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Research article

DECAY OF γ-H2AX FOCI CORRELATES WITH POTENTIALLY LETHAL DAMAGE REPAIR AND P53 STATUS IN HUMAN COLORECTAL CARCINOMA CELLS

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Abstract: The influence of p53 status on potentially lethal damage repair (PLDR) and DNA double-strand break (DSB) repair was studied in two isogenic human colorectal carcinoma cell lines: RKO (p53 wild-type) and RC10.1 (p53 null). They were treated with different doses of ionizing radiation, and survival and the induction of DNA-DSB were studied. PLDR was determined by using clonogenic assays and then comparing the survival of cells plated immediately with the survival of cells plated 24 h after irradiation. Doses varied from 0 to 8 Gy. Survival curves were analyzed using the linear-quadratic formula: $S(D)/S(0) = \exp-(\alpha D+\beta D^2)$. The γ-H2AX foci assay was used to study DNA DSB kinetics. Cells were irradiated with single doses of 0, 0.5, 1 and 2 Gy. Foci levels were studied in non-irradiated control cells and 30 min and 24 h after irradiation. Irradiation was performed with gamma rays from a ^{137}Cs source, with a dose rate of 0.5 Gy/min. The RKO cells show higher survival rates after

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Abbreviations used: BrdUrd – bromodeoxyuridine; Cs – cesium; DAPI – 4',6-diamidino-2-phenylindole; dp – delayed plating; DSB – double-strand breaks; FACS – fluorescent activated cell sorter; FCS – fetal bovine calfserum; HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HPV – human papilloma virus; ip – immediate plating; PBS – phosphate buffered saline; PLD – potentially lethal damage; PLDR – potentially lethal damage repair; PVDF – polyvinylidene fluoride; RIPA – radio immunoprecipitation assay; SDS PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis; TNBS – PBS containing 0.1% Triton X-100 and 1% FCS

delayed plating than after immediate plating, while no such difference was found for the RC10.1 cells. Functional p53 seems to be a relevant characteristic regarding PLDR for cell survival. Decay of γ-H2AX foci after exposure to ionizing radiation is associated with DSB repair. More residual foci are observed in RC10.1 than in RKO, indicating that decay of γ-H2AX foci correlates with p53 functionality and PLDR in RKO cells.

Key words: p53, Radiation sensitivity, Potentially lethal damage repair (PLDR), Linear-quadratic model, Clonogenic assay, Colon cancer cells, RKO cells, RC10.1 cells, γ-H2AX foci, Flow cytometry

INTRODUCTION

DNA double-strand breaks (DSBs) are biologically the most significant lesions produced by ionizing radiation. DSBs are the main threat to the genomic integrity of cells. When repaired inaccurately, DSBs may lead to e.g. chromosome breaks, translocations and deletions [1-3]. The presence of incorrectly repaired or persistent DSBs may result in genomic instability, which in turn can lead to carcinogenesis or cell death through the activation of oncogenes or inactivation of tumor-suppressor genes [4]. When present in the germ line, DSBs can be held responsible for congenital defects [1].

Histone H2AX is an important player in the DNA DSB repair cascade. After the exposure of cells to ionizing radiation, DSBs are induced and H2AX becomes rapidly phosphorylated (γ-H2AX) on serine 139 [5]. Immunostaining of γ-H2AX enables the detection of individual DSBs using fluorescence microscopy. The γ-H2AX foci appear as discrete nuclear foci within 1 min of cell exposure to ionizing radiation [6, 7]. Maximal values are reached about 30 min after irradiation. The number of these initial foci is similar to the number of expected DNA DSBs and decreases with the repair of these DSBs [7-9]. Since γ-H2AX is associated with DSB repair, the kinetics of the formation and loss of γ-H2AX foci are related to the efficiency of repair [10-12].

Efficient DNA repair and correct activation of cell cycle checkpoints upon induction of DNA damage are of crucial importance for the maintenance of genomic integrity. Checkpoints induce actively dividing cells to pause and repair DNA damage before segregation of the replicated genome into daughter cells [1]. The malfunction of proteins that are not involved in the actual repair processes can still result in non- and misrepair of DNA DSBs.

