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Published in:
Radiotherapy and Oncology

DOI:
10.1016/j.radonc.2013.06.016

Publication date:
2013

Radiotherapy and Oncology 108 (2013) 464–468

Contents lists available at SciVerse ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Normal tissue radiobiology

Endoglin haploinsufficiency attenuates radiation-induced deterioration of kidney function in mice

Marion Scharpfenecker [a,*], Ben Floot [a], Nicola S. Russell [b], Rob P. Coppes [c,d], Fiona A. Stewart [a]

[a] Division of Biological Stress Response; [b] Division of Radiotherapy, The Netherlands Cancer Institute, Amsterdam; [c] Department of Radiation Oncology; and [d] Department of Cell Biology, University Medical Centre Groningen, University of Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 15 April 2013
Received in revised form 7 June 2013
Accepted 11 June 2013
Available online 9 July 2013

Keywords:
Endoglin
Pericytes
Vasculature
Perfusion
Fibrosis
Kidney

ABSTRACT

Background and Purpose: Endoglin is a transforming growth receptor beta (TGF-β) co-receptor, which plays a crucial role in the development of late normal tissue damage. Mice with halved endoglin levels ($Eng^{+/-}$ mice) develop less inflammation, vascular damage and fibrosis after kidney irradiation compared to their wild type littermates ($Eng^{+/+}$ mice). This study was aimed at investigating whether reduced tissue damage in $Eng^{+/-}$ mice also results in superior kidney function.

Material and Methods: Kidneys of $Eng^{+/+}$ and $Eng^{+/-}$ mice were irradiated with a single dose of 14 Gy. Functional kidney parameters and kidney histology were analysed at 20, 30 and 40 weeks after irradiation.

Results: $Eng^{+/-}$ mice displayed improved kidney parameters (haematocrit, BUN) compared to $Eng^{+/+}$ mice at 40 weeks after irradiation. Irradiation of $Eng^{+/+}$ kidneys damaged the vascular network and led to an increase in PDGFR-β positive cells, indicative of fibrosis-promoting myofibroblasts. Compared to $Eng^{+/+}$ kidneys, vascular perfusion and number of PDGFR-β positive cells were reduced in $Eng^{+/-}$ control mice; however, this did not further deteriorate after irradiation.

Conclusions: Taken together, we show that not only kidney morphology, but also kidney function is improved after irradiation in $Eng^{+/-}$ compared to $Eng^{+/+}$ mice.

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Endoglin is a transforming growth factor beta (TGF-β) co-receptor, which is expressed at low levels in resting endothelial cells, but becomes upregulated in activated endothelium at sites of angiogenesis, vascular injury or inflammation [1]. Endoglin is not only expressed in endothelial cells, but also in macrophages, vascular smooth muscle cells, mesangial cells and fibroblasts. In the kidney, increased endoglin levels have been associated with fibrosis development in experimental models of renal disease [2,3]. Reduced endoglin levels have been linked with a decrease in endothelial cell activation, inflammation and kidney injury after ischaemia-reperfusion [4]. We showed in previous studies that endoglin also plays a crucial role in the development of irradiation-induced kidney damage. Mice with halved endoglin levels ($Eng^{+/-}$ mice) [5,6] displayed less vascular damage and fibrosis after kidney irradiation compared to their wild type littermates ($Eng^{+/+}$ mice) [7]. This was accompanied by decreased expression of pro-fibrotic TGF-β downstream target genes. We also showed that kidney irradiation triggered infiltration of macrophages in $Eng^{+/+}$ mice, which was reduced in $Eng^{+/-}$ mice [8]. As macrophages are able to promote development of kidney damage by producing excess pro-inflammatory, pro-fibrotic and anti-angiogenic cytokines, we suggested that reduced macrophage infiltration was probably – at least in part – the underlying cause for decreased development of normal tissue damage in the irradiated kidneys of $Eng^{+/-}$ mice.

