Late Residual γ-H2AX Foci in Murine Spinal Cord Might Facilitate Development of Response-modifying Strategies: A Research Hypothesis

NICOLAUS ANDRATSCHKE[1], TOBIAS BLAU[1,2], SABINE SCHILL[1] and CARSTEN NIEDER[3,4]

[1]*Department of Radiation Oncology, Klinikum rechts der Isar der Technischen Universität München, Munich, Germany;*
[2]*Department of Neuropathology, University Hospital of Cologne, Cologne, Germany;*
[3]*Department of Oncology and Palliative Medicine, Nordland Hospital, Bodø, Norway;*
[4]*Institute of Clinical Medicine, Faculty of Health Sciences, University of Tromsø, Tromsø, Norway*

Abstract. *The central nervous system (CNS) is among the critical late-responding normal tissues which influence radiation treatment planning and dose prescription. Both mouse and rat spinal cord are established radiobiological models. As late reactions in rodents develop many months after irradiation, long-term follow-up is necessary. Development of radioprotective strategies and refinement of optimum dose and administration schedules are therefore quite complicated and time consuming. Obviously, surrogate endpoints that allow for screening of radioprotective agents and optimisation of dosing with shorter follow-up would speed up and facilitate this type of radiobiological research. Such surrogate endpoints should be analyzable during the clinically asymptomatic latent period, ideally as early as possible. As unrepaired cellular damage is a prerequisite for overt side-effects of radiotherapy, detection of residual damage might predict for radiation myelopathy. Immunohistochemical evaluation of irradiated mouse spinal cord with phosphorylated histone H2AX (γ-H2AX) was performed. Preliminary data suggest that residual damage can be detected in mouse spinal cord and that such foci continue to be detectable over a long time period, which clearly extends into the phase where development of late reactions starts. Our initial findings justify further research which attempts to correlate γ-H2AX foci with the primary endpoint, i.e. radiation myelopathy. It can then be explored whether a given radioprotective agent reduces the number of foci and whether this translates into reduced incidence of radiation myelopathy. A rapid and reliable animal model would allow for screening of a large number of candidate drugs that might modify the radiation response of the spinal cord.*

Correspondence to: Carsten Nieder, MD, Department of Oncology and Palliative Medicine, Nordland Hospital, 8092 Bodø, Norway. Tel: +47 75578449, Fax: +47 75534209, e-mail: carsten.nieder@nlsh.no

Key Words: Radiation therapy, spinal cord, radiation myelopathy, γ-H2AX.

When treating cancer patients with ionising radiation, one has to achieve a favourable balance between tumour control and the probability of normal tissue complications. Recent developments, such as intensity-modulated radiotherapy, have improved the ability to better shape the dose distribution to the target volume and thus spare more of nearby critical organs. Despite these advances, development of strategies of response modification, in this case protection of healthy tissue, would further improve the therapeutic ratio. The central nervous system (CNS) is among the critical late-responding normal tissues which influence radiation treatment planning and dose prescription (1-3). Several groups have studied experimental CNS radiotherapy in rodents, and both mouse and rat spinal cord are among the established models (4-12). As late reactions in rodents develop many months after irradiation, long-term follow-up is necessary. Development of radioprotective strategies and refinement of optimum dose and administration schedules are therefore quite complicated and time consuming (5-7). Obviously, surrogate endpoints allowing for screening of radioprotective agents and optimisation of dosing with shorter follow-up would speed up and facilitate this type of radiobiological research.

Ionising radiation causes DNA damage in cells and tissues, which if left unrepaired might cause clinically apparent side-effects in healthy organs. DNA damage can be assayed by the formation and resolution of nuclear DNA repair foci (13, 14). These foci, *e.g.* phosphorylated histone H2AX (γ-H2AX), can be detected immunohistochemically in irradiated tissue (15-17). For normal mouse tissue, the initial formation of γ-H2AX shows a linear dose response at 30-60 min after irradiation. Many studies have used residual damage and γ-H2AX foci at 24 h as a measure of the capacity for double-strand break repair (18, 19). It has also been shown that γ-H2AX foci can be identified 7 days

after exposure of mice to ionising radiation (15). However, these data relate to keratinocytes within the epidermis. Studies in tumour patients have looked at intervals of up to 10 days (20, 21). From these, there are confirmatory data on the presence of γ-H2AX foci in irradiated cells at relatively long time intervals after exposure. Given the repair kinetics in irradiated cells and tissues, it has to be assumed that lesions persisting after 7-10 days will remain unrepaired (22). Unrepaired persistent damage is the prerequisite for development of chronic-progressive late normal tissue toxicity. In other words, the cascade of functional and structural tissue changes after irradiation will not start if all immediate radiation-induced damage can be repaired. We provide preliminary evidence that supports a new research hypothesis, namely that monitoring the time course of these foci might allow for prediction of late toxicity and development of protection strategies.