Protein p53 is one of the key proteins responsible for the correct activation of cell cycle checkpoints [13, 14]. It 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. Following exposure to radiation, cells normally arrest at the G1/S border. Cells with deficient p53 function continue into S-phase after exposure to radiation [17-19]. Due to its role in G1-phase arrest, p53 status is thought to influence the repair of potentially lethal damage.

Potentially lethal damage (PLD) is that part of radiation damage that can be altered by post-irradiation conditions [20, 21]. When cells are forced to go into mitosis, PLD causes cell reproductive death. If cells are held in suboptimal growth conditions for various times after irradiation, such as in cultures with G0/G1 contact inhibition or serum starvation, PLD repair (PLDR) is greatly increased, thus enhancing survival. Conditions that prevent cells from proliferating promote the repair of PLD, thereby influencing radiosensitivity [22, 23]. PLDR is studied in plateau phase cultures using a clonogenic assay setup, wherein the replating is performed immediately after treatment (immediate plating, ip) or with a 24-h delay (delayed plating, dp) to allow repair processes to occur [24]. The difference in survival rates of ip and dp cells is considered to represent the cells' capacity for repairing potentially lethal DNA damage.

Quantitative information about cultured cells can be analyzed in terms of mathematical dose-effect relationships based on the linear-quadratic (LQ) model of cell reproductive death as a function of the radiation dose [25-28]. The LQ-formula for the cell reproductive death of cells is described by an inverse exponential approximation:

$$S(D)/S(0) = \exp-(\alpha D+\beta D^2),$$

where D is the radiation dose, S(D) is the surviving fraction of the cells exposed to the radiation dose D, S(0) is the survival rate for non-irradiated cells, α (Gy^{-1}) is the parameter defining the initial slope of cell survival curves and the effectiveness at low doses, and β (Gy^{-2}) represents the increasing contribution from cumulative damage thought to be due to the interaction of two or more lesions induced by separate ionizing particles [27, 29].

Several studies have demonstrated that p53 status is important for PLDR [17-19]. However, other studies suggested that PLDR does not depend on functional p53 [30, 31]. This study was done to determine whether p53 status influences the repair of PLD in two isogenic human colorectal carcinoma cell strains. We also investigated whether PLDR correlates with DNA DSB repair.

MATERIALS AND METHODS

Cell cultures

The human colorectal carcinoma cell lines RKO and RC10.1 were provided by Dr. Kathleen Cho [32]. These cell lines differ in their p53 status: RKO has 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 [32]. Geneticin (200 μg/ml) was added to the culture flasks to ensure RC10.1 cultures would 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% CO_2 in air.

Irradiation

Irradiation of confluent cultures was performed at ambient temperature with gamma rays from a ^{137}Cs source. The dose rate was about 0.5 Gy/min. For determination of clonogenic survival, the cells were irradiated with single doses of 0, 2, 4, 6 and 8 Gy. To determine the amounts of γ-H2AX foci, cells were exposed to radiation doses of 0, 0.5, 1 and 2 Gy.

Clonogenic assay

Cellular survival subsequent to radiation treatment was assessed using clonogenic assays, which were performed as previously described [33]. The cells were treated in 60-mm culture dishes. To study differences in potentially lethal damage repair (PLDR) after exposure to ionizing radiation, cells were replated in appropriate dilutions immediately after irradiation (immediate plating; ip) or 24 h after irradiation (delayed plating; dp). After re-plating, the cells were cultured for 10 days to allow colonies to form. If sufficiently large colonies had formed, the medium was removed and colonies were fixed and stained with a mixture of 6% glutaraldehyde and 0.5% crystal violet for 30 min. After the staining solution was removed, the plates were rinsed with tap water and allowed to dry at ambient temperature. Colonies containing at least 50 cells were counted using a stereomicroscope and a colony counting pen.

The surviving fractions, defined by the formula S(D)/S(0), were calculated, and the survival curves were analyzed using SPSS statistical software via fit of data by weighted linear regression, according to the linear-quadratic formula: $S(D)/S(0) = \exp-(\alpha D+\beta D^2)$, as defined above [26, 27]. The ratio of the α-values of ip and dp was calculated as a measure of PLDR.

