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Biology Contribution

Thalidomide Ameliorates Inflammation and Vascular Injury but Aggravates Tubular Damage in the Irradiated Mouse Kidney

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Summary

Kidney-irradiated mice (14 Gy) were treated with thalidomide to prevent normal tissue toxicity. Thalidomide improved vessel perfusion and pericyte coverage at 40 weeks after irradiation. The drug also reduced irradiation-mediated inflammatory cell infiltration; however, it could not prevent the development of radiation-induced fibrosis. Renal function was also not rescued, probably because of extensive irradiation-induced tubular damage.

Purpose: The late side effects of kidney irradiation include vascular damage and fibrosis, which are promoted by an irradiation-induced inflammatory response. We therefore treated kidney-irradiated mice with the anti-inflammatory and angiogenesis-modulating drug thalidomide in an attempt to prevent the development of late normal tissue damage and radiation nephropathy in the mouse kidney.

Methods and Materials: Kidneys of C57Bl/6 mice were irradiated with a single dose of 14 Gy. Starting from week 16 after irradiation, the mice were fed with thalidomide-containing chow (100 mg/kg body weight/day). Gene expression and kidney histology were analyzed at 40 weeks and blood samples at 10, 20, 30, and 40 weeks after irradiation.

Results: Thalidomide improved the vascular structure and vessel perfusion after irradiation, associated with a normalization of pericyte coverage. The drug also reduced infiltration of inflammatory cells but could not suppress the development of fibrosis. Irradiation-induced changes in hematocrit and blood urea nitrogen levels were not rescued by thalidomide. Moreover, thalidomide worsened tubular damage after irradiation and also negatively affected basal tubular function.

Conclusions: Thalidomide improved the inflammatory and vascular side effects of kidney irradiation but could not reverse tubular toxicity, which probably prevented preservation of kidney function. © 2014 Elsevier Inc.

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Conflict of interest: none.

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Introduction

Nephropathy is a serious late complication in abdominal radiation therapy. It may appear after irradiation with fields in which one or both kidneys are exposed, and it can lead to chronic renal failure occurring months or years after irradiation. Radiation nephropathy is partially caused by irradiation-induced cellular damage affecting glomeruli, blood vessels, tubular epithelium, and interstitium (1). Irradiation also modifies the tissue microenvironment, leading to increased expression of proinflammatory and profibrotic factors, which not only aids the development of tissue fibrosis but probably also hampers the repair of damaged blood vessels (2, 3). An effective intervention to prevent or reduce radiation-induced renal failure in cancer survivors would increase the long-term therapeutic ratio of radiation therapy.

Thalidomide and its analogs are drugs that are known for their immunomodulatory, anti-inflammatory, and angiogenesis-regulating properties. Thalidomide was introduced in the 1960s as a sedative and to treat morning sickness in pregnant women worldwide (4). However, increased rates of spontaneous abortions, stillbirths, and infant mortality and the occurrence of severe limb malformations and other congenital defects led to its quick withdrawal from the market. Since then, many studies have addressed the biological activities of thalidomide. In 1994, D'Amato et al (5) postulated that the drugs' negative effects on the growing limb came from interfering with angiogenesis. Subsequent studies from different groups showed that thalidomide induces oxidative stress and affects angiogenesis, probably by inhibiting fibroblast growth factor, Wnt, and Akt signaling (6). Therapontos et al (7) demonstrated that thalidomide mainly affected endothelial tip cells, thereby inducing loss of newly formed but not of mature blood vessels in the developing chicken limb. Further insights into the vascular effects of thalidomide came from a study from Lebrin et al (8), who showed that in patients with the vascular disorder hereditary hemorrhagic telangiectasia (HHT) and in endoglin mutant mice ($Eng^{+/-}$), in which age-dependent vascular lesions develop that are similar to those seen in individuals with HHT, thalidomide normalized aberrant angiogenesis by improving mural cell coverage. This was mainly accomplished by upregulating platelet-derived growth factor B (*Pdgfb*) mRNA expression in endothelial cells, thereby stimulating the recruitment of vessel-stabilizing pericytes and also by inducing pericyte proliferation. Yet, neither of the studies conducted so far can sufficiently explain all the vascular effects of thalidomide nor the mechanisms of tissue and species specificity.

