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Molecular Imaging Detects Impairment in the Retrograde Axonal Transport Mechanism After Radiation Induced Spinal Cord Injury

Lucia G. LeRoux, Ph.D.^{+,*}, Sebastian Bredow, Ph.D.^{+,*}, David Grosshans, M.D. Ph.D.[†], and Dawid Schellingerhout, M.D.^{*,‡}

^{*}Department of Cancer Systems imaging, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[†]Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[‡]Department of Diagnostic Radiology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Abstract

Purpose—The goal of this study was to determine whether molecular imaging of retrograde axonal transport is a suitable technique to detect changes in the spinal cord in response to radiation injury.

Procedures—The lower thoracic spinal cords of adult female BALB/c mice were irradiated with single doses of 2, 10, or 80 Gy. An optical imaging method was used to observe the migration of the fluorescently labeled non-toxic C-fragment of tetanus toxin (TTc) from an injection site in the calf muscles to the spinal cord. Changes in migration patterns compared to baseline and controls allowed assessment of radiation-induced alterations in the retrograde neuronal axonal transport mechanism. Subsequently, tissues were harvested and histological examination of the spinal cords performed.

Results—Transport of TTc in the thoracic spinal cord was impaired in a dose-dependent manner. Transport was significantly decreased by 16 d in animals exposed to either 10 or 80 Gy, while animals exposed to 2 Gy were affected only minimally. Further, animals exposed to the highest dose also experienced significant weight loss by 9 d and developed posterior paralysis by 45 d. Marked histological changes including vacuolization and white matter necrosis were observed in radiated cords after 30 d for mice exposed to 80 Gy.

Conclusion—Radiation of the spinal cord induces dose-dependent changes in retrograde axonal transport, which can be monitored by molecular imaging. This approach suggests a novel diagnostic modality to assess nerve injury and monitor therapeutic interventions.

Corresponding Author: Dawid Schellingerhout, M.D., 1400 Pressler Street, Unit 1482, Houston, TX 77030, Phone: (713) 794-5673, Fax: (713) 794-4629, dawid.schellingerhout@mdanderson.org.

⁺These authors contributed equally

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Keywords

Retrograde axonal transport; Spinal cord; Radiation injury; Molecular imaging; Tetanus toxin fragment C; Demyelination

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Introduction

The central nervous system (CNS) remains one of the major dose-limiting tissues in radiation therapy [1]. Clinical symptoms of radiation-induced CNS toxicity develop from several months to years and include focal cerebral necrosis, neurocognitive deficits, and less frequently cerebrovascular disease, myelopathy or secondary neoplasms [2]. Both cerebral necrosis and myelopathy are particularly devastating to the individual patient.

As the use of high dose-per-fraction stereotactic treatments increase [3], methods that allow for the detection and monitoring of potential adverse effects of clinical radiation therapy are urgently needed. While animal models have been used to establish dose-response curves and to investigate the irradiation conditions that modify the response, there is still little known about the human spinal cord tolerance to single-fraction irradiation. Variables such as dose rate, irradiated length, irradiated lateral cross-section, dose to adjacent spinal cord, previous irradiation and age are variables known to influence clinical outcomes [4-6]. Such dosimetric information is valuable for use in the treatment planning process and may predict outcomes when applied to groups of patients. However, to date, our capacity to predict the susceptibility of an individual patient or to monitor potential preventative or therapeutic interventions remains rudimentary. Moreover, while demyelination is known to be a late histopathologic correlate of radiation injury, much remains to be learned about the pathologic mechanisms underlying radiation injury [1].

Retrograde axonal transport is an important neuronal maintenance pathway. Growth factor signaling from the distal axon terminus informs the cell soma of the health of the distant synapse and end organ, and maintains neuronal health [7].

We have devised a method to image retrograde axonal transport in live animals by means of a molecular tracer, the C-fragment of tetanus toxin (TTc) [8-9]. Utilizing the retrograde axonal transport mechanism in nerves we previously demonstrated that this non-toxic polypeptide is a suitable imaging agent for the spinal cord [10], and useful for detecting and monitoring neurotoxicity in response to chemotherapy [11]. Based on these findings we *hypothesized* that a similar imaging approach would be able to measure changes in the spinal cord in response to single dose irradiation.