Immunohistochemical detection of γ-H2AX

The γ-H2AX foci assay was used to study the induction and repair of DNA DSBs. Cells were grown on sterile cover slips (21 x 26 mm) placed in culture dishes [2, 34]. The cells were reseeded at a density of 2.5×10^5 cells per cover slip. When the cells had attached to the underlying surface, medium was added to a total volume of 3 ml per culture dish. The dishes were incubated until a confluent layer was obtained and then irradiated. To study the formation and decay of γ-H2AX foci, the number of foci was determined 30 min and 24 h after irradiation.

After washing with PBS, the cells were fixated with 2% paraformaldehyde for 15 min. After three further washes with PBS, cells were treated with TNBS (PBS containing 0.1% Triton X-100 and 1% FCS) for 30 min to permeabilize the cells. To stain for γ-H2AX foci, first a primary mouse monoclonal anti-γ-H2AX antibody (Millipore, diluted 1:100 in TNBS) was used. TNBS was removed and the cells were incubated with 50 µl primary antibody for every cover slip under a parafilm strip for 90 min at ambient temperature. Then the parafilm strip was removed and the cells were washed with PBS for about 5 min, followed by two further washes with TNBS. The secondary antibody used was Goat anti-Mouse Cy3 (Jackson-Immunoresearch, diluted 1:100 in TNBS). Cells

on cover slips were incubated with 50 µl secondary antibody under a parafilm strip for 30 min at ambient temperature. During incubation, the culture dishes containing the cover slips with cells were kept in the dark. Again the parafilm strip was removed and the cells were washed three times with TNBS for about 5 min. Finally, the nuclei were stained with two droplets of DAPI (2.5 µg/ml) per cover slip. One droplet of Vectashield antifade solution was added to all of the microscope slides. The cover slips were removed from the dishes and mounted to microscope slides. Rubber cement was used as a sealant.

Scoring of γ-H2AX foci

In order to determine the number of γ-H2AX foci induced by ionizing radiation, the microscope slides were examined using a Leica DM RA HC fluorescence microscope equipped with a CCD camera under a 100x objective lens [9, 34]. Fluorescent photomicrographs of γ-H2AX foci were obtained using custom-made software. Stack images of at least 100 cells per sample were taken. One stack image consisted of 20 slices with a 300 nm interval between the slices along the z-axis. The images were processed and the number of foci in cells was manually scored and checked. The ratio of the number of γ-H2AX foci 30 min and 24 h after irradiation was calculated as a measure of foci decay resulting from repair of DNA double-strand breaks.

The obtained data are presented as the means and standard error of the mean (SEM). To assess whether the amount of γ-H2AX foci significantly differed between cell lines, means were compared using Student's T-test. Results were considered significantly different at $p < 0.05$.

Western blotting

Levels of p53 were determined with Western blotting. Controls and irradiated cells were washed with PBS and harvested 4 h after treatment. Pellets were lysed in ice-cold RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) for 30 min on ice with protein inhibitors [9, 19, 35-38]. Laemmli buffer with 2-mercaptoethanol (355 mM) was added to the supernatant (1:1) and heated in boiling water for 2 to 5 min. Then the samples were sonificated (Sonics & Materials Inc.). Finally, 1 µg of protein was resolved by 10% SDS-PAGE precast gels (BioRad) and transferred to PVDF membranes. Equal protein loading was checked using Ponceau S staining.

Immunodetection was performed for p53 using mAb Do-7 (Dako) in combination with a horseradish peroxidase-conjugated secondary anti-mouse IgG (Dako). The housekeeping protein ERK2 was detected using mAb (Bethyl Laboratories) and a secondary anti-rabbit (mAb, Invitrogen). All of the antibodies were enhanced chemiluminescence (Amersham Pharmacia Biotech). Finally, the blots were analyzed using an ImageQuant LAS 4000 (GE, Healthcare Life Sciences).

Flow cytometric analysis

The distribution of cells over the cell cycle after irradiation was determined in exponentially growing cells using bivariate flow cytometric analyses of DNA content and BrdUrd incorporation, 16 h after treatment. Controls and irradiated cells (4 Gy) were allowed to incorporate BrdUrd (10 μM) for 1 h [19]. Then cells were fixed, and, after washing, immunofluorescence staining was carried out with rat-anti-BrdUrd IgG (Harlan SeraLab Ltd.) and fluorescein-conjugated goat-anti- rat IgG (Jackson Laboratories). Total DNA was stained with propidium iodide (0.5 μg/ml). Flow cytometry was performed using the FACS Canto (Becton Dickinson Biosciences). Dot blots were analyzed with FlowJo (v 10.0, TreeStar). The statistical analysis was carried out with Student's T-test.