Thalidomide and its derivatives are also potent immunomodulators, with effects on cytokine production and T cell activity (9). Accordingly, thalidomide has been shown to have anti-inflammatory and antifibrotic properties in animal models of myocardial, peritoneal, and pulmonary fibrosis (10-14). This was accomplished by suppressing the production of proinflammatory and profibrotic cytokines and growth factors such as tumor necrosis factor-α, interleukin-1β, interleukin-6, or transforming growth factor-β (TGF-β). Furthermore, the drug also reduced fibroblast proliferation and transdifferentiation into collagen-producing myofibroblasts and thus production of collagen. In the kidney, thalidomide was shown to decrease proteinuria and to lower glomerular and tubular damage in lupus-prone mice (15). It also protected against tubulointerstitial injury in adenine-induced chronic kidney disease in mice (16). Owing to its immunomodulatory, anti-inflammatory, and antiangiogenic characteristics, thalidomide and its derivatives are once again in clinical use to treat multiple myeloma and inflammatory complications of leprosy (erythema nodosum leprosum) and also various other inflammatory and oncologic conditions (4).

We showed in previous studies that kidney irradiation with a single irradiation dose of 14 or 16 Gy triggers proinflammatory cytokine expression, infiltration of inflammatory cells, and the development of fibrosis (17, 18). In addition, irradiated mice developed multiple vascular defects, including reduced vessel perfusion, loss of capillaries, and telangiectasia (17, 19). We also observed focal accumulation of pericytes, which was interpreted as differentiation of pericytes into fibrosis-promoting myofibroblasts (19). In the current study, we therefore investigated whether thalidomide treatment could reduce these side effects of irradiation and prevent radiation-induced nephropathy.

Methods and Materials

All experiments were in accordance with the Dutch Act on Animal experimentation and approved by the Animal Experiments Committee of the Netherlands Cancer Institute (10009_100309_VRA). Groups of 9 to 12 female C57BL/6 mice with a starting weight of at least 21 g were irradiated with a single dose of 14 Gy (2.07 Gy/min). Every 10 weeks after irradiation, blood samples were taken for determination of hematocrit and blood urea nitrogen (BUN) (Pointe Scientific). On the basis of our previous studies, thalidomide treatment was started 16 weeks after irradiation, which is 4 weeks before the onset of detectable tissue damage and impairment of kidney function. The mice were receiving approximately 100 mg/kg body weight/day (300 mg/m^2/day) of the drug. One week before being killed, the mice underwent single photon emission computed tomography (SPECT) of the kidney with the use of technetium-labeled mercaptoacetyltriglycine (^{99m}Tc MAG3, Covidien) as a renal imaging agent. The anesthetized mice were injected with 200 μL (∼14 MBq) ^{99m}Tc MAG3. Images of the renal region were then acquired at 15 s/frame for 20 frames. Five minutes before being killed, the mice were injected with the perfusion marker fluorescein isothiocyanate (FITC)-dextran (2000 kDa, Sigma-

Aldrich). At 40 weeks, the mice were killed, and the kidneys were either snap-frozen for cryosectioning and mRNA isolation or formalin-fixed and processed for paraffin embedding. Digital image analysis of the Sirius Red (collagen) and the CD45 (leukocytes) staining was performed with Leica QWin image analysis and processing software (Leica microsystems). Dextran perfusion and pericyte accumulation were measured with ImageJ (Wayne Rasband, NIH). All image analyses were conducted blindly. The SPECT images were analyzed with InVivoScope software (Bioscan). The Kruskal-Wallis and Mann-Whitney tests were used to determine differences between treatment groups. Graphs display the averaged group values ± standard equivalent of the mean. *P* values smaller than .05 were considered to be statistically significant.

For detailed materials and methods, see Supplementary Data 1 (available online at www.redjournal.org).

Results

Thalidomide reduces inflammation but not fibrosis in the irradiated kidney

First, we investigated whether thalidomide affected inflammatory cell numbers in the irradiated kidney. Staining of kidney sections with the pan leukocyte marker CD45 showed that irradiation strongly upregulated the infiltration of inflammatory cells in comparison with the nonirradiated controls ($P<.002$) (Fig. 1A and B). Thalidomide treatment significantly reduced the number of $CD45^+$ cells after kidney irradiation ($P<.011$), implying a reduced inflammatory response.

