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CHAPTER 2

Decay of Gamma-H2AX foci Correlates with Potentially Lethal Damage Repair and P53 Status

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ABSTRACT

Potential lethal damage repair (PLDR) is an important cellular response after radiation treatment and influences tumor radiosensitivity and curability. Here, the influence of the p53 status on the repair of potentially lethal damage and DNA double strand breaks (DSBs) was studied in two isogenic colorectal carcinoma cell lines: RKO (p53 wild-type) and RC10.1 (p53 null), and three prostate cancer cell lines: LNCaP (p53 wt), DU145 (mut p53), and PC3 (p53 null).

Cell survival was determined by clonogenic assay, directly and 24h after irradiation with doses ranging from 0 to 8Gy. Survival curves were analyzed using the linear-quadratic formula: S(D)/S(0) = exp-(αD+βD²). To study DNA-DSB kinetics, induction and decay of γ-H2AX foci was measured 30 min and 24h post treatment. For this assay, cells were irradiated with single doses of 0, 0.5, 1 and 2Gy. Functional p53 status was assessed with cell cycle analysis and western blot.

Cells with functional p53 (RKO and LNCaP) clearly demonstrated PLDR, which was assessed as increased survival levels after delayed plating (24h) as compared to cells plated immediately after irradiation. Mutated p53 DU145 cells demonstrated only slight PLDR, and p53 null RC10.1 and PC3 cells did not show PLDR at all. For all cell lines, levels of γ-H2AX foci 24h after radiation were tremendously decreased compared to levels found 30 min after treatment. Interestingly, significantly less γ-H2AX foci were detected at 24h in wild-type p53 cells compared to mutated or p53 null cells. In addition, cells which demonstrated a clear PLDR also showed the largest decay in the number of γ-H2AX foci. In conclusion, functional p53 seems to be necessary for both cell survival and γ-H2AX foci decay, i.e. DNA DSB repair. Higher levels of PLDR and less residual foci were found in p53 wild type cells compared to p53 mutated or null cells. This study demonstrated a clear correlation between the degree γ-H2AX foci decay, and p53 status and potentially lethal damage repair.
INTRODUCTION

DNA double-strand breaks (DSBs) are biologically the most lethal lesions produced by ionizing radiation. The induction of DSBs can lead to cellular death and when repaired incorrectly, they might lead to chromosomal breaks, genomic instability and translocations [1, 2]. Persistent breaks could eventually lead to carcinogenesis, through activation of oncogenes or inactivation of tumor-suppressor genes [3]. Phosphorylation of the histone protein H2AX (γ-H2AX) on serine 139[4] is one of the earliest markers of DNA DSBs. The γ-H2AX ionizing radiation induced foci (IRIF) appear minutes after exposure with a maximum at 20-30 min, and are supposed to mark the locations of DNA double strand breaks [5-9]. After the breaks are rejoined, γ-H2AX is dephosphorylated again and thus the disappearance of the foci is related to repair of the DNA[10]. The repair of the potentially lethal DNA DSBs is an important factor in responses of cells to irradiation. Normally, the potential lethal damage induced by radiation causes cell reproductive death if cells are forced to go into mitosis. Preventing cells from proliferation will promote the repair of potential lethal damage and could subsequently lead to a decrease in radio sensitivity [11]. PLDR can be studied in plateau phase cultures using a clonogenic assay setup, wherein the replating is performed immediately after treatment (ip) or with a delay of 24h after treatment (dp) to allow repair processes to occur [12]. The difference in survival of immediately and delayed plated cells is considered to exhibit the cells’ capacity of repairing lethal DNA damage.

Following exposure to radiation, cells with potential lethal DNA damage are normally arrested at the G1/S border. Protein p53 is one of the key proteins responsible for correct activation of cell-cycle checkpoints [13, 14], and also plays an important role in the regulation of apoptosis [15, 16]. Abrogation of p53 is associated with a loss in G1 cell cycle checkpoint control, thereby allowing cells with DNA damage to progress into S-phase [17, 18]. Because of this, functional p53 status is thought to influence the repair of potentially lethal damage [11, 19]. However, some studies have shown that PLDR does not depend on functional p53 [20, 21].