RESULTS

P53 function and cell cycle arrest

The protein p53 functionality of the two cell lines is illustrated in Fig. 1. Western blot analysis shows that there was an accumulation of p53 at 4 h after 4 Gy irradiation in RKO cells, but p53 could not be detected in RC10.1 cells (Fig. 1A). G1 cycle arrest was found 16 h after 4 Gy radiation dose in RKO cells and not in RC10.1 cells (Fig. 1B). The percentage of S-phase cells remaining 16 h post irradiation was only 30 ± 6%. In the RC10.1 cells, G2 arrest was observed after exposure to ionizing radiation. The percentage of S-phase for RC10.1 cells was 45 ± 2%.

Radiation cell survival

The survival curves of both cell lines are presented in Fig. 2. RKO cells are more sensitive to ionizing radiation than RC10.1 cells when plated immediately after irradiation. This is highlighted by the values of α in the linear-quadratic model of ip cells (Table 1). The RKO cells clearly show increased survival after delayed plating compared to immediately plated cells, indicating repair of potentially lethal damage (PLDR). The difference between the ip and dp survival curves for the RKO cells is significant ($p < 0.02$). For the RC10.1 cells, this was not the case. In Table 1, the values of the linear and quadratic parameters, α and β, and the PLDR-α ratio (α_{ip}/α_{dp}) are presented as a measure of PLDR. The respective PLDR ratios for RKO cells and RC10.1 cells are 1.7 ± 0.3 and 1.1 ± 0.2.

DNA DSBs (γ-H2AX foci)

Fluorescent photomicrographs of γ-H2AX foci in RKO (p53 wild-type) and RC10.1 (p53 null) colon carcinoma cells after irradiation with 1 Gy and the mean number of γ-H2AX foci 30 min and 24 h after 0, 0.5, 1 and 2 Gy irradiation are shown in Fig. 3. There are no significant differences between the cell lines in terms of the amount of foci prior to irradiation and the amount 30 min after irradiation. At 24 h after irradiation, the p53 null RC10.1 cell line appeared to have more remaining foci than the wt p53 RKO cell line for all radiation