Next, we investigated whether the reduced irradiation-induced inflammatory response after thalidomide treatment also affected fibrosis development. Collagen deposition, as readout for fibrosis, was visualized with a Sirius red staining (Fig. 2A). Quantification of positively stained areas showed that irradiation significantly increased collagen deposition ($P<.013$); however, this was not altered by thalidomide (Fig. 2A and B). Taking these results together, we showed that despite significantly reduced inflammatory cell infiltration, thalidomide could not prevent profibrotic changes after kidney irradiation.

Improved vessel perfusion and pericyte numbers after irradiation and thalidomide treatment

The effects of thalidomide on the vascular system were assessed by investigating vessel perfusion. To this end, mice were injected with the fluorescently labeled polysaccharide dextran. Larger dextrans as used for this study (2000 kDa) are excreted poorly from the kidney and therefore remain in the blood until they are metabolized. Vascular perfusion was analyzed by measuring the FITC-positive area per section showing that irradiation obstructs vessel perfusion, probably through capillary loss ($P<.021$) (Fig. 3A upper panels and 3B). Thalidomide treatment protected the honeycomb-like vascular network in the irradiated kidneys and prevented irradiation-induced reduction in vessel perfusion (Fig. 3A lower panels and 3B). Yet, thalidomide also affected the nonirradiated control group: perfusion was slightly lower in that group than in the nonirradiated animals without thalidomide.

Previous studies from our group had shown that kidney irradiation induces a multifocal increase in the number of pericytes, suggesting pericyte-to-myofibroblast differentiation (19). To assess the impact of thalidomide on pericyte localization after irradiation, kidney sections were stained for the pericyte marker platelet-derived growth factor receptor-β (PDGFR-β). Positively stained structures with a

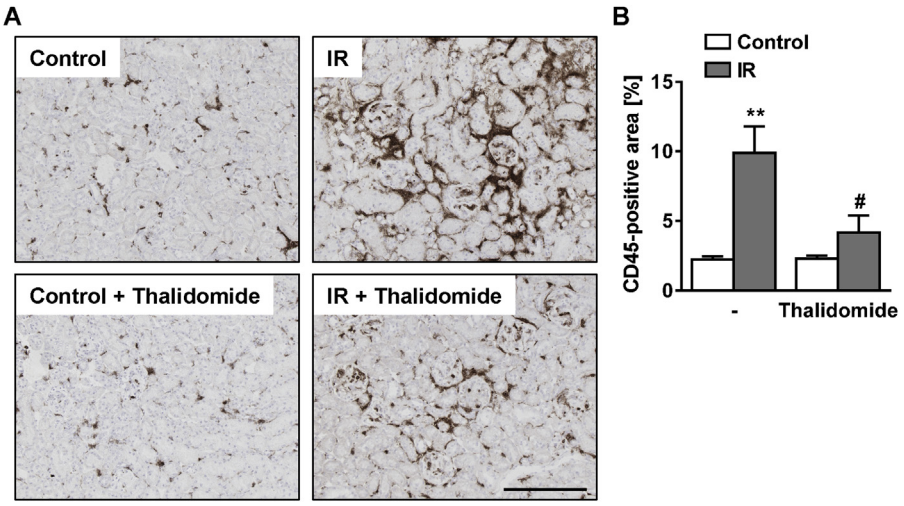

Fig. 1. (A) Kidney infiltration of inflammatory cells stained for CD45 at 40 weeks after irradiation. (B) Determination of CD45-positive area. Bars represent the mean group value ± standard error of the mean. IR = ionizing radiation. x200 magnification, scale bar = 200 μm. ∗,#Significant difference compared with the corresponding nonirradiated control or compared to mice without thalidomide treatment, respectively.