To test this hypothesis, adult female BALB/c mice received single fraction radiation treatments to the lower thoracic spinal cord at doses of 2, 10, or 80 Gy. The animals were serially imaged at weekly intervals utilizing intramuscular injections of fluorescently labeled TTc. We found that radiation caused a dose-dependent impairment of retrograde transport.

To the best of our knowledge, this is the first study to quantitate the impact of radiation on retrograde axonal transport. Our findings may be of great importance in defining the

mechanisms of radiation induced spinal cord injury. Potential clinical applications might include the prediction and early detection of radiation myelopathy, and monitoring treatments to alleviate or prevent the disease.

Methods and Materials

Animals

Three month-old female BALB/c mice (20 – 22 g) were purchased from Charles River Laboratories (Wilmington, MA). Prior to experiments animals were housed under pathogen free conditions on a 12-h light/dark cycle for at least 3 weeks in the institutional American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility with food and water available *ad libitum*. All experiments involving the animals were approved by the Institutional Animal Care and Use Committee (IACUC) at our institution and conducted in compliance with the regulations of the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Spinal cord irradiation

Groups of 4 animals were irradiated in our institution's veterinary facility utilizing a dedicated ^{60}Co radiotherapy unit. Animals were anesthetized using a 2% isoflurane/air mixture at 2 L/min (Butler Animal Health Supply; Dublin, OH). Animals were positioned to receive about 1 Gy/min. Radiation was delivered in a single session to total doses of 2, 10 and 80 Gy, levels which would be expected to give injury ranging from insignificant to severe [12]. In order to avoid bowel toxicities, animals were treated with opposing lateral beams with each beam delivering 50% of dose. Custom lead shielding was used to shield normal tissues. The irradiation portal was designed to encompass a 2 cm long segment of the thoracic spinal cord. Prior to treatment, radiation dosimetry was confirmed using thermoluminescent dosimeters and an animal phantom.

In vivo imaging

In vivo imaging was performed as previously described [10]. In brief, recombinant TTc, fluorescently labeled with Alexa Fluor 790 dye (Invitrogen; Carlsbad, CA) was injected into the left calf muscle of depilated, anaesthetized animals. Images were obtained using the Xenogen IVIS 200 small animal imager (Caliper Life Sciences; Hopkinton, MA) using a band pass fluorescent filter set matched to the fluorophore (710-760 nm excitation/810-875 nm emission). Images were acquired before injection ($t=0$ min) and at 60 min post-injection and consisted of a white light image (for orientation) paired with a fluorescent image (to show TTc distribution). A Region of Interest (ROI) was drawn on the lower thoracic spine, and the uptake and transport of TTc was calculated per animal by subtracting the radiance (photons/sec/cm²/sr) at $t=0$ min from that measured at 60 min post-injection. Animals were imaged in groups of 4 once per week commencing 5 d prior to irradiation (Baseline) and subsequently at 2, 9, 16 and 30 d post-Radiation.

Histology

Tissue was harvested 30 days post irradiation with 80Gy. Complete spinal columns were dissected out *en bloc* and divided into smaller segments of approximately 1 cm, with special

attention to identify and harvest tissues in the radiation field. The material was fixed in 4% paraformaldehyde at room temperature, overnight. The material was prepared for paraffin sectioning using standard methods [13]. Microtomy sections (5µm) were made at thoracic level T8-9 [4] and the sections were stained with hematoxylin-eosin. Digital micrographs were captured using an Olympus BX51 microscope.

Statistical analysis

Data were analyzed with Excel (Microsoft, Redmond, WA) and Prism (Graphpad Software, San Diego, CA). Each irradiated group is presented as mean $\pm$ standard deviation. For each group one-way analysis of variance (ANOVA) was performed on transport data measured at specific days after radiation, with multiple comparisons of means corrected using Tukey's test. We also performed ANOVA to test for a linear trend in transport means as a function of time. *P*-values of <0.05 were considered statistically significant.