Materials and Methods

This study was performed as part of a larger preclinical research project where mouse kidney and spinal cord were irradiated with the aim of exploring radioprotective strategies (23, 24). In the initial spinal cord experiments, the radiation dose response relationship for myelopathy was explored and in parallel, a pilot study of erythropoietin administration was conducted.

Female adult C3H/N mice (12 weeks old) were purchased from Charles River Laboratories, Sulzfeld, Germany. Animals were housed in conventional rodent facilities at the Department of Experimental Oncology, Klinikum rechts der Isar, Munich, Germany, as described elsewhere (23, 24). These facilities are accredited by the Government of Upper-Bavaria and are operated in accordance with state standards and laws. The Governmental Animal Care and Use Committee approved the experimental protocol. Mice were treated in a prone position with 70 kV X-rays (Philips RT100) to a 1-cm wide single field that included the cervical spinal cord (at a dose rate of approximately 6 Gy per minute and source to skin distance of 10 cm). The animals were anaesthetized during irradiation by inhalation of 1.5-2.0% halothane (plus oxygen 0.5 l/min) using a semi-circuit inhalation anaesthesia system to immobilize them in the desired position. In the main experiment (evaluation of the dose response relationship) graded doses of radiation were administered. The first radiation fraction was kept constant at 17 Gy. Seven days later, the second fraction was administered (lowest dose level 11 Gy, 2-Gy increments). Each dose group included 5-6 mice.

Previous studies had suggested that erythropoietin administration starting immediately before irradiation might reduce the extent of brain necrosis in rats exposed to a single dose of 100 Gy (25). These data provided a rationale for the study of two different erythropoietin doses in the spinal cord model. In parallel, a pilot study was run where 4 additional groups received radiotherapy (17 Gy plus 15 Gy and 17 Gy plus 19 Gy, respectively) and Epoetin alfa (Janssen-Cilag, Neuss, Germany) at two different dose levels (10 IE and 40 IE, respectively). The drug was injected subcutaneously 24 h before, 1 h after and 24 h after each dose of radiation, *i.e.* for a total of 6 injections per mouse. Follow-up was one year after irradiation. The animals were then sacrificed and the spinal cord of selected mice was examined histologically.

For this purpose, paraffin-embedded histological slides from irradiated spinal cord were prepared. We used all surviving mice that did not develop neurological damage within one year after irradiation with 17+19 Gy, *i.e.* the dose where the dose-response curve started to bend upward. We assumed that mice treated with high radiation doses would be more likely to harbour unrepaired damage than those treated with lower doses. Slides were prepared from 4 unirradiated control mice, 5 irradiated mice and 5 mice treated with radiation plus two different doses of erythropoietin. Immunohistochemical examination was performed with phospho-histone H2AX (Ser139) antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA). The stained slides were evaluated under a microscope (Olympus BX51, ×40) by counting the stains in 10 high power fields (HPF). The results were compared with the Wilcoxon, Mann-Whitney *U*-test. All reported *p*-values are two-sided. A *p*-value <0.05 was considered statistically significant.

Results

Unexpectedly, none of the animals developed neurological deficits after irradiation with doses between 17+11 Gy and 17 Gy + 17 Gy. Only in the 17+19 Gy groups did two animals develop signs of spinal cord damage. One mouse belonged to the control group and one to the 40 IE erythropoietin-treated group. Thus, the experiment did not allow for computation of a complete radiation dose-response curve, which ranges from 0-100% incidence of myelopathy. In unirradiated spinal cord, γ-H2AX foci were virtually absent. In the group of 5 irradiated mice, a mean of 155 foci were counted (range 45-216, median 205). In the lower dose erythropoietin group, a mean of 98 foci were counted (range 4-187, median 61), $p=0.15$. In the higher dose erythropoietin group, the mean number was 85 (range 40-123, median 89), $p=0.25$. Figure 1 shows representative histological slides, demonstrating that the γ-H2AX foci were found predominantly in blood vessel endothelium.