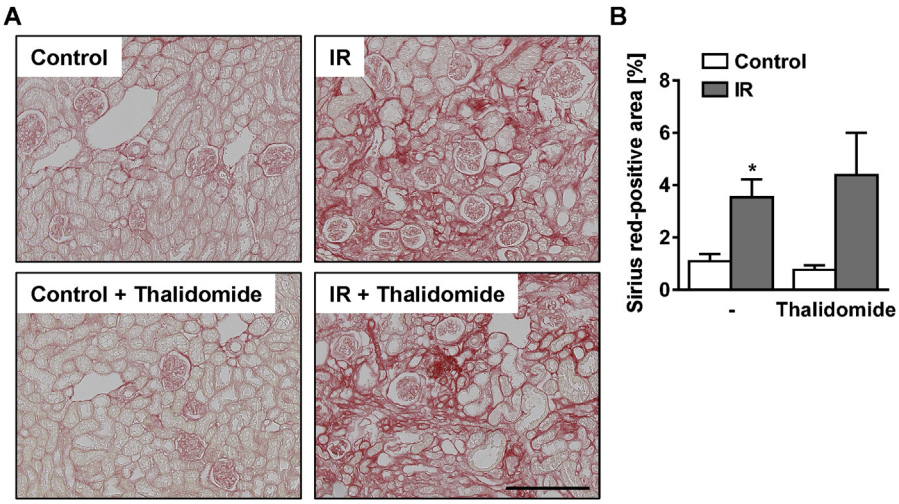

Fig. 2. (A) Staining for collagen deposition (Sirius red) at 40 weeks after kidney irradiation. (B) Quantification of Sirius red−positive areas. Bars represent the mean group value ± standard error of the mean. IR = ionizing radiation. x200 magnification, scale bar = 200 μm. ∗Significant difference compared with the corresponding nonirradiated control.

size larger than 150^2 pixels were considered to be pericyte accumulation. Quantification demonstrated that kidney irradiation induced the accumulation of pericytes ($P<.008$) and that this was blocked by thalidomide ($P<.023$) (Fig. 3C and D). Pericytes are recruited to the vasculature by endothelial cell-expressed *Pdgfb* (20). In addition, thalidomide has been shown to modulate *Pdgfb* levels and in this way pericyte coverage of the vasculature (8). Determination of *Pdgfb* levels in pooled whole kidney lysates showed that thalidomide slightly upregulates its expression in the irradiated kidneys but not in control kidneys (Supplementary Data 2, available online at ww.redjournal.). In summary, thalidomide prevents deterioration of vessel perfusion after irradiation and normalizes pericyte accumulation.

Thalidomide does not prevent tubular damage in the irradiated kidney

We then asked whether reduced inflammation, better vessel perfusion, and normalized pericyte numbers after thalidomide treatment also have an impact on kidney function after irradiation. One possibility to assess radiation-induced tubular damage is to measure uptake and excretion of the tracer ^{99m}Tc MAG3 with SPECT. In the resulting renogram, the ascending part of the curve (until ~9 minutes after tracer injection) reflects extraction of the tracer from the blood and tubular uptake. Tracer excretion can be deduced from the descending part of the curve. The steeper the curve, the better is tubular function. Our data show that ^{99m}Tc MAG3 uptake and excretion were compromised in both irradiated groups compared with the respective controls ($P<.0001$) (Fig. 4A). They also demonstrate that thalidomide worsens tracer excretion after irradiation ($P<.0001$). Interestingly, thalidomide also affected the control group: nonirradiated thalidomide-treated mice displayed impaired tracer uptake and excretion compared with the control group without thalidomide ($P<.0001$).

In agreement with the functional data, analysis of sections stained with hematoxylin and eosin showed that irradiation induced extensive tubular damage in both irradiated groups, especially in the renal cortex. The tubuli were almost depleted by irradiation, indicating that no or only very little regeneration had taken place (Fig. 4B, I). Irradiation also induced glomerular injury, although this was less severe compared with the tubular damage. We observed enlargement of Bowman's space (Fig. 4B, I), thickening of the glomerular basement membrane, and hypertrophy (Fig. 4B, II) and hyperplasia (Fig. 4B, III) of the parietal cell layer of Bowman's capsule. In both irradiated groups, changes in glomerular tufts with reduction in capillary lumen (Fig. 4B, IV), but also capillary dilation (telangiectasia) (Fig. 4B, V), occurred. Interestingly, despite the functional impairment of tubuli described above, there was no microscopically detectable morphologic damage in the thalidomide-treated control group. Taken together, these results show that thalidomide is unable to improve ^{99m}Tc MAG3 uptake and excretion after irradiation, probably as a result of persistent tubular damage.