Results

Animals and Radiation

In order to reliably induce myelopathy, a group of adult mice ($n=4$) were first irradiated with a dose of 80 Gy, which well exceeds the dose at which injury is expected [14]. Within 9 d, 3 of the 4 adult animals had lost nearly 23% of body mass (Fig. 1; from an average of 21 to 16.2 g), causing the death of one animal. Although one animal regained weight by 30 d (from 15.7 to 20.2 g), overall weight loss in this group was approximately 10% by the end of the experiment (see Fig. 2). Two animals in this dose group developed severe posterior paralysis by 45 days post-radiation, in keeping with severe radiation injury to the cord.

The parameters developed during the imaging of this group were subsequently applied to animals irradiated with either 10 Gy (the maximum clinical threshold dose to human cord tissue recommended for stereotactic treatments [15]) or 2 Gy (a dose level consistent with a typical single fractionated radiation treatment, and thought to be well below the threshold for any injury). Animals exposed to the lower doses did not lose weight over the course of the experiment and, by 30 d after radiation, had gained approximately 10% body weight (data not shown). Six months later there were no phenotypical indications that the lower doses may have caused adverse effects in these animals.

Imaging

It has been previously demonstrated that uptake and transport of fluorescently labeled TTc in the spinal cord is specific and quantifiable [10]. Here we used a similar imaging approach to detect and monitor dose-dependent changes in the retrograde axonal transport mechanism in response to single radiation doses by serially measuring neural transport after intramuscular TTc-injection.

Baseline TTc uptake and transport (as measured by the increase in fluorescence over the lower thoracic spine 60 minutes after intramuscular injection of TTc in the calf muscles, see Fig. 3) was a radiance of $5.1 \times 10^7 \pm 6.4 \times 10^6$ photons/sec/cm²/sr (mean $\pm$ standard deviation) for the 80 Gy group, $4.1 \times 10^7 \pm 5.7 \times 10^6$ photons/sec/cm²/sr for the 10 Gy group and

$7.2 \times 10^7 \pm 1.4 \times 10^6$ photons/sec/cm²/sr for the 2 Gy group. The variability observed is typical of optical imaging experiments, and are controlled for by using the same animals in longitudinal serial observations. Values were normalized by setting these baselines to unity, and are shown in Fig 4.

For animals radiated at 80 Gy, Imaging demonstrated that TTc-transport in the thoracic spine began to decline by 16 d after the radiation procedure and did not recover by 30 d after radiation (Fig. 3 & Fig 4). Interestingly, quantification of thoracic cord transport of this group revealed that transport seemingly increased by approximately 20% nine days after radiation (Fig. 4, left columns). This is most likely a dosing effect, since the amount of TTc injected was not adjusted to compensate for the significant weight loss that had occurred in these animals. Subsequently, thoracic signal demonstrated a 51% decline in transport between 9 and 16 d after the radiation procedure, followed by a further decline to end at 31% of Baseline at 30 d post-radiation, the end of the experiment. Differences between baseline-30 days, 2 days-30 days, 9 days-16 days, and 9 days-30 days were statistically significant (Tukey's multiple comparisons test, $p < 0.05$, see Fig 4). ANOVA to test for a linear trend revealed a slope of -8.98×10^6 with r^2 value of 0.52 with high significance ($p < 0.0001$).

For animals radiated at 10 Gy, transport declined monotonously from 2 d after radiation (Fig 4), and the transient increase in transport noted in the 80 Gy animals did not occur. The initial drop from baseline was about 10%, followed by 13% between 2 and 9 d, and 27% between 9 and 16 d to come to a final of 47% of baseline at 30 d. The changes from baseline-16 days, baseline-30 days, 2 days-16 days and 2 days-30 days were statistically significant (Tukey's multiple comparisons test, $p < 0.05$, see Fig 4). ANOVA to test for a linear trend revealed a slope of -5.9×10^6 with r^2 value of 0.67 with high significance ($p < 0.0001$).

For animals radiated at 2 Gy, transport again declined monotonously from 2 d after radiation (Fig 4), and again, the transient increase in transport observed in the 80 Gy group was not observed. The initial drop from baseline was 20% from baseline, but with lesser subsequent declines to come eventually to 66% of baseline at the end of the experiment. None of the multiple comparisons between time points reached statistical significance. ANOVA to test for a linear trend showed a slope of -4.8×10^6 and an r^2 of 0.18 which did not reach statistical significance.