The current study was aimed at investigating whether the observed differences in histology between irradiated $Eng^{+/+}$ and $Eng^{+/-}$ mice were also reflected in differences in functional kidney parameters. Mouse kidneys were irradiated with a single dose of 14 Gy and kidney function was investigated after 20, 30 and 40 weeks. We show that at 40 weeks after irradiation, kidney function was significantly better in $Eng^{+/-}$ compared to $Eng^{+/+}$ mice. Improved function also coincided with superior kidney morphology and reduced fibrosis formation in $Eng^{+/-}$ mice. Taken together we show that endoglin regulates normal tissue damage development in the irradiated mouse kidney on morphological and functional level.

Materials and methods

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). Female $Eng^{+/+}$ and $Eng^{+/-}$ C57BL/6 mice (originally obtained from H. Arthur, Institute of Human Genetics, International Centre for Life, Newcastle upon Tyne, UK and subsequently bred in our institute) were irradiated with a single dose of

* Corresponding author. Address: The Netherlands Cancer Institute (NKI), Division of Biological Stress Response, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.
E-mail address: m.scharpfenecker@nki.nl (M. Scharpfenecker).

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http://dx.doi.org/10.1016/j.radonc.2013.06.016

14 Gy (age 10–14 weeks). Groups of 8–14 mice were sacrificed at 20, 30 and 40 weeks after irradiation. One week before sacrifice, the 40 weeks group was subjected to kidney single photon emission computed tomography (SPECT) using technetium-labelled mercaptoacetyltriglycine (^{99m}Tc MAG3, Covidien) as renal imaging agent. At sacrifice, blood samples were taken from all groups and haematocrit and blood urea nitrogen (BUN) (Pointe Scientific) were determined. Five minutes before sacrifice, mice were injected with the perfusion marker FITC-dextran (2000 kDa, Sigma-Aldrich). Kidneys were harvested and either snap-frozen or processed for paraffin embedding. The Leica QWin image analysis and processing software (Leica microsystems) was used to analyse the Sirius red and the CD45 staining. ImageJ (Wayne Rasband, NIH) was applied to analyse dextran perfusion and the PDGFR-β staining. SPECT images were analysed using the InVivoScope software (Bioscan). Graphs display the averaged group values +/− SEM. *P* values smaller than 0.05 were considered as statistically significant. $^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, $^{***,\#\#\#}P < 0.001$, $^{****,\#\#\#\#}P < 0.0001$. For detailed materials and methods: see supplementary data.

Results

Improved functional kidney parameters in irradiated Eng$^{+/-}$ mice

In order to determine whether kidney irradiation was accompanied by a decline in general health, we determined the average weight change of each treatment group over periods of 10 weeks. Control and irradiated *Eng*$^{+/+}$ and *Eng*$^{+/-}$ mice equally gained weight during the first 20 weeks of the experiment (Supplementary Fig. 1A). However, by 40 weeks, irradiated and non-irradiated *Eng*$^{+/-}$ mice were significantly heavier than their respective *Eng*$^{+/+}$ littermates.

Haematocrit is the volume percentage of red blood cells in blood. Reduction of haematocrit is a readout for anaemia reflecting the inability of the failing kidneys to secrete the hormone erythropoietin, which stimulates bone marrow to produce red blood cells.

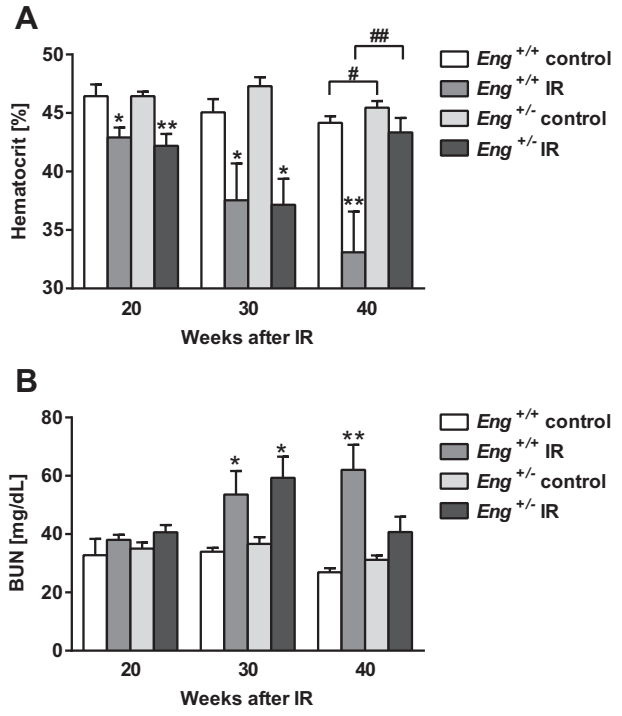

Fig. 1. (A) Determination of the percentage of red blood cells in blood plasma (haematocrit). (B) Analysis of BUN levels in serum. IR: irradiation. *,#Significant difference compared to the control or compared to *Eng*$^{+/+}$ mice with the same treatment, respectively.