Thalidomide does not affect functional kidney parameters after irradiation

The effects of thalidomide on radiation-induced changes in kidney function were also determined by measuring hematocrit and BUN. Hematocrit is the volume percentage of red blood cells in the blood and a readout for anemia. Irradiation led to a significant reduction in hematocrit values in both irradiated groups at 40 weeks ($P<.013$ and $P<.017$) (Fig. 5A). This could not be reversed by thalidomide treatment. BUN also gives an indication of renal

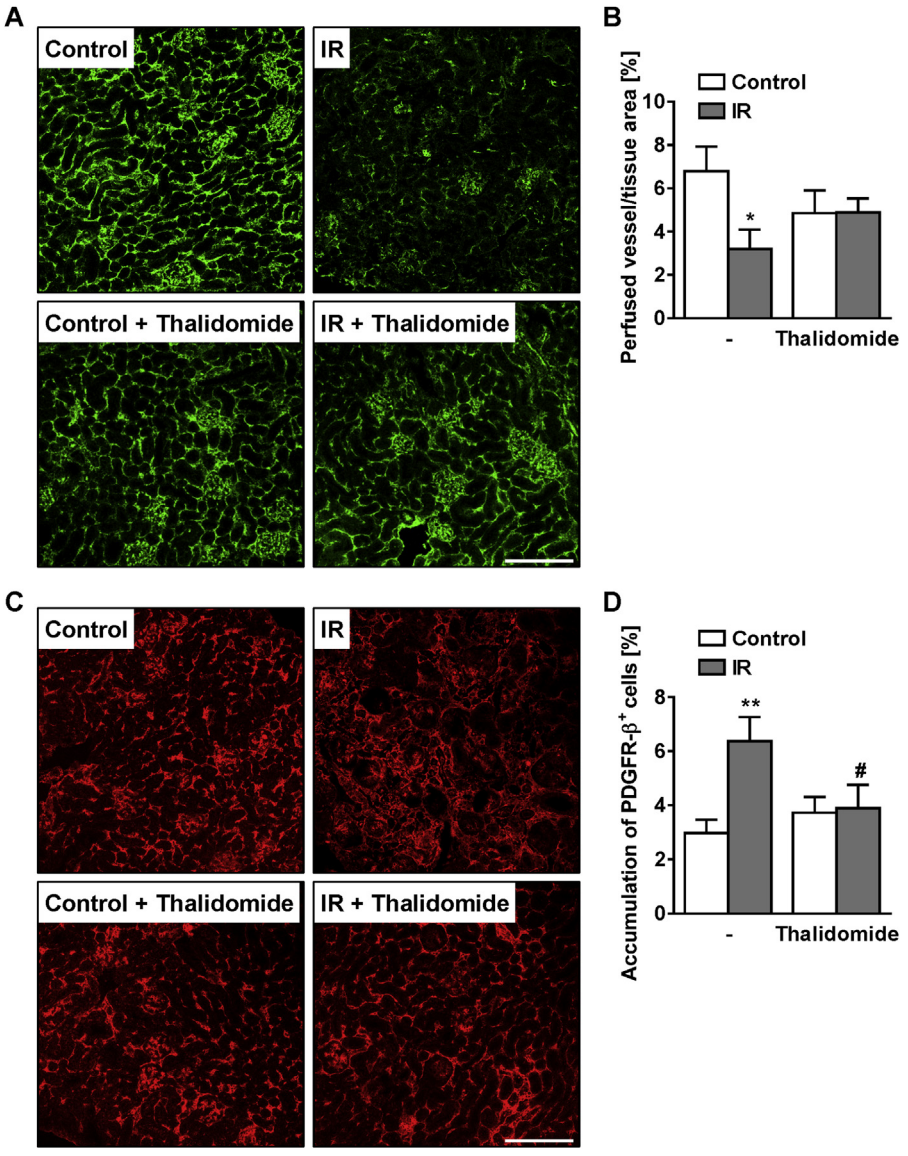

Fig. 3. (A) Injection of the perfusion marker fluorescein isothiocyanate (FITC) dextran (2000 kDa) at 40 weeks after irradiation. (B) Quantification of FITC-dextran perfusion. (C) Staining for the pericyte marker platelet-derived growth factor receptor-β (PDGFR-β). (D) Quantification of the PDGFR-β−positive area. IR = ionizing radiation. x200 magnification, scale bar = 200 μm. ∗,#Significant difference compared with the corresponding nonirradiated control or compared with mice without thalidomide treatment, respectively.

function. If glomerular filtration rate is disturbed, BUN levels will increase. Irradiated mice showed a significant increase in BUN levels starting from 20 to 30 weeks after irradiation ($P<.019$ and $P<.001$), which further increased at 40 weeks ($P<.002$) (Fig. 5B). Yet, thalidomide treatment could not circumvent an increase in BUN levels after irradiation. In summary, thalidomide does not ameliorate changes in hematocrit and BUN after irradiation.

Discussion

We showed in previous studies that kidney irradiation triggered inflammatory cell infiltration and the development of fibrosis (17, 18). Kidney irradiation also resulted in reduced vessel perfusion, loss of small blood vessels, and capillary dilation (19). In this study, we therefore treated mice with the drug thalidomide, which is known for its anti-inflammatory and angiogenesis-modulating properties (9). We showed that thalidomide indeed inhibited the inflammatory processes after irradiation in the kidney; however, it could not impede the development of fibrosis. Irradiation-induced fibrosis is mediated by fibroblast differentiation and continuous activation of profibrotic signaling pathways (21). This can be further aggravated by inflammatory cells through the production of proinflammatory and profibrotic cytokines (22, 23). Considering the strong reduction in the radiation-