Discussion

As known from previous experiments and confirmed here, the evaluation of radioprotective strategies in late-responding normal tissues such as spinal cord is a very time-consuming process. Even rationally designed experiments might lead to unexpected results necessitating subsequent studies with equally long follow-up. It would thus be helpful to identify surrogate endpoints of radiation myelopathy. Such surrogate endpoints should be analyzable during the clinically asymptomatic latent period, ideally as early as possible. As unrepaired cellular damage is a prerequisite for overt side effects of radiotherapy, we hypothesize that detection of residual damage might predict for myelopathy. We provide immunohistochemical data suggesting that residual damage can be detected in mouse spinal cord. This finding is not unexpected, as other groups previously have identified γ-H2AX foci in different irradiated mouse tissues several days after exposure (15, 18). Our own data also suggest that such foci continue to be detectable over a long time period, which clearly

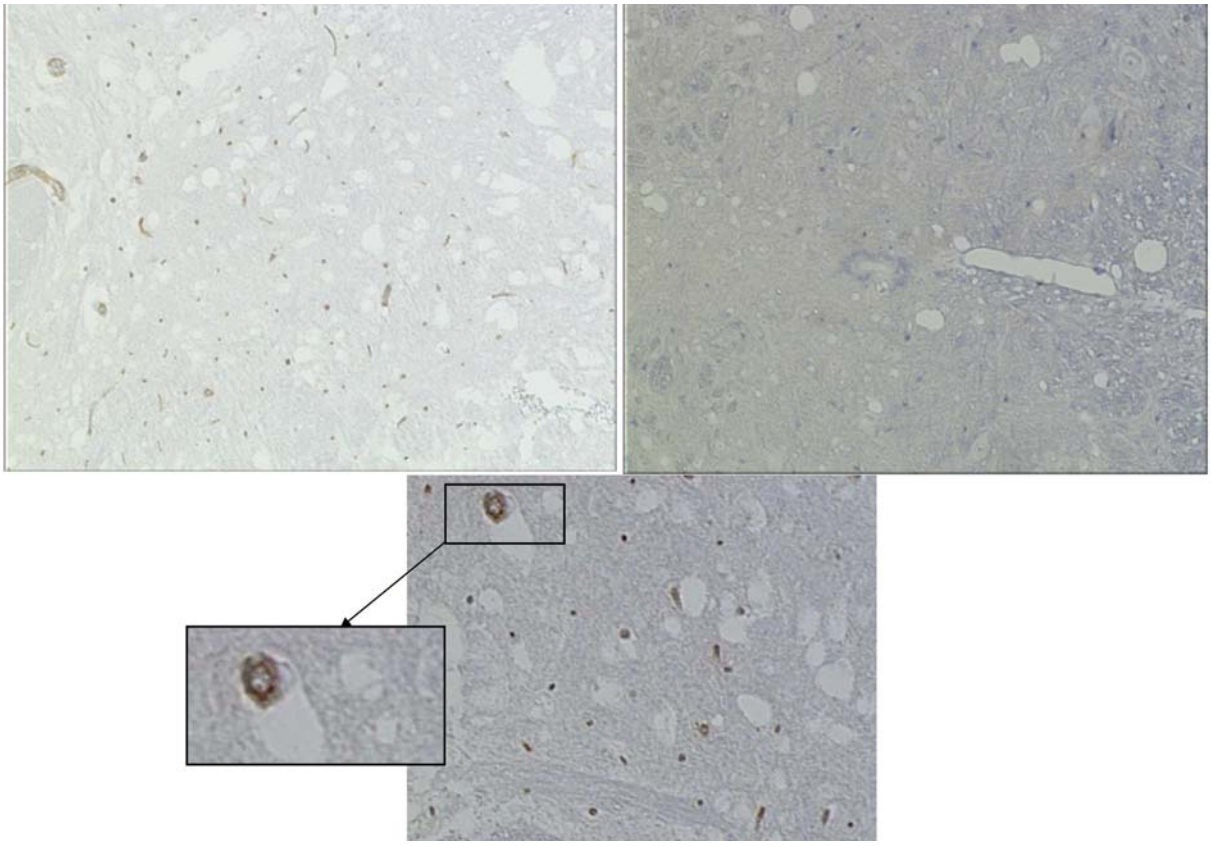

Figure 1. Representative immunohistochemical results. Irradiated mouse spinal cord (upper left) contains γ-H2AX foci predominantly in the blood vessel endothelium (lower panel). Unirradiated control is shown in the upper right corner.