In both irradiated *Eng*$^{+/+}$ and *Eng*$^{+/-}$ mice, haematocrit was significantly lower compared to the respective controls at 20 and 30 weeks (Fig. 1A). At 40 weeks, haematocrit was further reduced in irradiated *Eng*$^{+/+}$ mice, but not in irradiated *Eng*$^{+/-}$ mice compared to the respective control groups. We also measured blood urea nitrogen (BUN) levels. Urea is a by-product of protein metabolism in the liver, which is cleared from the bloodstream by the kidneys. If kidney function is disturbed, BUN levels in the blood rise. BUN levels were significantly elevated in irradiated *Eng*$^{+/+}$ and *Eng*$^{+/-}$ mice at 30 weeks after irradiation compared to the non-irradiated controls (Fig. 1B). At 40 weeks, BUN levels further increased in irradiated *Eng*$^{+/+}$ mice, but not in Eng$^{+/-}$ animals compared to the respective controls. In summary, we show that kidney parameters in *Eng*$^{+/-}$ mice are superior to those in *Eng*$^{+/+}$ mice at 40 weeks after irradiation.

Reduced decline of tubule function, vascular perfusion and pericyte coverage in irradiated Eng$^{+/-}$ mice

We further assessed kidney function by SPECT using the tracer ^{99m}Tc MAG3. This imaging agent is almost exclusively excreted by secretion in the proximal tubule and therefore a good readout for renal tubular function. In the resulting renogram, the ascending part of the curve is due to extraction of the tracer from the blood and thus reflects renal blood flow and tubular uptake. The descending part of the curve is due to tubular excretion and reflects drainage function of the kidney. A steeply sloping curve indicates proper kidney function whereas flattening of the curve shows tubular damage. Quantification of SPECT measurements demonstrated that irradiation significantly reduced ^{99m}Tc MAG3 uptake in kidneys of *Eng*$^{+/+}$ and *Eng*$^{+/-}$ mice. However, the difference between non-irradiated and irradiated kidneys was much less in *Eng*$^{+/-}$ than in *Eng*$^{+/+}$ mice (Fig. 2A). The flattened descending part of the curve showed that irradiation hampered tubular secretion of the tracer in both irradiated groups. Interestingly, in the control groups, tracer uptake was higher in *Eng*$^{+/+}$ compared to *Eng*$^{+/-}$ mice, although tracer excretion was superior in the *Eng*$^{+/-}$ control group ($P < 0.0001$).

The effect of irradiation on blood flow and vessel structure was further investigated by injecting fluorescently-labelled polysaccharide dextran. In kidney glomeruli, molecules of low molecular weight (<60,000 Dalton) are filtered out of the blood. We used dextran with a molecular weight of 2,000,000 Dalton, which cannot be excreted by the kidneys but needs to be metabolised to be removed from the blood. Analysis of renal perfusion on frozen sections showed that non-irradiated *Eng*$^{+/+}$ animals displayed a well-perfused honeycomb-like vascular network (Fig. 2B). Of note, this network was disturbed in *Eng*$^{+/-}$ control mice and the perfused vessel area was significantly reduced (Fig. 2B and D). Irradiation severely damaged the vasculature in *Eng*$^{+/+}$ mice as evidenced by a significant decrease in the number of FITC-dextran perfused vessels. In contrast to irradiated *Eng*$^{+/+}$ mice, the vascular network was only slightly affected in irradiated *Eng*$^{+/-}$ mice.