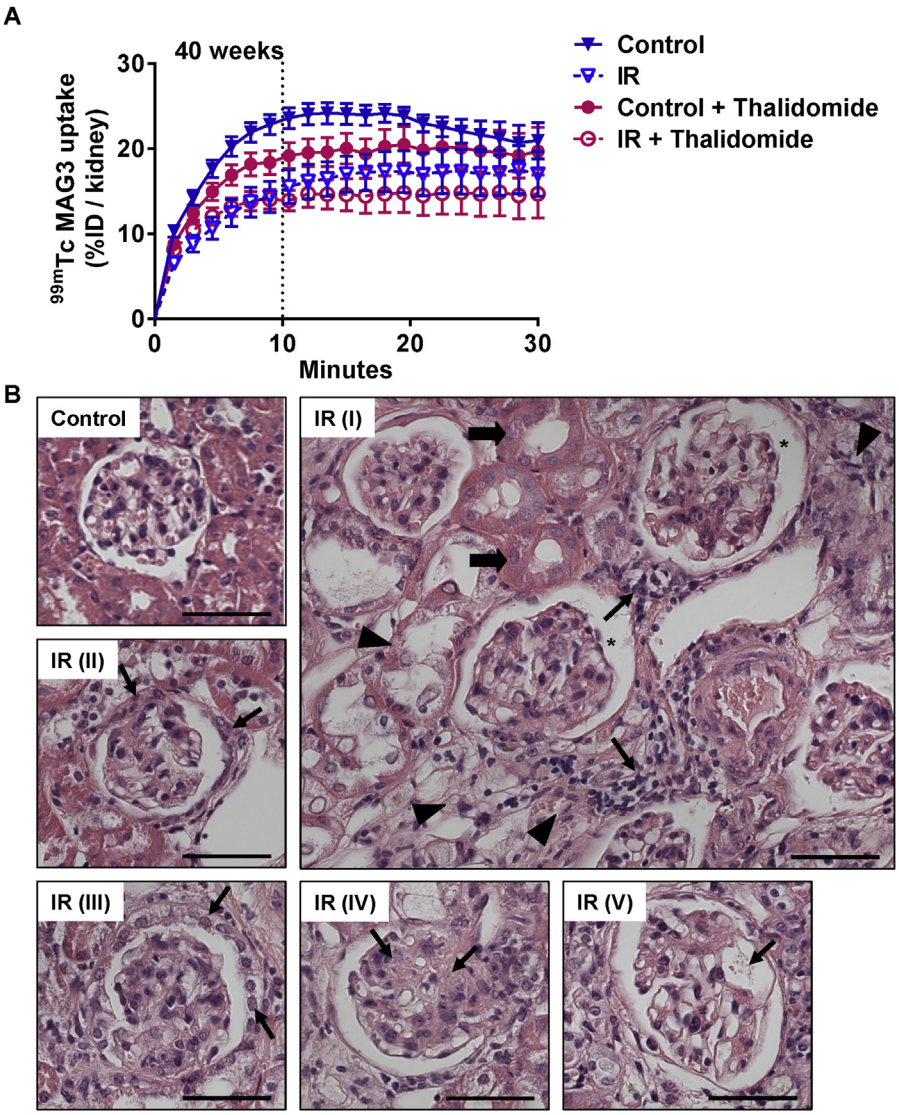

Fig. 4. (A) Single photon emission computed tomographic image of kidney with use of the tracer ^{99m}Tc MAG3 at 40 weeks after irradiation. Graphs show the uptake of the radioactive tracer corrected for the injected dose (%ID) over time. Dashed line separates ascending from the descending part of the curve and thus extraction of the tracer from the blood and tubular uptake from tracer excretion. (B) Kidney morphology (hematoxylin and eosin) at 40 weeks in the non-irradiated control group and irradiated (I–V) animals at × 400 magnification. (I) Degeneration and depletion of renal tubuli (*arrowheads*). Remaining intact tubuli are marked with wide arrows. Thin arrows depict inflammatory infiltrate. Asterisks designate enlarged Bowman's space. Hyperplasia (II) and hypertrophy (III) of the parietal cell layer in the Bowman's capsule (*arrows*). Glomerulus with narrowed capillaries (IV) or with a telangiectatic capillary (V) (*arrows*). Scale bar = 50 µm. IR = ionizing radiation.

induced inflammatory response after thalidomide treatment, this may suggest that CD45$^+$ cells are not the main source of profibrotic cytokines in the irradiated kidney. It is also possible that the observed reduction in the number of inflammatory cells would have slowed the progression of kidney fibrosis at time points