extends into the phase where development of late reactions starts. It is therefore justifiable to study the time course of γ-H2AX detection in greater detail. Such studies might start 30-60 min after irradiation and include groups with intermediate (weeks) and long follow-up (months). It is also necessary to correlate γ-H2AX foci with the primary endpoint, *i.e.* radiation myelopathy, in animals irradiated to higher doses than the ones used in our experiment. After successful completion of these steps, it can be explored whether a given intervention or radioprotective agent reduces the number of foci and whether this translates into reduced incidence of radiation myelopathy. In this context, it is important to understand that DNA damage plays a crucial role in the development of radiation-induced reactions. However, other types of damage also exist. As these might not contribute to a large extent to radiation-induced myelopathy, the γ-H2AX method appears promising. The erythropoietin data shown in the present report were not statistically different from those of the control group, but the statistical power is low with groups of 5-6 animals. The fact that fewer foci were detectable in erythropoietin-treated mice is compatible with the protective effect observed in a previous study of brain irradiation (25); one should also be aware of the fact that the brain study differs from the present one with regard to longer application of the drug, *i.e.* 10 days after single-fraction irradiation. Future studies might therefore include prolonged drug treatment.

The most plausible explanation for the unexpectedly high tolerance of the spinal cord to irradiation in our mouse model is that we underestimated the volume effect. We chose the radiation dose levels from previous publications (10-12). However, these authors had irradiated larger spinal cord areas and the irradiated volume is an important determinant of response, besides the radiation dose. Nevertheless, the radiation doses used in our model are adequate for examination of subclinical long-term consequences of radiation.

Current models of radiation-induced CNS changes include a cascade of complex and dynamic interactions between mature parenchymal cells (oligodendrocytes, astrocytes, microglia, neurons), stem and progenitor cells and the vascular system, also resulting in important alterations of the local microenvironment (2, 3, 26). Radiobiological studies of boron neutron-capture therapy (BNCT) support the view that

vascular damage is one of the crucial components leading to radiation-induced myelopathy after higher doses (4). By choosing boron compounds which are unable to cross the blood-brain barrier, a largely selective irradiation of the vessel walls can be accomplished with BNCT. Compared to conventional non-selective radiotherapy methods, spinal cord lesions with similar histological appearance were induced. Latency time also was comparable between damage induced by BNCT and conventional radiotherapy. It has also been shown that vasoactive drugs might reduce radiation-induced spinal cord damage (27). These data fit well with our observation that γ-H2AX foci were found predominantly in blood vessel endothelium. In conclusion, we present a hypothesis that might facilitate screening of radioprotective agents and optimisation of dosing with shorter follow-up.

References

1 Schultheiss TE, Kun LE, Ang KK and Stephens LC: Radiation response of the central nervous system. Int J Radiat Oncol Biol Phys *31*: 1093-1112, 1995.

2 Tofilon PJ and Fike JR: The radioresponse of the central nervous system: a dynamic process. Radiat Res *153*: 357-370, 2000.

3 Coderre JA, Morris GM, Micca PL, Hopewell JW, Verhagen I, Kleiboer BJ and van der Kogel AJ: Late effects of radiation on the central nervous system: role of vascular endothelial damage and glial stem cell survival. Radiat Res *166*: 495-503, 2006.

4 Morris GM, Coderre JA and Bywaters A: Boron neutron-capture irradiation of the rat spinal cord: histopathological evidence of a vascular-mediated pathogenesis. Radiat Res *46*: 313-320, 1996.

5 Andratschke NH, Nieder C, Price RE, Rivera B, Tucker SL and Ang KK: Modulation of rodent spinal cord radiation tolerance by administration of platelet-derived growth factor. Int J Radiat Oncol Biol Phys *60*: 1257-1263, 2004.

6 Nieder C, Andratschke N, Price RE and Ang KK: Evaluation of insulin-like growth factor-1 for prevention of radiation-induced myelopathy. Growth Factors *23*: 15-18, 2005.

7 Spence AM, Krohn KA and Edmondson SW: Radioprotection in rat spinal cord with WR-2721 following cerebral lateral intraventricular injection. Int J Radiat Oncol Biol Phys *12*: 1479-1482, 1986.

8 Ruifrok AC, Stephens LC and van der Kogel AJ: Radiation response of the rat cervical spinal cord after irradiation at different ages: tolerance, latency and pathology. Int J Radiat Oncol Biol Phys *29*: 73-79, 1994.

9 Wong CS and Hao Y: Long-term recovery kinetics of radiation damage in rat spinal cord. Int J Radiat Oncol Biol Phys *37*: 171-179, 1997.

10 Lo YC, McBride WH and Withers HR: The effect of single doses of radiation on mouse spinal cord. Int J Radiat Oncol Biol Phys *22*: 57-63, 1992.