Survival curves following clonogenic assay are commonly described and analyzed using the linear-quadratic model: \( S(D)/S(0) = \exp \left(-\alpha D - \beta D^2\right)\) [22, 23]. The advantage of using the LQ model is that changes in PLDR can be quantitatively determined by analyses of the linear parameter alpha, describing the low dose range of the survival curve, separately from the parameter \( \beta \) dominating the high dose range [24-26]. Analysis of survival curves from many studies has shown that PLDR is most clearly demonstrated by changes of the linear parameter \( \alpha \) [22, 27].

The relation between PLDR, radiosensitivity and the induction and disappearance of γ-H2AX IRIF is not always clear. The induction of γ-H2AX foci is not directly correlated with the overall survival after radiation [7, 28]. However, it has been demonstrated that there is a correlation between the number of residual DNA DSBs at 10hr after irradiation and cell survival [29, 30]. Residual γ-H2AX foci, i.e. DNA DSBs, after irradiation could indicate that cells were not capable of
PLDR. The present study was initiated to examine the importance of p53 in the PLDR and DNA DSBs repair. The induction and disappearance of γ-H2AX IRIF were scored as a measure of DNA DSB repair, and clonogenic assay were performed to detect PLDR. Results were obtained in prostate cancer cells and two colorectal cancer cells with different p53 status.

**MATERIALS AND METHODS**

**Cell cultures.** The human colorectal carcinoma cells RKO and RC10.1 were kindly provided by Dr. Kathleen Cho [31]. These isogenic cell lines only differ in p53 status: RKO is wild-type p53 and RC10.1 is p53 null. Abrogation of TP53, resulting in a p53 null status, was caused by transfection with HPV16-E6. Geneticin (200 μg/ml) was added to the culture flasks to ensure RC10.1 cultures consist of transfected cells only. Both cell lines were cultured in McCoy’s 5a medium with 25 mM HEPES, supplemented with 10% fetal calf serum, 1 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ in air. Human prostate cancer cell lines LNCaP (p53 wild type), PC3 (p53 null) and DU145 (p53 mutant) were obtained from American Type Culture Collection (ATCC, Rockville). All three cell lines were cultured in RPMI 1640 (Gibco, Invitrogen) medium supplemented with 10% fetal calf serum, 100 U/ml penicillin/streptomycin and 1 mM glutamine in a humidified atmosphere of 5% CO₂/95% air.

**Irradiation.** Irradiation of colorectal cancer cells was performed with γ-irradiation using a 137Cs source at a dose rate of about 0.5 Gy/min. Prostate cancer cells were irradiated with a Siemens Stabilipan Orthovolt at a dose rate of 3 Gy/min (15 mA, 250 kV) with a 0.5 mm Cu filter. For determination of clonogenic survival, the cells were radiated with single doses of 0, 2, 4, 6 and 8 Gy. Detection and scoring of γ-H2AX IRIF was performed after irradiation with 0, 0.5, 1 and 2 Gy (RKO and RC10.1 cells) or 2 Gy (LNCaP, PC3 and DU145 cells). A radiation dose of 4 Gy was applied for the cell cycle analysis.