beyond 40 weeks. Another option would be that fibrosis development had already been irreversibly initiated before the start of thalidomide treatment, thereby limiting the impact of the reduced inflammatory response.

Vascular damage is a major factor contributing to the decline in renal function after kidney damage (24). Analysis of vascular injury with the perfusion marker FITC-dextran demonstrated that irradiation-induced destruction of the honeycomb-like vascular network was prevented by thalidomide, resulting in better vessel perfusion. This might have been a direct effect of thalidomide on endothelial cell proliferation and survival. It is also possible that the impaired inflammatory response positively affected blood vessels through reduced production of proinflammatory mediators that might otherwise hamper vessel repair after irradiation.

Pericytes are embedded within the basement membrane of microvessels, where they tightly surround the endothelium (20). During angiogenesis, *Pdgfb* recruits pericytes to the nascent endothelium, where they promote endothelial

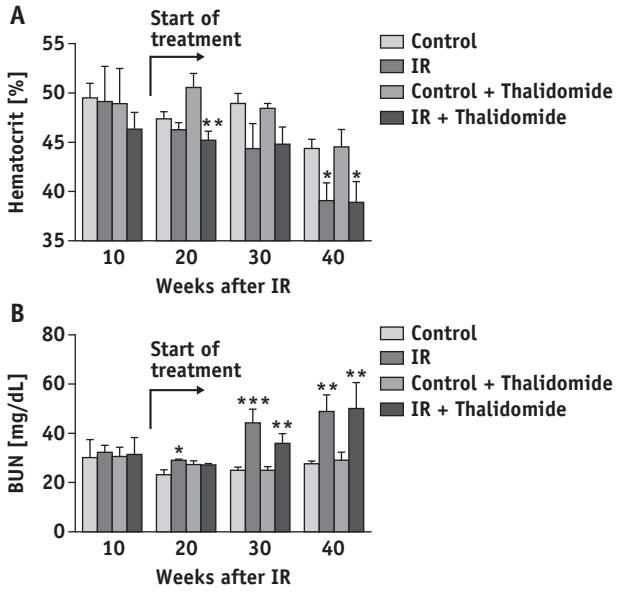

Fig. 5. (A) Determination of the percentage of red blood cells in blood plasma (hematocrit). (B) Analysis of BUN levels in serum. IR = ionizing radiation. ∗Significant difference compared with the corresponding nonirradiated control.