Platelet-derived growth factor receptor beta (PDGFR-β) is a marker for pericytes, which surround small blood vessels and regulate blood flow and endothelial cell homoeostasis [9]. During kidney injury, pericytes may lose contact with endothelial cells thereby destabilising capillaries. Moreover, pericytes form a major source of scar-producing cells by differentiating, via a PDGFR-β dependent process, into collagen-producing myofibroblasts [10,11]. Kidney irradiation of *Eng*$^{+/+}$ mice induced a strong multifocal increase in PDGFR-β positive cells, which was not observed in irradiated *Eng*$^{+/-}$ mice (Fig. 2C(i)). However, irradiation of *Eng*$^{+/+}$ mice also induced pericyte loss in other kidney areas (Fig. 2C(ii)). The combination of these two phenotypes led to non-significant differences between the control and the irradiated *Eng*$^{+/+}$ group when the PDGFR-β positive area was quantified (Fig. 2C and E

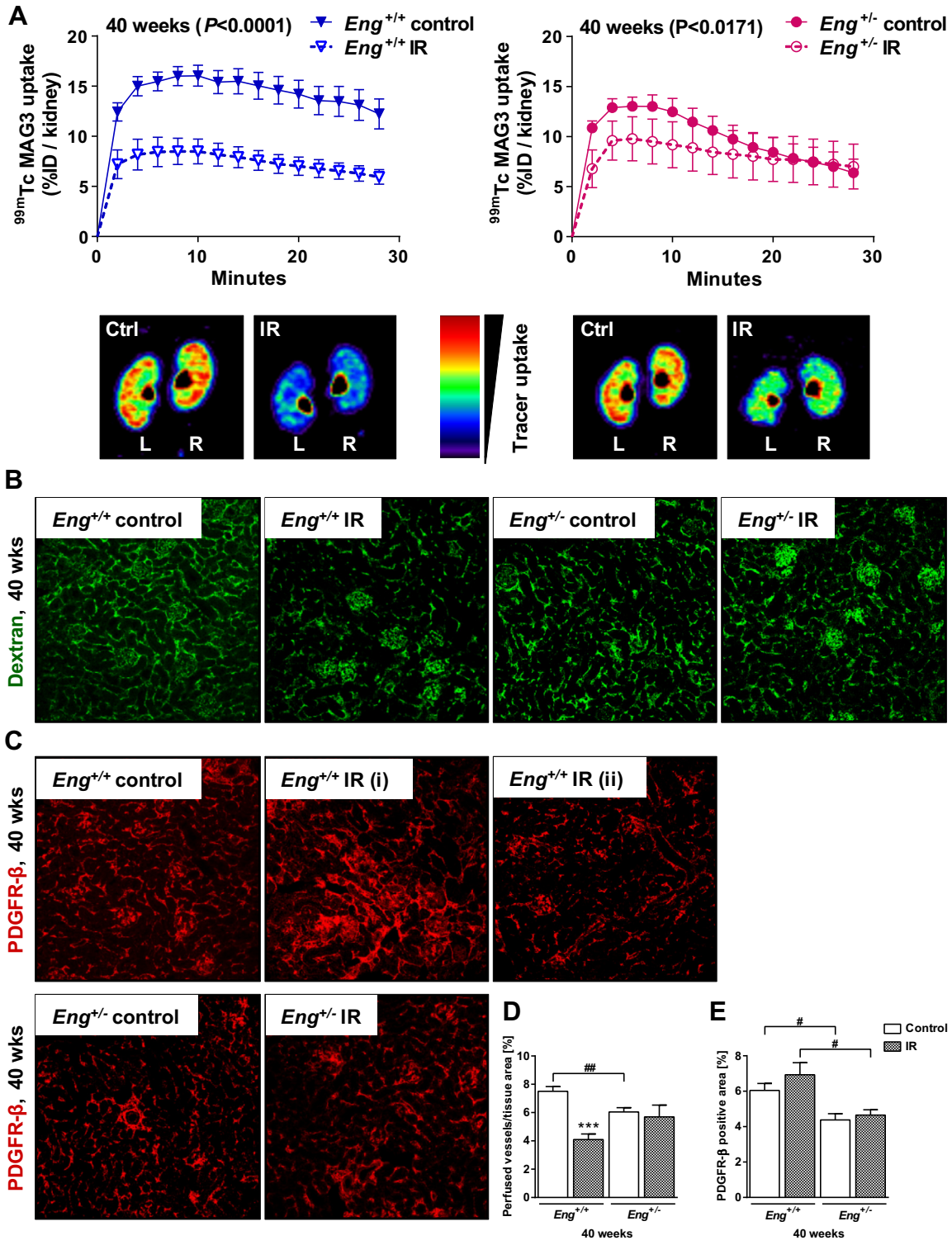

Fig. 2. (A) Kidney SPECT using the tracer ^{99m}Tc MAG3 at 40 weeks after irradiation. Graphs show the uptake of the radioactive tracer corrected for the injected dose (% ID) over time. Pictures depict representative SPECT images at 4 min after imaging. L, R: left and right kidney. (B) Injection of the perfusion marker FITC-dextran (2000 kDa). (C) Pericyte staining for the marker PDGFR-β. (i): focal increase and (ii) loss of pericytes. (D) Quantification of FITC-dextran perfusion. (E) Quantification of PDGFR-β positive area. IR: irradiation. ∗,#Significant difference compared to the control or compared to *Eng*$^{+/+}$ mice with the same treatment, respectively.

and supplementary Fig. 1B). Interestingly, in the control groups, we detected less PDGFR-β positive cells in *Eng*$^{+/-}$ mice compared to *Eng*$^{+/+}$ mice. Taken together, we show that irradiation impairs tubule function and vascular perfusion and focally increases the number of PDGFR-β positive cells in *Eng*$^{+/+}$, but not in *Eng*$^{+/-}$ mice.

Decreased tissue damage in irradiated Eng$^{+/-}$ *mice*

Development of normal tissue damage after kidney irradiation was also determined by histology. Analysis of collagen deposition after Sirius red staining showed little fibrosis in both *Eng*$^{+/+}$ and *Eng*$^{+/-}$ mice at 20 and 30 weeks after irradiation (Fig. 3B). At

