Radiotherapy and Oncology 88 (2008) 388–397
www.thegreenjournal.com

*Low-dose hypersensitivity*

# Low-dose hypersensitive γH2AX response and infrequent apoptosis in epidermis from radiotherapy patients

Martin Simonsson[a,*,1], Fredrik Qvarnström[a,*,1], Jan Nyman[b], Karl-Axel Johansson[c], Hans Garmo[d], Ingela Turesson[a]

[a] *Department of Oncology, Radiology and Clinical Immunology, Uppsala University, Uppsala, Sweden,* [b]*Department of Oncology, and* [c]*Department of Radiophysics, Göteborg University, Sahlgrenska University Hospital, Gothenburg, Sweden,* [d]*Regionalt Onkologiskt Centrum, Akademiska Sjukhuset, Uppsala, Sweden*

## Abstract

***Background and purpose*: A low-dose hypersensitivity to radiation can be observed in vitro for many human cell types in terms of increased cell kill per dose unit for doses below 0.5 Gy.**

**Quantification of the double-strand break marker γH2AX in samples taken in clinical radiotherapy practice has the potential to provide important information about how induction and repair of severe DNA damage and apoptosis are linked to low-dose hypersensitivity.**

***Material and methods*: The effects of exposure to low doses (0.05–1.1 Gy) were investigated in skin biopsies taken from prostate cancer patients undergoing the first week of radiotherapy.**

**γH2AX foci and apoptotic cells were visualised by immunohistochemistry and quantified by image analysis.**

***Results*: The γH2AX foci pattern in biopsies taken 30 min after a single fraction revealed a low-dose hypersensitivity below 0.3 Gy ($p = 0.0009$). The result was consistent for repeated fractions ($p = 0.00001$).**

**No decrease in foci numbers could be detected when comparing biopsies taken 30 min and 2 h after single fractions of 0.4 and 1.2 Gy. The result was consistent for repeated fractions.**

**Only 43 of 168,000 cells in total were identified as apoptotic, yet a dose dependency could be detected after 1 week of radiotherapy ($p = 0.003$).**

***Conclusions*: We describe a method based on γH2AX to study DNA damage response and apoptosis in a clinical setting. A γH2AX hypersensitive response to low doses can be observed in epidermal skin, already 30 min following delivered fraction.**

**A very low frequency of apoptosis in normal epithelium suggests that this effect is not an important part of the in vivo response to low doses.**

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*Keywords:* γH2AX; Hypersensitivity; Apoptosis; Epidermis; Normal tissue; Parp-1; DNA damage; DNA repair; Double strand breaks; DSB; Digital image analysis; Foci

The cellular response to DNA damage by means of its various complex mechanisms is a corner stone for many current cancer therapies. In radiotherapy, different responses to DNA damage can be utilised to eliminate cancer cells with malfunctioning damage signalling, while preserving normal healthy cells with an intact repair mechanism. However, despite normal repair capacity, damage induced in the healthy cells can result in both short- and long-term side effects, which are important considerations for treatment planning. A deeper understanding of the molecular mechanisms underlying normal tissue response to DNA damage can provide new strategies to reduce side effects and further adapt treatments to individual variations.

To study the response in normal epithelium, repeated skin biopsies were taken from prostate cancer patients undergoing their first week of radiotherapy. This study focuses on three different stages of the response to radiation-induced DNA double-strand break (DSB) damage: First, the early response, reflected by the damage level at 30 min following insult. Second, the repair phase which reflects the cells repair capacity and kinetics by measuring residual DSB damage after 2 h. Finally, the long-term effect of repeated DSB damage, by the evaluation of cell death in terms of apoptosis, at the end of the first week of treatment.

[1] These authors contributed equally to this work.

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New molecular markers capable of detecting different parts of the DNA repair machinery have provided extensive information about the molecular pathways that regulate cell and tissue responses to low levels of DNA damage [1,2]. One of these markers, the phosphorylated histone γH2AX, has proved to be a good surrogate marker for the detection of DSBs [3,4]. γH2AX is detectable as discrete foci within minutes of radiation-induced DSBs. These foci co-localise with other proteins at the site of DSBs, and there is evidence that one DSB is enough to induce a γH2AX focus in non-dividing cells [5,6]. Furthermore, the dephosphorylation of γH2AX and disappearance of foci have been shown to follow DSB repair kinetics at clinically relevant levels of damage [7,8].