**Clonogenic assay.** Directly and 24h after irradiation cells were trypsinized and replated for clonogenic survival assay in appropriate cell numbers in 6-well plates [32]. Subsequently, cells were incubated for 10 days. Surviving colonies were fixed and stained with glutaraldehyde-crystal violet solution and counted. Survival curves were analyzed using IBM SPSS 20 (Chicago, USA) by means of fit of data by weighted linear regression, according to the linear-quadratic formula: \( S(D)/S(0)=\exp(-\alpha D+\beta D^2) \) [22, 23]. In the formula the \( S(D) \) is the survival at dose \( D \) and \( S(0) \) is the survival at dose 0. As a measure of PLDR, the ratio PLD-α is calculated which is the ratio of the value of linear parameter α of cells immediately plated (ip) after irradiation and cells delayed plated (dp) 24 hours after irradiation.
Immunohistochemical detection of γ-H2AX. The γ-H2AX foci assay was used to study the induction and repair of DNA-DSBs. For this purpose cells were grown on sterile cover slips (21 x 26 mm) placed in 60 mm cell culture dishes [2, 33]. Cells were irradiated when a confluent layer was obtained. The number of γ-H2AX foci was determined 30 min and 24 h after irradiation. After irradiation, cells were washed with phosphate buffered saline (PBS) and fixed in PBS containing 2% paraformaldehyde for 15 min. After three further washes with PBS, cells were treated with PBS containing 0.1% Triton X-100 & 1% FCS (TNBS) for 30 min to permeabilize the cells. A primary mouse monoclonal anti-γ-H2AX antibody (Millipore) was diluted 1:100 in TNBS. Fixed, permeabilized cells on the cover slides were incubated with 50µl primary antibody under a parafilm strip for 90 min at room temperature. Cells were then washed with PBS for about 5 min and the parafilm strip was removed. After this, cells were washed 2 times with TNBS. Cells on cover slides were incubated with 50µl secondary antibody anti-Mouse Cy3 (Jackson) (1:100 in TNBS) under a parafilm strip for 30 min at room temperature. Cells were then washed 2-3 times with TNBS for about 5 min and the parafilm strip was removed. Nuclei were stained with DAPI (2.5µg/ml) for 5 min and embedded in vectashield. Then cover slides were sealed to microscope slides. Rubber cement was used to seal the whole construct.

Scoring of γ-H2AX foci. Digital image analysis was performed to determine the number of γ-H2AX IRIF. Fluorescent photomicrographs of γ-H2AX foci were obtained using Image Pro Plus software. Stack images of at least 50 cells were obtained using a Leica DM RA HC Upright Microscope equipped with a CCD camera. One stack image consists of 23 slices with a 300 nm interval between the slices along the z-axis. Images were then processed and the number of foci per nuclei was scored using custom made software [5]. All experiments were carried out in triplicates, independently from each other. Numbers of foci in unirradiated control cells were subtracted from numbers in irradiated samples. S-phase cells were excluded using an EDU (5-ethyl-2'-deoxyuridine) staining (Invitrogen, Eugene, Oregon USA) to mark these cells. The ratio of the number of γ-H2AX foci at 30 min and 24 h after irradiation was calculated as a measure of foci decay resulting from repair of DNA double strand breaks.

Western Blotting. Levels of p53 were determined with Western blotting. Controls and irradiated cells were washed with PBS and harvested at 4 h after treatment. Pellets were lysed in ice-cold RIPA buffer (20mM Tris-HCl, 150mM NaCl, 1mM Na2EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na3VO4, 1μg/ml leupeptin) for 30 min on ice with protein inhibitors [34]. Laemmli buffer with 2-mercaptoethanol (355mM) was added to the supernatant (1:1) and heated in boiling water 2-5 min. Finally samples were sonificated (Sonics & Materials Inc). 1 μg of protein was resolved by 10% SDS-PAGE precast gels (BioRad) and transferred to PVDF membranes. Equal protein loading was checked by Ponceau S staining. Immunodetection was performed for p53 (mAb Do-7, Dako) in combination with a
horseradish peroxidase-conjugated secondary anti-mouse IgG (Dako). Housekeeping protein ERK2 was detected using (mAb, Bethyl Laboratories) and a secondary anti-rabbit (mAb, Invitrogen, California, USA). All were enhanced chemiluminescence (Amersham Pharmacia Biotech). Finally, blots were analyzed using LAS4000 (GE, Healthcare life sciences).