40 weeks after irradiation, some of the $Eng^{+/+}$ mice displayed a strong increase in Sirius red-positive areas compared to the non-irradiated controls, but the group mean values were not significantly increased (Fig. 3A and B and supplementary Fig. 1C). Fibrosis formation was less in $Eng^{+/-}$ than in $Eng^{+/+}$ animals, although this was not significant.

We next assessed the irradiation-induced inflammatory response, which might contribute to fibrosis formation. Staining for the pan-leukocyte marker CD45 showed increased leukocyte infiltration at 20–40 weeks after irradiation in $Eng^{+/+}$ and $Eng^{+/-}$ mice (Fig. 3A and C and supplementary Fig. 1C). At 40 weeks after irradiation, the increase in CD45-positive cells was stronger in $Eng^{+/+}$ compared to $Eng^{+/-}$ mice, although this was only borderline significant in the wild type group ($P < 0.06$).

Finally, we analysed H&E-stained sections for tissue damage after irradiation. At 40 weeks, irradiated $Eng^{+/+}$ animals displayed strong tubular and glomerular damage (Fig. 3A and supplementary Fig. 1C). Kidney morphology in irradiated $Eng^{+/-}$ animals was superior to the $Eng^{+/+}$ mice, although these mice also displayed some glomerular and tubular changes.

In summary, we demonstrate that at 40 weeks after irradiation, kidney morphology is better in $Eng^{+/-}$ mice compared to $Eng^{+/+}$ mice and that there is also a trend towards reduced fibrosis and inflammatory cells infiltration in this group.

Discussion

Previous studies from our lab showed that $Eng^{+/-}$ mice develop less inflammation, vascular injury and fibrosis after kidney irradiation compared to their wild type littermates [7,8]. Now we demonstrate that halved endoglin levels also protect from radiation-induced deterioration of kidney function. At 40 weeks after a single dose of 14 Gy, $Eng^{+/+}$ mice had lower haematocrit (indicative of anaemia) and higher BUN levels (indicative of reduced filtration ability of the kidney) compared to irradiated $Eng^{+/-}$ mice. $Eng^{+/+}$ animals also lost weight around 40 weeks after irradiation – indicative of progression of normal tissue damage development – which was not observed in the irradiated $Eng^{+/-}$ group.