stability and maturation (25). A recent study showed that thalidomide enhances pericyte recruitment in the postnatal mouse retina—an environment actively undergoing angiogenesis—by the upregulation of *Pdgfb* expression in endothelial tip cells (8). We observed an upregulation of approximately 2-fold of *Pdgfb* expression after irradiation in whole kidney lysates. Yet, whether this increase promoted pericyte recruitment and thus vascular function needs to be determined. In the injured kidney, pericytes may also contribute to scar formation and fibrosis (26). They become activated by a combination of growth factors, including PDGF-β and TGF-β, that are produced during inflammation. As a consequence, pericytes detach, move away from capillaries, and differentiate by a PDGFR-β–dependent process into collagen-producing myofibroblasts (25-27). We recently reported multifocal cortical accumulation of pericytes after kidney irradiation at sites distant from blood vessels (19). We now demonstrate that irradiation-induced pericyte accumulation is normalized by thalidomide. Yet, increased *Pdgfb* levels after irradiation and thalidomide treatment suggest that this was not accomplished by interference with PDGF-B signaling. Instead, we propose that by altering the inflammatory response, thalidomide downregulates the production of growth factors such as TGF-β, thereby leading to reduced pericyte differentiation and accumulation.

Despite normalized vascular and inflammatory parameters, renal function (BUN, hematocrit) was not enhanced by thalidomide treatment after irradiation. Accordingly, kidney imaging demonstrated that thalidomide failed to rescue irradiation-mediated impairment of tracer uptake and excretion. Inasmuch as renography is a readout for both vascular and tubular function, this strongly suggests that persistent tubular damage was the main contributor to reduced renal function after irradiation. This hypothesis is supported by histologic evidence of clear tubular injury in both irradiated groups regardless of thalidomide treatment. The mechanism by which this was accomplished is not clear. In the kidney, thalidomide may promote apoptosis by suppressing NF-κB activation, which is mediated by oxidative stress or proinflammatory stimuli (28, 29). Given that irradiation induces both oxidative stress and activation of NF-κB (30), it is likely that aggravation of irradiation-induced renal damage by thalidomide was achieved by promoting tubular apoptosis. Yet, these studies also demonstrated that thalidomide did not affect NF-κB activation in the control groups.

Thalidomide elicits a multitude of different effects (9), which might be potentiated when the drug is used at higher doses. Thus, reducing the thalidomide dose might have prevented deterioration of kidney function in the nonirradiated control group. Chade et al (31) demonstrated that in the hypercholesteremic pig kidney, low doses of thalidomide 4 mg/kg/day reduced pathologic angiogenesis and inflammation but also negatively affected basal renal blood flow and glomerular filtration rate. By contrast, in mouse models of lupus or chronic kidney disease, thalidomide doses of 10 to 30 mg/kg/day were shown to successfully reduce macrophage infiltration, BUN levels, proteinuria, glomerular damage, and tubular damage without causing further toxicities (15, 16). Taken together these results suggest that the side effects of thalidomide are not only determined by drug dose but also highly dependent on the animal or disease model used.

In summary, we show that thalidomide reduces kidney inflammation and rescues vascular function but that it cannot prevent fibrosis and irradiation-induced tubular damage. Our data also demonstrate that well-perfused blood vessels are essential, but not sufficient, to maintain kidney function and that tubular damage also needs to be minimized. Yet, the deleterious effect of thalidomide on nonirradiated tubuli precludes its use in the treatment of late irradiation nephropathy. Future studies are needed to assess whether lowering of drug dose, changing the start of treatment, or shortening the treatment time will reduce the negative side effects in the irradiated kidney.

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