**Figure 1. Function of p53 in colorectal cancer cells and prostate cancer cells.**

**A:** Western blot analysis of p53 levels in whole cell lysates of RKO (wild-type), RC10.1 (p53 null), LNCaP (wild-type), DU145 (p53 mutated) and PC3 (p53 null) cells. **B:** Flow cytometric analysis of cell cycle distributions in RKO and RC10.1 cells. PI fluorescence indicates the amount of DNA and FITC fluorescence the incorporation of BrdU. Representative data from untreated and treated cells (16h after 4Gy) are shown.

**Flow Cytometric Analysis.** Cell cycle distribution after irradiation was determined in exponentially growing cells by flow cytometric analyses of DNA content and BrdU incorporation. After 16h post treatment, untreated and irradiated cells were incubated with BrdU (10 μM) for 1h [11, 35], harvested and fixed in 70% ethanol in PBS. After 30 min incubation in 1 ml pepsin-
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HCl (0.4 mg/ml 0.1N HCl), washing with PBS containing 0.05% Tween20 (PBT), 30 min incubation in 1ml 2N HCl at 37°C and washing with PBT with 20mg/ml BSA (PBTb), cells were incubated with 100 μl rat-anti-BrdU IgG (Harlan SeraLab LTD) in PBTb for 60 min at 37°C. For secondary antibody step, cells were washed with PBTg (PBT with 1% v/v normal goat serum (DAKO)) followed by 60 min incubation at 37°C with 0.1 ml fluorescein conjugated goat anti-rat IgG (Jackson Immunoresearch) diluted 1:100 in PBTg. PI was added to a final concentration of 20μg/ml in PBS and samples were stored at 4°C before flowcytometric (FACS Canto, BD Biosciences) analysis.

RESULTS

P53 function and cell cycle arrest
To assess p53 status of the used cell lines, western blot analysis was performed for p53 induction at 4h after 4Gy radiation treatment (see Figure 1 A). A clear induction of p53 after irradiation is demonstrated for both p53 wildtype cells RKO and LNCaP. In contrast, no p53 could be detected in RC10.1 and PC3 cells (p53 null). The p53 mutated DU145 cells showed similar levels of p53 in untreated and treated conditions, so radiation did not result in an induction. In addition, cell cycle analysis revealed a G1-cycle arrest 16 h after radiation in wildtype RKO cells compared to p53 null RC10.1 cells (Figure 1 B). For RC10.1 cells only a G2 arrest was observed after radiation treatment. The percentage of RKO cells remaining in S-phase cells is only 21 ± 6%, whereas the percentage of S-phase in RC10.1 is 46 ± 2%.

Functional p53 result influences cell survival and PLDR capacity
PLDR capacity of all cell lines is measured by comparing the survival curves of immediate (ip) and delayed plated (dp) cells. As can be depicted from Figure 2 A-B, RKO cells are more sensitive to ionizing radiation than the RC10.1 cells when plated immediately after irradiation. This is highlighted by the α values of the linear-quadratic model of ip plated cells (table I). The RKO cells clearly shows increased survival after delayed plating compared to immediately plated cells after irradiation, indicating repair of potentially lethal damage (PLDR). The difference between the ip and dp survival curves of the RKO cells is significant (p<0.02). For the RC10.1 cells, no differences between ip and dp survival curves were observed. Similar results were obtained for the prostate cancer cell lines: LNCaP and DU145 cells clearly showed PLDR, but the p53 null PC3 cells didn’t show any difference in survival after ip or dp conditions (Figure 2 C-E). In Table 1, the values of the linear and quadratic parameters, α and β, and the PLDR-α ratio (= α_{ip}/α_{dp}) as a measure of PLDR are presented.
Figure 2. Survival curves of the different cancer cell lines, plated immediately (ip) and 24h after (dp) irradiation. PLDR is the increase in survival after dp compared to ip, and is most pronounced in p53 wildtype RKO (A) and LNCaP (C). Mutated p53 DU145 (D) only shows a slight PLDR, and p53 null cells RC10.1 (B) and PC3 (E) show no PLDR at all. Error bars indicate standard error of the mean (SEM), N=3.