SPECT analysis confirmed that deterioration of kidney function was more pronounced in irradiated $Eng^{+/+}$ than in $Eng^{+/-}$ mice. As

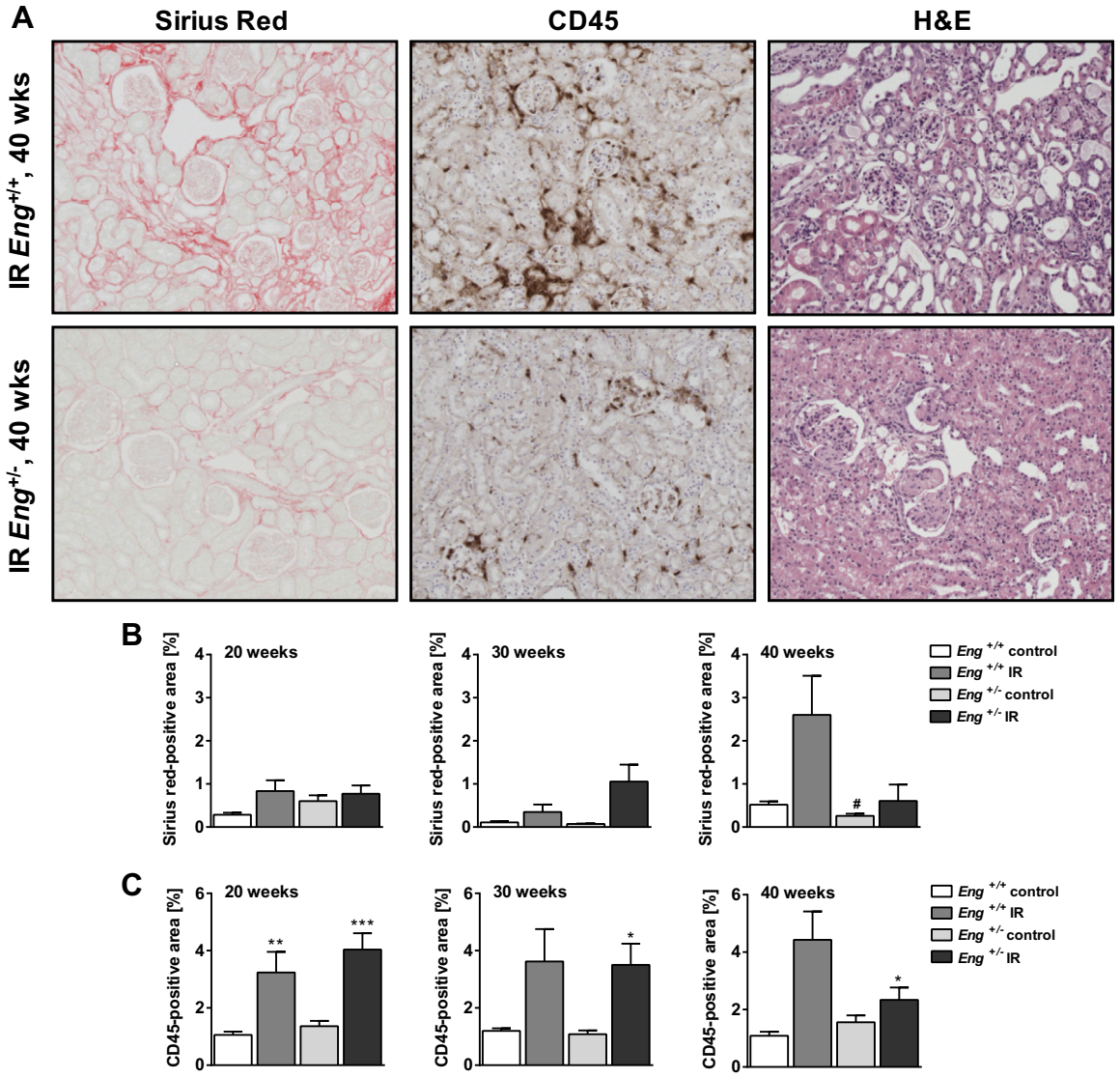

Fig. 3. (A) Fibrosis (Sirius red), inflammation (CD45) and kidney morphology (H&E) at 40 weeks after irradiation. Quantification of (B) Sirius red positive area and (C) CD45-positive area at 20, 30 and 40 weeks after irradiation. IR: irradiation. *,#Significant difference compared to the control or compared to $Eng^{+/+}$ mice with the same treatment, respectively.