11 Habermalz HJ, Valley B and Habermalz E: Radiation myelopathy of the mouse spinal cord – isoeffect correlations after fractionated radiation. Strahlenther Onkol *163*: 626-632, 1987.

12 Lavey RS, Taylor JMG, Tward JD, Li LT, Nguyen AA, Chon Y and McBride WH: The extent, time course, and fraction size dependence of mouse spinal cord recovery from radiation injury. Int J Radiat Oncol Biol Phys *30*: 609-617, 1994.

13 Rogakou EP, Pilch DR, Orr AH, Iwanova VS and Bonner WM: DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem *273*: 5858-5868, 1998.

14 Foster ER and Downs JA: Histone H2A phosphorylation in DNA double-strand break repair. FEBS J *272*: 3231-3240, 2005.

15 Bhogal N, Kaspler P, Jalali F, Hyrien O, Chen R, Hill RP and Bristow RG: Late residual γ-H2AX foci in murine skin are dose responsive and predict radiosensitivity *in vivo*. Radiat Res *173*: 1-9, 2010.

16 Bhogal N, Jalali F and Bristow RG: Microscopic imaging of DNA repair foci in irradiated normal tissues. Int J Radiat Biol *85*: 732-746, 2009.

17 Liu S, Olive P and Bristow R: Biomarkers for DNA DSB inhibitors and radiotherapy clinical trials. Cancer Metastasis Rev *27*: 445-458, 2008.

18 Koike M, Mashino M, Sugasawa J and Koike A: Histone H2AX phosphorylation independent of ATM after X-irradiation in mouse liver and kidney *in situ*. Radiat Res (Tokyo) *49*: 445-449, 2008.

19 Rübe CE, Grudzenski S, Kühne M, Dong X, Rief N, Löbrich M and Rübe C: DNA double-strand break repair of blood lymphocytes and normal tissues analysed in a preclinical mouse model: implications for radiosensitivity testing. Clin Cancer Res *14*: 6546-6555, 2008.

20 Sak A, Grehl S, Erichsen P, Engelhard M, Grannass A, Levegrün S, Pöttgen C, Groneberg M and Stuschke M: Gamma-H2AX foci formation in peripheral blood lymphocytes of tumor patients after local radiotherapy to different sites of the body: dependence on the dose distribution, irradiated site and time from start of treatment. Int J Radiat Biol *83*: 639-652, 2007.

21 Sak A, Grehl S, Engelhard M, Wierlemann A, Kaelberlah HP, Erichsen P, Pöttgen C, Groneberg M and Stuschke M: Long-term *in vivo* effects of cisplatin on gamma-H2AX foci signaling in peripheral lymphocytes of tumor patients after irradiation. Clin Cancer Res *15*: 2927-2934, 2009.

22 Landuyt W, Fowler J, Ruifrok A, Stüben G, van der Kogel A and van der Schueren E: Kinetics of repair in the spinal cord of the rat. Radiother Oncol *45*: 55-62, 1997.

23 Nieder C, Cai L, Weber WA, Schill S, Andratschke N, Schwaiger M and Molls M: Evaluation of insulin-like growth factor-1 in a mouse model of long-term abdominal radiation toxicity. Anticancer Res *27*: 183-187, 2007.

24 Andratschke N, Schnaiter A, Weber WA, Cai L, Schill S, Wiedenmann N, Schwaiger M, Molls M and Nieder C: Preclinical evaluation of erythropoietin administration in a model of radiation-induced kidney dysfunction. Int J Radiat Oncol Biol Phys *64*: 1513-1518, 2006.

25 Erbayraktar S, de Lanerolle N, de Lotbiniere A, Knisely JP, Erbayraktar Z, Yilmaz O, Cerami A, Coleman TR and Brines M: Carbamylated erythropoietin reduces radiosurgically induced brain injury. Mol Med *12*: 74-80, 2006.

26 Siegal T, Pfeffer MR, Meltzer A, Shezen E, Nimrod A, Ezov N and Ovadia H: Cellular and secretory mechanisms related to delayed radiation-induced microvessel dysfunction in the spinal cord of rats. Int J Radiat Oncol Biol Phys *36*: 649-659, 1996.

27 Hornsey S, Myers R and Jenkinson T: The reduction of radiation damage to the spinal cord by post-irradiation administration of vasoactive drugs. Int J Radiat Oncol Biol Phys *18*: 1437-1442, 1990.

Received December 1, 2010
Revised January 11, 2011
Accepted January 12, 2011