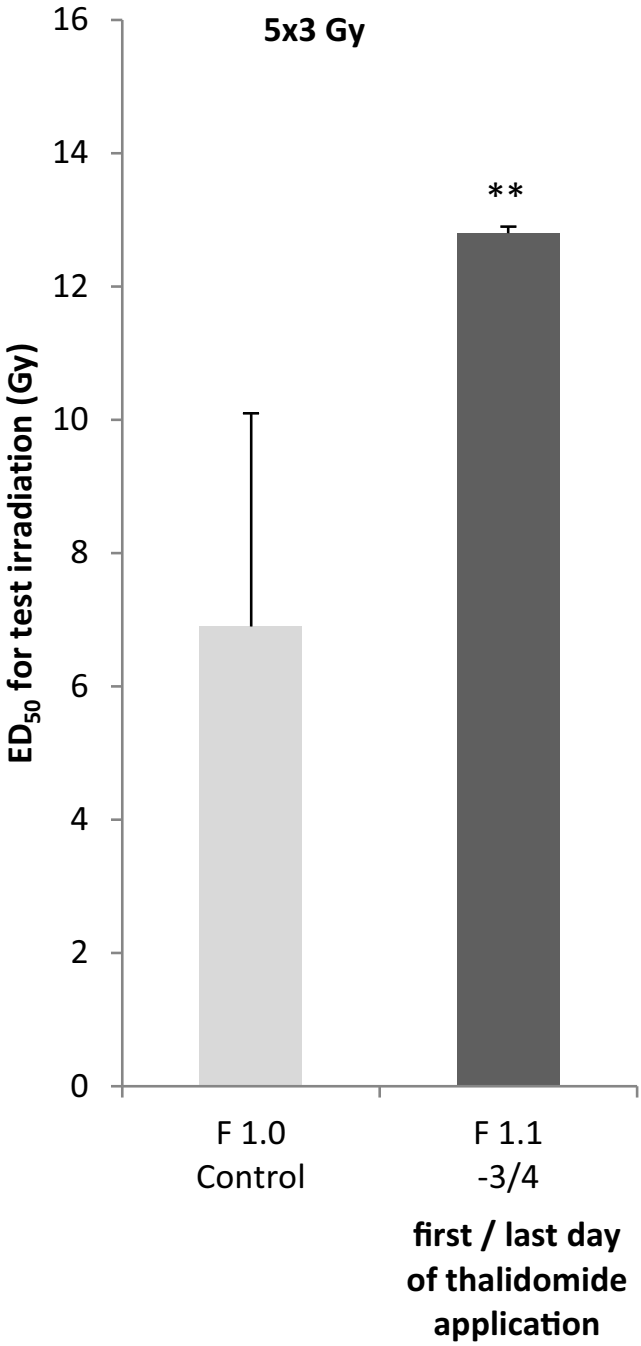

Fig. 2 Effect of thalidomide in combination with one week of fractionated irradiation followed by local irradiation with 5 graded dose groups and 10 animals each. Thalidomide was applied daily from day –3 until day 4 (F 1.1). The *light grey bar* (F 1.0) indicates the control group. $^{**}p < 0.0001$