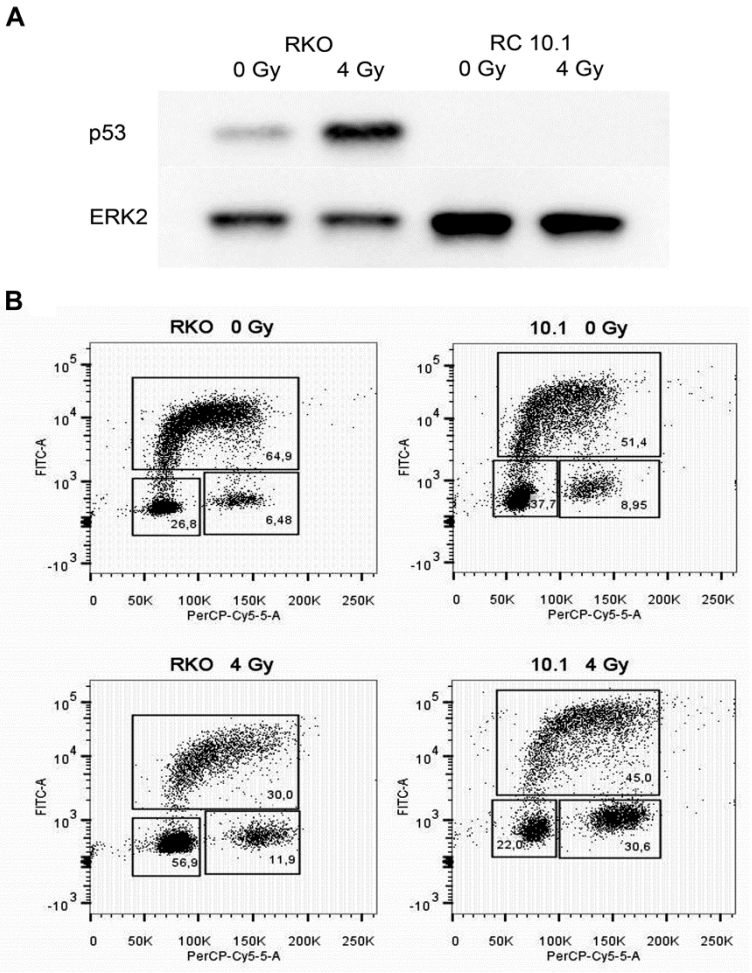

Fig. 1. Western blot and flow cytometric analyses illustrating the functionality of p53 in RKO and RC10.1 cells. A – Western blot analyses of p53 levels in whole cell lysates of RKO (wild-type p53) and RC10.1 (p53 null) cells. The accumulation of p53 after irradiation indicates wild-type p53 function [19]. The housekeeping protein ERK2 was used as a loading control. B – Flow cytometric analyses of RKO and RC10.1 cells. PI fluorescence on the X-axis indicating the amount of DNA and the amount of FITC-labeled BrdUrd (10 μM for 1 h) on the Y axis. Representative examples of data from control and irradiated cells (16 h after exposure to 4 Gy) are shown. Western blots and flow cytometry were carried out at least 3 times.

doses. Only for the 2 Gy dose is the difference in number of foci significant ($p < 0.01$). For 0.5 and 1 Gy, the differences were only marginally significant ($p < 0.1$). Mean values and SEM derived from the γ-H2AX experiments are shown in Fig. 3C-E. Dose response curves for both cell lines for the number of γ-H2AX foci 30 min and 24 h after irradiation are presented in Fig. 3F. The

number of irradiation-induced foci 30 min after 1 and 2 Gy exposure is 16 ± 2 and 25 ± 2, respectively. The ratio of decay of foci after 2 Gy is 5.2 ± 0.6 for RKO cells and 3.5 ± 0.4 for RC10.1 cells. (Table 1). This corresponds with the p53 status for RKO and RC10.1 and the PLDR ratio as described above. This suggests that the decay of foci is associated with PLDR and p53 status.

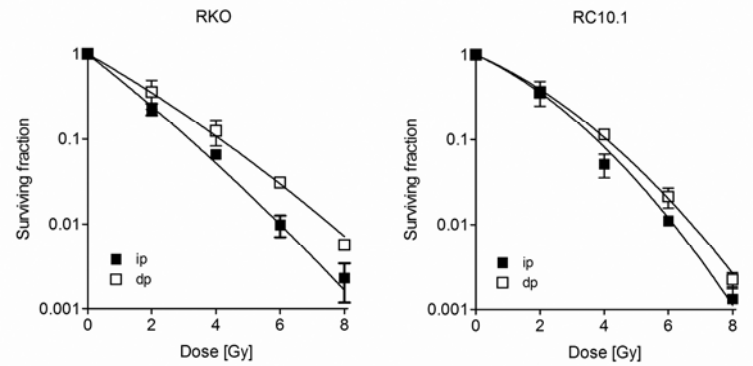

Fig. 2. Survival curves of the different colorectal cancer cell lines that were plated immediately (ip) and 24 h after (dp) irradiation. PLDR is most pronounced in RKO. Clonogenic survival experiments were carried out at least 3 times.

Table 1. Values of the LQ parameters α and β, PLDR-α, the ratio of foci decay after 2 Gy irradiation, and the p53 status of the colorectal cancer cell lines.

LQ parameter	α, Gy^{-1}	β, Gy^{-2}	PLDR-α	Ratio of foci decay after 2 Gy irradiation	p53 status
Cell line					
RKO ip	0.68 ± 0.063	0.02 ± 0.015	1.3	5.2 ± 0.6	p53 wt
RKO dp	0.40 ± 0.07	0.02 ± 0.019			
RC10.1 ip	0.40 ± 0.074	0.06 ± 0.015	1.1 ± 0.2	3.5 ± 0.4	p53 null
RC10.1 dp	0.38 ± 0.037	0.04 ± 0.007			

DISCUSSION

The level of PLDR was investigated in two cell lines, one with proficient p53 function (RKO) and one with abrogated p53 function (RC10.1). As reported in earlier studies, an intact p53 status is required for the repair of potentially lethal damage [17-19]. We also studied whether or not formation and decay of γ-H2AX foci correlates with the repair of PLD observed in survival assay. PLDR was studied using the linear-quadratic model (LQ model). The LQ model provides a biologically plausible and experimentally established method to quantitatively describe the dose-response to irradiation in terms of clonogenic survival. In the basic LQ formula: $S(D)/S(0) = \exp-(\alpha D+\beta D^2)$, α and β are