only uptake, but not excretion of the tracer was differentially affected between the irradiated groups, this strongly suggests that disturbances in vascular perfusion were the underlying cause for the observed differences in kidney function. Assessment of vessel perfusion with dextran corroborated this hypothesis. Aberrant blood flow was probably also the reason for differences in tracer uptake between the control groups, as non-irradiated $Eng^{+/-}$ mice displayed less perfused blood vessels compared to $Eng^{+/+}$ mice. Yet, we could not explain superior excretion of the tracer in non-irradiated $Eng^{+/-}$ compared to $Eng^{+/+}$ animals. It is possible that halved endoglin levels in the vasculature of $Eng^{+/-}$ mice indirectly led to an improvement in tubular excretion or prevented an age-related decline in tubular function. Taken together these findings underline the importance of the vascular system in the development of kidney damage after irradiation.

Reduced numbers of perfused vessels were probably also one of the reasons for lower pericyte counts in non-irradiated $Eng^{+/-}$ compared to $Eng^{+/+}$ kidneys. Moreover, it is known that mice with homozygous or heterozygous endoglin deficiency display a defect in pericyte and smooth muscle cell coverage [12,13]. Irradiation had opposite effects on pericytes in $Eng^{+/+}$ kidneys resulting in increased pericyte numbers in some and loss of pericytes in other tissue areas. As reduction in pericyte numbers leads to vascular instability [9] and as vascular dysfunction is one of the underlying causes for chronic kidney problems [14,15], it is very likely that irradiation-induced pericyte loss in $Eng^{+/+}$ animals contributed to vascular damage and thus deterioration of kidney function.

In the injured kidney, differentiated pericytes are the major source of myofibroblasts, which can contribute to fibrosis by producing collagen [11]. Differentiated pericytes/myofibroblasts also secrete the chemokine (C–C motif) ligand 2 (CCL2), which recruits macrophage precursors [10]. We showed in previous studies that *Ccl2* expression and macrophage infiltration are increased in irradiated $Eng^{+/+}$, but not in $Eng^{+/-}$ mice [8]. This strongly suggests that an increase in PDGFR-β positive cells promotes macrophage infiltration and thereby fibrosis after kidney irradiation. Accordingly, we detected less inflammatory cells and collagen deposition at 40 weeks after irradiation in $Eng^{+/-}$ compared to $Eng^{+/+}$ mice. The later onset and reduced magnitude of inflammation and fibrosis compared to previous studies was probably due to the lower irradiation dose (14 Gy compared to 16 Gy in previous studies). The adjustment in dose had been necessary as irradiation with 16 Gy had caused non-tolerable skin toxicity and mortality in some of the mice. As a consequence of this dose modification, differences in fibrosis development probably only became significant beyond 40 weeks. H&E-stained sections confirmed reduced normal tissue damage development in irradiated $Eng^{+/-}$ mice, although these mice also displayed some, yet milder tubular and glomerular alterations.

Taken together we show that kidney function and morphology after irradiation are superior in $Eng^{+/-}$ mice compared to $Eng^{+/+}$ mice. We suggest that this was accomplished by reduced pericyte to myofibroblast differentiation and inflammatory cell infiltration after kidney irradiation in this way reducing vascular injury and fibrosis formation. In summary, we suggest that by altering endothelial functions – such as proliferation, permeability, antithrombotic properties, and secretion of leukocyte recruiting factors – reduced endoglin levels do not directly promote repair, but rather prevent the manifestation of tissue damage in this way leading to improved kidney function after irradiation.

Conflict of interest

None declared.

Funding

This study was financed by grants from the Dutch Cancer Society (NKI-2009-4480), http://www.kwfkankerbestrijding.nl/Pages/Home.aspx. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

From the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital (NKI–AVL), Amsterdam, we would like to thank Nils Visser and Bert Pool for technical advice and assistance with setting up the functional imaging experiments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2013.06.016.

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