Previously, we had described a method to detect and quantify single DSBs in paraffin-embedded tissue sections with immunohistochemistry, fluorescence microscopy and digital image analysis [9]. Since then several similar approaches to foci quantification has been described [10,11]. Our method relies on γH2AX foci for DSB detection and since the method is applied to tissue sections the induced DSB damage is quantified as number of foci per nuclear area. By taking the nuclear area into account, the foci density measurement attained is less affected by variations in cell size, due to the presence of only partial cells in the section, and cell cycle-dependent variations in DNA content. Counting number of foci instead of foci area reduces the possible influence of method-based errors caused by variations in staining and microscopy light source intensities between different staining occasions. This method had previously been used in a clinical setting to measure low levels of DNA damage in normal tissue induced by radiotherapy and has been further developed and standardised for this study to improve resolution in the low-dose range. Such improvements are of particular relevance for low-dose applications such as radiation protection, dosimetry, X-ray diagnostic, radioimmunotherapy, radionuclide therapy and radiotherapy.

Apoptosis is known to cause abundant phosphorylation of H2AX [12]. This phenomenon is related to the fragmentation of DNA that occurs during apoptotic cell death. In this study, we show that a subset of γH2AX-positive cells with no foci pattern and completely stained nuclei could easily be discriminated in the immunostained skin biopsies. These cells displayed several features of apoptosis such as nuclear condensation, rounding and formation of apoptotic bodies. The apoptotic staining pattern was also shown to co-localise with cleaved PARP-1, a previously defined marker for apoptosis [13]. Furthermore, the γH2AX apoptotic staining pattern corresponding to DNA fragmentation can function as an apoptotic marker and may provide a surrogate end point for mitotic failure by detecting secondary apoptotic decisions [14].

Twenty years ago Joiner and colleagues described an increased sensitivity of tissues to small doses of X-rays. The phenomenon was observed for acute skin reactions and kidney function [15]. Since then, this topic has been the subject of intensive research. We had previously reported a low-dose hypersensitivity in cell kill by measuring the reduction of basal keratinocytes in human skin over 4 weeks of fractionated radiotherapy. The reduction was dose dependent for fraction sizes between 0.05 and 1.2 Gy, with a hypersensitivity below 0.4 Gy [16]. A continuation of that study has further confirmed hypersensitivity in keratinocyte reduction throughout 7 weeks of daily fractions below 0.4 Gy (data in preparation).

In our previous publication, we quantified foci in skin biopsies taken from two patients undergoing radiotherapy [9]. With that setup we could detect a dose-dependent response which could generally be described as linear. In this publication, we have examined the dose–response in more detail, using an extensive set of samples from a larger group of patients. Looking at the dose–response in more detail, we are able to detect an early hypersensitivity effect observed in the initial response to DSB damage. This effect is observed already 30 min after radiation, and could possibly be one of the mechanisms behind the hypersensitivity seen in cell kill and skin reactions.

The present study illustrates how γH2AX can be used to study DSB kinetics and apoptotic responses in vivo by using paraffin-embedded tissue samples taken from patients in a clinical setting. The results demonstrate, for the first time, an early low-dose hypersensitivity effect that is reflected by fraction size-dependent differences in DSB levels. We also found that γH2AX is a robust marker for apoptosis, giving confident estimates even at very low frequencies.

## Materials and methods

### Patient material

Skin biopsies were taken from five prostate cancer patients in clinical practice undergoing radiotherapy with a curative intent [16]. Biopsies were taken under local anaesthesia (Lidocaine, 5 mg/ml). Approval was obtained from the Ethical Committee at Göteborg University, and written informed consent was received from all patients prior to participation.

The skin biopsies were taken as 3 mm punch biopsies at various regions of the skin, as described previously, after careful determination of the dose distribution [16]. All patients started their radiotherapy treatment on a Monday or Tuesday. The patients received one fraction per day, prescribed to 2 Gy to the prostate according to ICRU, adding to a total of 4 or 5 fractions at the end of the first treatment week depending on starting day. All patients had at this stage received 8 or 10 Gy in the tumour target area, depending on whether they started on a Monday or Tuesday.

During the first treatment day, four biopsies were taken 30 min after the first dose fraction at locations of the skin corresponding to approximately 0.1, 0.2, 0.45 and 1.1 Gy. Two additional biopsies, corresponding to 0.45 and 1.1 Gy, were taken 2 h after the first fraction. A second set of biopsies with the same doses and time points as the first set were collected at the end of the first treatment week.

All biopsies were fixed immediately after collection in 5% formaldehyde. Two different fixations were used due to logistical reasons. The biopsies taken after the first fraction were fixed for 1 day and biopsies taken at the end of the first treatment week, after multiple fractions, were fixed for 3 days. A total of 64 biopsies were collected from the five patients.

Due to the differences in fixation times between the biopsies a separate test was set up in order to investigate the influence of fixation time on the γH2AX staining. Four

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