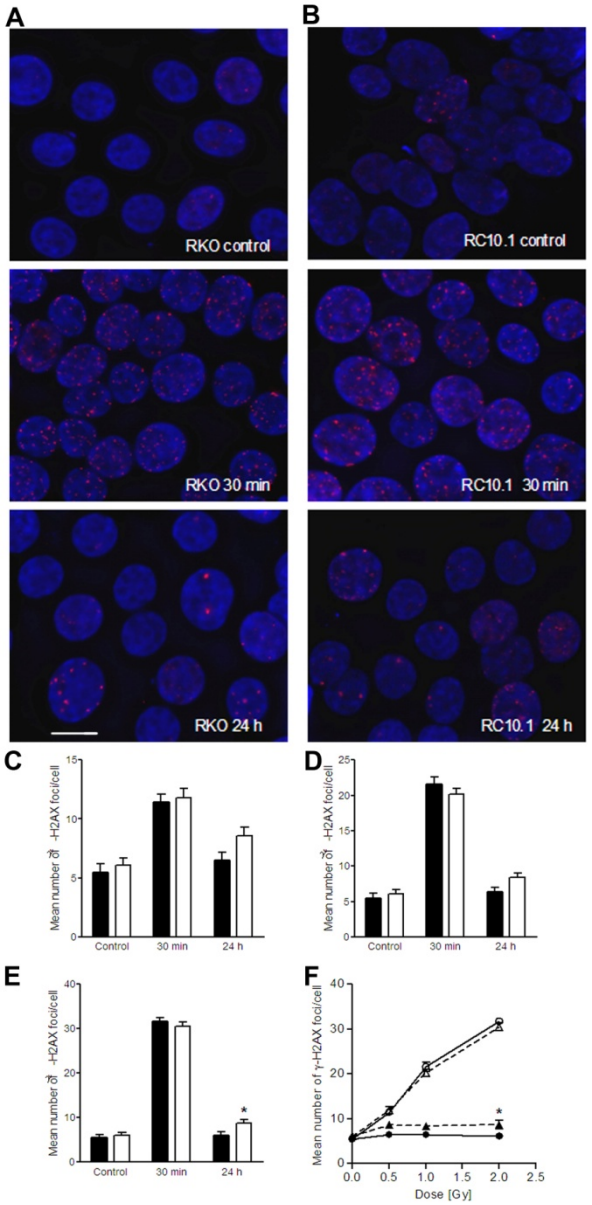

Fig. 3. Radiation-induced γ-H2AX foci in control and irradiated RKO and RC10.1 colon carcinoma cells. Cells were irradiated with 0, 0.5, 1 and 2 Gy and foci were scored 30 min or 24 h after irradiation. A and B – Fluorescent photomicrographs of RKO and 10.1 cells after 1 Gy; scale bar is 5 μm. C, D and E – Quantitative representation of the radiation-induced levels of γ-H2AX foci per cell in RKO cells (black bars) and RC10.1 (white bars) in control cells and in cells 30 min and 2 h after 0.5 Gy (C), 1 Gy (D) or 2 Gy irradiation (E). F – Dose-response curves for γ-H2AX foci 30 min (open symbols) and 24 h (closed symbols) after irradiation, for RKO (solid lines) and RC10.1 (dotted lines) cells. At least 100 cells from 2 different experiments were counted. The error bars represent SEM.

experimentally derived parameters for the linear and quadratic terms, respectively, and PLDR could also be expressed as the ratios of α-values.

The results demonstrate that p53 status influences PLDR. RKO cells with wild-type p53 protein clearly demonstrated PLDR while the RC10.1 cell line with a p53 null status showed reduced PLDR. These findings correspond to data presented earlier [19, 25]. The obtained PLDR-α values were 1.4 ± 0.2 and 1.0 ± 0.2 for RKO and RC10.1 cells, respectively, which are almost identical to the values observed in a previous study 1.7 ± 0.3 and 1.1 ± 0.2 [19]. RKO cells appeared to be more radiosensitive compared to RC10.1 cells when plated immediately after irradiation. This might be due to the induction of apoptosis [15, 16] in the p53 wild type cells. On the other hand, in these cells the p53-induced cell cycle arrest allows the cells to recover from the radiation damage and consequently repair their potentially lethal damage. This resulted in higher survival levels when the cells were replated 24 h after irradiation [21].