Histology

We confirmed radiation damage to the cord, showing white matter necrosis with vacuolization, diffuse tissue loss and gliosis in the cords of high-dose radiated animals. This is consistent with clinical findings of posterior paralysis and also with prior literature reports [16] (Fig. 5).

Discussion

We show, for the first time, that radiation injury of the spinal cord induces dose-dependent reductions in axonal transport, and that these changes can be monitored by molecular

imaging in a live animal model. The changes in retrograde transport occur relatively early in the course of injury, prior to the expected development of overt necrosis and the late pathological findings of radiation injury. Imaging findings display a dose-response relationship with the amount of radiation administered, and also paralleled clinical findings and histological findings. Importantly though, imaging changes preceded the development of clinical findings by weeks to months, suggesting a predictive element that might be exploited in the triage and execution of clinical interventions to prevent injury prior to its full expression. Furthermore, monitoring retrograde transport suggests a means of monitoring the success of preventative measures against radiation injury, or guiding the need for, and success of, post-exposure treatment measures.

Current literature (reviewed in [12]) suggests 20 Gy as the dose at which 50% of subjects will suffer cord damage for single dose radiation treatment, with similar values for many species: 19 Gy for mice [17], 20 Gy for rats [18] and 20 Gy for pigs [19]. A maximum safe limit of 10 Gy was suggested for clinical stereotactic treatments [15], as a dose limit below which clinical pathology would not be expected to develop. For standard fractionated radiation treatments, single fractions of 2Gy are commonly employed. We selected our radiation doses accordingly. One dose well above the safe limit (80 Gy, almost certain to develop injury), at the clinical maximum threshold dose (10 Gy, as suggested by [15], and at 2 Gy, a typical single fractionated dose, thus covering the range of expected cord exposures that might occur clinically.

Our findings show reductions in retrograde transport at 80 Gy, a dose at which injury is expected, however, this impairment was detected far earlier than the clinical manifestations of injury. We found transport clearly impaired at 30 days (and likely earlier, but masked by weight loss), while clinically detectable injury usually has a lag time of many months [12]. This suggests that retrograde transport might serve as an early marker of radiation injury. We confirmed histologic injury on subsequent follow up, with findings similar to published data [12].

Surprisingly, at 10 Gy, a dose for which no effects were expected, there was an impairment of retrograde neural transport, suggesting that impairment of neural transport might be a sensitive measure of subtle injury below the threshold of clinical detection. We found no impairment of transport at 2 Gy, commensurate with this dose being far below the level of immediate biologic effects.

These findings are relevant given the increasing use of stereotactic hypo-fractionated radiation treatments for spinal metastases. Such treatments offer the hope of improved disease control, but with potentially increased risk of adverse effects. The potential for superior control rates along with monetary incentives, has driven the increasing utilization of single dose therapies to spinal targets [3, 20]. This trend is expected to continue as the population of cancer patients and cancer-survivors increases, raising interest in the potential adverse effects of such dose regimens.

The nature of radiation myelopathy is intrinsically probabilistic [21], and the biological thresholds to induce it differ between individuals. This means that predictions can be made

for groups of patients, but not for individuals. There is a real and increasing need for a biological readout that would give useful data on individual patients rather than statistical groups. Such a marker would need to be: a) biologically relevant to the subsequent development of injury, and b) show changes early enough to alter the course of therapy, or guide remedial measures. We show proof-of-concept that imaging retrograde neural transport might serve as such a candidate imaging biomarker.

There is very little known about the effects of radiation on retrograde neural transport. However, defective axonal transport has been implicated in many neuropathies such as those caused by diabetes [22], chemotherapeutics [23-24] and toxins [25-26]. There is increasing evidence that defects of axonal transport may be a common factor in many neuropathies [27]. It is reasonable to pose a similar role for radiation induced nerve damage, but unfortunately very little data is available on this question. Until the development of live animal molecular imaging techniques to measure retrograde neural transport [10], this biological parameter was not accessible for investigation.