Lowest number of residual γ-H2AX foci after radiation in p53 wildtype cells

To measure the efficiency of DNA DSB repair, γ-H2AX foci were measured 30 min and 24h after radiation treatment in all cell lines (Figure 3 A). The initial numbers of foci at 30 min elucidated differences in radiation response between the different cancer cell types (Figure 3 B-C). Prostate cancer cells seem to be less sensitive to radiation (11 to 17 foci/nucleus) as compared to the colorectal cancer cells (approximately 25 foci/nucleus) after 2Gy radiation. For this study however, the decay of γ-H2AX foci 24h after treatment is more important. By comparing the initial induced foci numbers with the residual foci numbers, differences in repair capacity between cell types can be observed. Significant more residual γ-H2AX foci were detected 24h after radiation in RC10.1 cells compared to the RKO cells (p<0.01). As the initial foci numbers are the same for both cell lines, the largest foci decay is therefore observed in wildtype p53 RKO cells. Similar for the prostate cancer cells, the highest decline in foci number is detected for LNCaP cells (p53 wildtype) and the lowest for PC3 cells (p53 null). Foci decay ratios (initial γ-H2AX foci numbers divided by residual γ-H2AX foci numbers) and p53 status of the different cell lines are presented in Table 1.
DISCUSSION

The level of PLDR and the decay of γ-H2AX foci was investigated in two isogenic colorectal cell lines and in three prostate cancer cell lines with different p53 status (wildtype, null or mutated). Results obtained in this study show that both PLDR capacity and the efficient decay of γ-H2AX foci are dependent on functional p53 status. Additionally, a correlation is observed between the decay of γ-H2AX foci and levels of PLDR. Cells with high residual γ-H2AX foci numbers did not show PLDR and vice versa. This indicates that the repair of potential lethal damage is linked to DNA DSBs repair. Survival analysis demonstrated that p53 status influences PLDR. RKO and LNCaP cells with wild-type p53 protein clearly demonstrated PLDR while the p53 null PC3 and RC10.1 cell lines showed no PLDR at all. These findings correspond to earlier studies, which demonstrated that an intact TP53 status is required for repair of potentially lethal damage [13, 18]. PLDR-α values of 1.4 ± 0.2 and 1.0 ± 0.2 for RKO and RC10.1 cells respectively were found, which are almost identical to the values observed in the study of Franken et al. [11]. In addition,
p53 wild type cells appeared to be more radiosensitive compared to p53 null or mutated cells when plated immediately after irradiation. This might be due to the induction of apoptosis [15, 16] in the p53 wildtype cells. On the other hand, in these cells the p53 induced cell cycle arrest allows the cells to recover the radiation damage and consequently the repair of their potentially lethal damage. This results in higher survival levels when plated 24h after irradiation [24]. Furthermore, the presented data demonstrated that decay of γ-H2AX foci after exposure to ionizing radiation is also dependent on p53, and conjointly associated with PLDR. Results show that cells with the highest PLDR also had the largest decay in number of foci, resembling a more proficient repair of DNA DSB. This is in agreement with results obtained by MacPhail et al. [36], which showed that the decay of γ-H2AX foci is associated with cell survival and repair of DSB. Moreover, it was reported that cervical cancer cells with wild-type p53 showed a significantly faster γ-H2AX decay rate after irradiation than cells deficient in p53 [30]. Taneja et al. [37] also found a correlation between radiosensitivity and residual amounts of γ-H2AX. However, other studies did not find a correlation between residual foci and radio sensitivity [28, 38]. A difference in study set up might explain these different findings, as they only studied survival of cells plated immediately after irradiation. To add, p53 functionality was not examined in these studies. Here, cell reproductive death was examined both directly as 24h after irradiation in order to study PLDR, which was correlated with foci decay and p53 status. Banath [30] stated that residual γ-H2AX foci at 24h after irradiation are indicative for lethal DNA damage. Although, the initial number of γ-H2AX foci closely correlates with the amount of DSBs, the disappearance of γ-H2AX may however differ from the actual DSB repair [39, 40]. As dephosphorylation of γ-H2AX is slower than DSB repair, disappearance of γ-H2AX foci could occur while the break is not repaired yet [41]. On the other hand, when phosphorylation is hindered and γ-H2AX foci remain visible, it is possible that the DNA DSB is already still repaired. Residual foci could also be an indication for misrepaired chromosomes [42]. Nevertheless, the decay of γ-H2AX foci is generally used as a measure for DSB repair.