The presented data demonstrate that decay of γ-H2AX foci after exposure to ionizing radiation is associated with PLDR. Correlation between the decay of γ-H2AX foci and p53 status was shown earlier for prostate tumor cell lines [9]. Furthermore, 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 [10]. Here, it is demonstrated that the level of remaining foci at 24 h was higher in p53 null cells than in p53 wt cells. It is possible that after this time period, further decay of foci might also occur in the p53 null cells. The γ-H2AX foci have been suggested to be a valid measure for radiosensitivity, and the disappearance of foci is correlated to the repair of DNA damage following radiation treatment [9, 11]. Several studies demonstrate that the decay of γ-H2AX foci is associated with cell survival and repair of DSB [9, 39]. A correlation between residual levels of γ-H2AX foci 24 h after irradiation and clonogenic survival at a dose of 2 Gy was observed in six human cervical cancer cell lines [10]. Taneja *et al.* [40] also found a correlation between radiosensitivity and residual amounts of γ-H2AX. By contrast, no correlation between residual foci and radiosensitivity in some tumor cell lines was observed by Mahrhofer *et al*. and Yoshikawa *et al.* [41, 42]. A difference in study setup might explain these different findings, as they only studied survival of cells plated immediately after irradiation and correlated this with numbers of foci. However, p53 functionality was not examined in these studies. In our study, cell reproductive death was examined both directly and 24 h after irradiation in order to study PLDR, which was correlated with foci decay and p53 status.

Obviously, residual foci are still present in both cell lines 24 h after irradiation. In contrast with this are the results of Vandersickel *et al*. [8], who did not observe residual foci in MCF10A cells and MCF10A cells silenced for Ku70 and Ku80 24 h after treatment. It might be that the cells with residual foci had already died and therefore were not scored. Banath [10] stated that residual γ-H2AX foci 24 h after irradiation are indicative of lethal DNA damage. However, also the kinetics of dephosphorylation of γ-H2AX can be different in

the various cell lines. Furthermore, endogenous γ-H2AX can be an indication of chromosomal instability. P53 mutations that promote chromosomal instability could also cause high background levels of γ-H2AX foci [43]. Suzuki *et al*. [44] suggested that residual foci are an indication of misrepaired chromosomes. It was stated that the rate of γ-H2AX decay and relative residual damage after exposure to ionizing radiation may be useful as indicators of intrinsic radiosensitivity, with more rapid loss and less retention in the more radioresistant cell types [11].

However, a comparison of DSB repair and the kinetics of γ-H2AX foci should be performed with care. While the initial number of γ-H2AX foci correlate with DSB, the disappearance of γ-H2AX may differ significantly from DSB repair [45], as dephosphorylation of γ-H2AX is slower than DSB repair [46]. It has even been described that in the presence of calyculin A, dephosphorylation of γ-H2AX is blocked but DSB are still repaired [46].

The number of radiation-induced foci after exposure to 1 Gy is about 16 ± 2, which should correlate with the number of DSB. This is significantly lower than 40 DSB/Gy, which is usually mentioned. A lower number than 40 DSB/Gy is frequently observed [2, 9, 45, 47]. In SW1573 lung cancer cells, between 20 and 25 foci/Gy were observed [2] and in several prostate cancer cell lines, between 5 and 15 foci/ Gy were observed [9]. Markova *et al.* [45] observed 20 foci/Gy in VH-10 cells and Kuefner *et al.* [47] observed about 20 foci/Gy in lymphocytes,

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 sub-lethal damage from two independent particles. DNA DSBs are considered the most important lesions. These results on the decay of γ-H2AX foci as indicators of DNA DSBs and the correlation with PLDR are in agreement with this. In previous studies, we demonstrated a correlation between survival, chromosomal aberrations and PLDR, which was shown by a decrease in the value of α with higher survival and lower number of chromosomal aberrations [2, 48]. In this study, we further demonstrated that there is an association between PLDRα and a decrease of DNA DSBs.

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