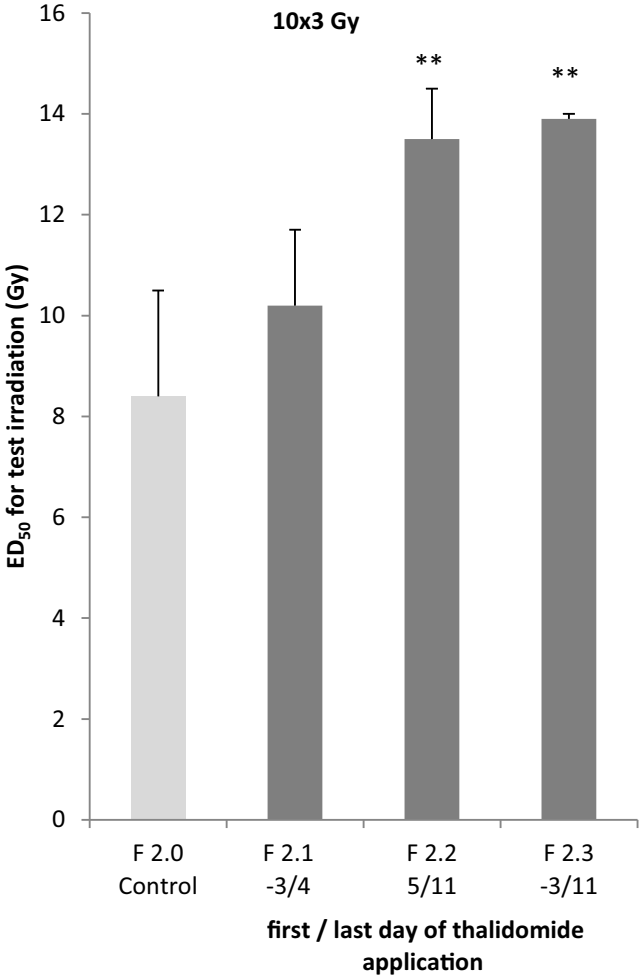

Fig. 3 Effect of thalidomide in combination with two weeks of fractionated irradiation followed by local irradiation with 5 graded dose groups and 10 animals each. Thalidomide was applied daily from day –3 until day 4 (F 2.1), from day 5 until day 11 (F 2.2), or from day –3 until 11 (F 2.3). The *light grey bar* (F 2.0) indicates the control group. $^{**}p < 0.0001$

sponse, and therefore COX-2 activation may not initiate but rather modulate already existing mucosal reactions [12]. In line with these considerations, selective inhibition of COX-2 did not affect the incidence of mouse tongue ulcers [8]. Moreover, a randomized double-blind placebo-controlled trial of celecoxib for oral mucositis in patients receiving radiation therapy for head-and-neck cancer also failed to prove a beneficial effect on the severity and/or the morbidity of mucositis [26]. Thalidomide is known to directly and indirectly (through NF-κB modulation) inhibit COX-2 expression and enhances the rate of COX-2 mRNA degradation [27]. It is therefore possible that this mechanism contributes to the mucositis-ameliorating properties of thalidomide.

Jaal et al. [28] observed a clear increase in endothelial ICAM-1 expression in the submucosa during fractionated irradiation of mouse tongue. In response to inflammatory stimuli, ICAM-1 expression is increased on multiple cell types, for example, human epithelial and endothelial cells and facilitates transendothelial migration of leukocytes [29–31]. Lin et al. [32] showed that thalidomide suppresses TNF-α induced ICAM-1 expression through inhibition of

NF-κB binding to the ICAM-1 promoter. The anti-inflammatory effect of thalidomide could hence also be (partly) due to the indirect inhibition of this adhesion molecule.

In oral mucosa regenerative processes in response to fractionated irradiation ("repopulation") start at the end of the first treatment week, and subsequently are responsible for the increase in radiation tolerance with increasing overall treatment time. The highly complex process consists of three major mechanisms: acceleration of stem cell proliferation, asymmetry loss of stem cell divisions, and abortive divisions of sterilized cells [33–35]. The significant radiation protection by thalidomide could be related to an interaction with any of these three mechanisms. Thalidomide application during the first week of fractionated irradiation may lead to an earlier onset of the compensatory regenerative response, thus, resulting in a decreased incidence of ulcerations. Furthermore, the interrelation with inhibition of NF-κB and the inflammatory signaling cascade may additionally stimulate, indirectly, one or more of the underlying mechanisms of repopulation.

Conclusion

In this study, a mucoprotective potential of thalidomide in radiation-induced oral mucositis during fractionated radiotherapy was demonstrated, presumably by inhibiting NF-κB and supporting epithelial repopulation. Since thalidomide is already approved for various therapeutic indications, it seems a promising drug for future clinical studies. However, further mechanistic studies are needed to clarify the biological mechanisms underlying the mucoprotective efficacy of this drug.

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Compliance with ethical guidelines

Conflict of interest K. Frings, S. Gruber, P. Kuess, M. Kleiter, and W. Dörr state that there are no conflicts of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed and necessary approval was obtained from the relevant authorities (BMWF, file no. 66.009/0038-II/3b/2014).

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