The linear-quadratic model is based on the observation that cell reproductive death results from lesions induced by single-particle tracks of ionizing particles or from interaction of sublethal damage from two independent particles. As DNA DSBs are considered to be the most lethal lesions, PLDR capacity should represent the repair of DNA DSBs. The present results on the decay of γ-H2AX foci as indicators of DNA DSBs and the correlation with PLDR are consistent with this hypothesis. Furthermore, the results show that functional p53 status is important for efficient repair of potential lethal damage and thus DNA DSBs. Efficient PLDR capacity undermines the effect of radiation treatment. Therefore studies investigating genes or factors which influence PLDR activity are important and may lead to a better tumor control or treatment strategy.
Decay of γ-H2AX correlates with Potential Lethal Damage Repair

Table I. LQ parameters α and β, PLDR-α, foci decay ratio and p53 status of the different cancer cell lines.

<table>
<thead>
<tr>
<th>LQ Parameters</th>
<th>Cell line</th>
<th>α, Gy$^{-1}$</th>
<th>β, Gy$^{-2}$</th>
<th>PLDR-α</th>
<th>Ratio foci decay</th>
<th>p53 status</th>
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<tr>
<td></td>
<td>RKO ip</td>
<td>0.68 ± 0.06</td>
<td>0.02 ± 0.02</td>
<td>1.7 ± 0.3</td>
<td>5.2 ± 0.6</td>
<td>wild-type</td>
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<td></td>
<td>RKO dp</td>
<td>0.40 ± 0.07</td>
<td>0.02 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RC10.1 ip</td>
<td>0.40 ± 0.07</td>
<td>0.06 ± 0.02</td>
<td>1.1 ± 0.2</td>
<td>3.5 ± 0.4</td>
<td>null</td>
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<td></td>
<td>RC10.1 dp</td>
<td>0.38 ± 0.04</td>
<td>0.04 ± 0.01</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LNCaP ip</td>
<td>0.31 ± 0.09</td>
<td>0.08 ± 0.03</td>
<td>10.3 ± 3.9</td>
<td>6.9 ± 0.3</td>
<td>wild-type</td>
</tr>
<tr>
<td></td>
<td>LNCaP dp</td>
<td>0.03 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td></td>
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<td>null</td>
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<tr>
<td></td>
<td>DU-145 ip</td>
<td>0.22 ± 0.06</td>
<td>0.04 ± 0.01</td>
<td>3.1 ± 1.6</td>
<td>4.2 ± 0.7</td>
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<td></td>
<td>DU-145 dp</td>
<td>0.07 ± 0.03</td>
<td>0.05 ± 0.01</td>
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<td></td>
<td>PC3 ip</td>
<td>0.39 ± 0.04</td>
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<td></td>
<td>PC3 dp</td>
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<td>0.04 ± 0.01</td>
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</table>

LQ, linear-quadratic; PLDR, potentially lethal damage repair; ip, cells immediately plated after irradiation; dp, cells delayed plated 24h after irradiation. Error indicates standard error of the mean (SEM), n=3.

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