Our study showed proof-of-concept by demonstrating the dose-dependent impact of radiation on retrograde transport in cord radiation injury, but has several limitations. First are the difficulties inherent in quantifying planar optical imaging datasets. Photon attenuation in tissue is known to be a significant problem, and is partially addressed by choosing fluorophores in the Near Infrared, where such attenuation effects are minimized, and by liberal use of dissection, ex vivo imaging and histology such as was previously done in our group for TTc [10-11]. Radiolabeled versions of TTc are under development to provide more robust quantification and bio-distribution data in future. Secondly, the full complexity of radiation induced myelopathy are not fully addressed in this study, which was focused on demonstrating effects on retrograde transport. Multiple other effects, such as perfusion abnormalities, immune modulation and others likely are all players in the process, and will require future controlled experiments to fully assess. However, we do have prior data with albumin controls, and histologically verified, that cord delivery is mainly via retrograde transport and specific to TTc [10]. Given this route of agent delivery, we suspect that radiation induced perfusion abnormalities will not be a main effect. Thirdly, the radiation doses chosen for this proof-of-concept study covered a wide range, and was not concentrated about the clinically relevant IC50 value of 19 gray (for humans). Clearly more detailed study is required with finer dose calibrations centered at the IC50. With proof-of-concept now established, we can motivate for, and execute such studies. Such future work would also need to include standard fractionated treatment regimens (with multiple 2 gray fractions), to address the needs of the large patient segment undergoing such treatments, along with those receiving stereotactic regimens (utilizing a single very high radiation dose).

We found a dose-dependent decrease in the amount of axonal transport that occurs in radiated cord, and that even “safe” doses of 10 Gy, had an impact on retrograde transport. There is growing data that suggests the retrograde transport mechanism to be a central part of neuronal maintenance, and that injury to this mechanism leads to neuropathy [11]. There is also data to suggest that chemo-protective therapies that can successfully shield animals from the development of Oxaliplatin-induced neuropathy [28], show maintenance of their retrograde axonal transport rates (unpublished data). The role of retrograde neural transport

in radiation injury is unknown, but the current study suggests that it may be important, and invites further investigation.

Live molecular imaging of retrograde axonal transport provides a novel and interesting view of a potential mechanism of radiation injury to the cord. Further investigation into the usefulness of such imaging in the prediction, prevention, triage and treatment of radiation injury is justified.

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post-Radiation:**BASELINE** **Figure 1.**

Animals irradiated with a single cumulative dose of 80 Gy to the lower thoracic spinal cord experience >20% weight loss within 9 d of exposure. A representative animal is shown at Baseline prior to radiation and 2, 9, 16, and 30 d after radiation with bilateral opposing beams. The rectangular box indicates the position of the radiation portal.

Figure 2. Weight change with radiation therapy. Animals at the highest radiation dose (80 Gy) variably lost more weight than controls and lesser radiation doses.

Figure 3.

Radiation-induced loss of neural transport is illustrated by fluorescent imaging in a representative animal after 80 Gy. Animals (BalbC mice) were injected with 20 μ g of TTe790 in the calf muscles, and imaged 60 minute later with Region of Interest (ROI) measurements over the lower thoracic spine. At baseline, neural transport of TTe790 from the left calf muscles, along the sciatic nerve and into the cord is normal, and mediated by fast axonal transport (for histological descriptions see [10]). As the experiment progresses there is a clear loss of TTe transport in radiated animals, with visible and quantifiable impairment from 16-30 days post radiation.

Figure 4.

Quantification of transport with ROI measurements over the lower thoracic spine 60 min after TTe injection into the calf muscles. Radiance per animal (in p/s/cm²/sr) was measured by quantifying identical ROIs. The average radiance per group (n=4; n=3 for 80 Gy at 16 and 30 d post-radiation) was normalized to a baseline taken prior to radiation, which was set to 1. Significant impairment of transport are shown for the 80 and 10 Gy doses but not for the 2 Gy dose (ANOVA; *p<0.05, **p<0.001).

Figure 5. Mouse cord sections at the T8-T9 thoracic level [4] stained with Hematoxylin and Eosin. A normal thoracic cord section (A) is compared to a cord after an 80G single fraction radiation injury (B). Please note the generalized volume loss in the radiated cord, with areas of vacuolization and white matter necrosis. Scale bar – 300 μ m.