Radiation-induced mitotic catastrophe in PARG-deficient cells

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Summary

Poly(ADP-ribosyl)ation is a post-translational modification of proteins involved in the regulation of chromatin structure, DNA metabolism, cell division and cell death. By contrast, PARG-deficient cells because it protected cells from spontaneous single-strand breaks deleterious to undamaged cells and was in fact rather beneficial, PARG depletion leading to PAR accumulation was not knocked down expression of PARG isoforms in HeLa cells. PARG in response to irradiation, we constitutively and stably subcellular localizations. To gain insight into the function of isoforms encoded by a single gene and displaying various subcellular localizations. To gain insight into the function of PARG in response to irradiation, we constitutively and stably knocked down expression of PARG isoforms in HeLa cells. PARG depletion leading to PAR accumulation was not deleterious to undamaged cells and was in fact rather beneficial, because it protected cells from spontaneous single-strand breaks and telomeric abnormalities. By contrast, PARG-deficient cells showed increased radiosensitivity, caused by defects in the repair of single- and double-strand breaks and in mitotic spindle checkpoint, leading to alteration of progression of mitosis. Irradiated PARG-deficient cells displayed centrosome amplification leading to mitotic supernumerary spindle poles, and accumulated aberrant mitotic figures, which induced either polyploidy or cell death by mitotic catastrophe. Our results suggest that PARG could be a novel potential therapeutic target for radiotherapy.

Key words: NAD+ metabolism, PARP, DNA repair, Mitosis, Genome stability

Introduction

Poly(ADP-ribosyl)ation is a NAD+-dependent post-translational modification of proteins mediated by poly(ADP-ribose) polymerases (PARPs). It is involved in various biological processes such as DNA repair, transcription, mitotic segregation, telomere homeostasis and cell death (Schreiber et al., 2006). PARP1, the founding member of the PARP family, can detect DNA strand breaks and can become activated and poly(ADP-ribosyl)ate itself or acceptor proteins involved in chromatin structure and DNA metabolism. PARP1 activation and PAR synthesis have several outcomes: (1) labelling of the damaged site by the PAR molecules; (2) local opening of the chromatin structure by the transient heteromodification of histones; (3) recruitment to the damage site of repair factors with strong affinity for PAR, such as the X-ray repair cross-complementing protein 1 (XRCC1); and (4) signaling of the severity of the DNA insult for the cell to adapt its response accordingly (reviewed by Schreiber et al., 2006).

PAR accumulation is transient. PAR is rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG), an enzyme with both exo- and endoglycosidase activities that generate large amounts of free ADP-ribose. In contrast to the 17 members of the human PARP family (Amé et al., 2004; Schreiber et al., 2006), human PARP is encoded by a single gene, but is present within the cell through different isoforms displaying various subcellular localizations: nuclear (PARG111), cytoplasmic (PARG102 and PARG99) and mitochondrial (PARG60) (Meyer et al., 2007). Study of the functional role of PARG through the generation of mutant mouse models has been hampered by the existence of these isoforms. A hypomorphic mutation targeting exons 2 and 3 (PARG H9004/H9004 mice) is viable, but the mice are sensitive to ionizing radiation and alkylating agents (Cortes et al., 2004). However, these mutant mice displayed a 3.3-fold increase of the mitochondrial PARG activity, which could partially compensate for the absence of the longer PARG isoforms (Cortes et al., 2004). Targeting exon 4 of the PARP gene abolished any isoform production and led to early embryonic lethality (Koh et al., 2004). Trophoblast PARG−/− cells were shown to be highly sensitive to alkylating agents, to accumulate PAR and to die by apoptosis, highlighting the crucial role of PARG in the response to DNA damage. Similarly, a loss-of-function mutation in the Drosophila melanogaster PARP gene led to increased lethality associated with PAR-accumulation-induced neurodegeneration at the larval stages (Hanai et al., 2004). Attempts to transiently silence PARG expression with siRNA led to controversial results: either protection (Blenn et al., 2006) or sensitivity (Fisher et al., 2007) towards H2O2-induced cell death, which might be explained by differences in cell types analyzed (HeLa versus A549 cells).

Many studies revealed the importance of PARP activity and PAR levels in the control of the life-and-death balance following DNA damage. PAR synthesis in response to DNA strand breaks allows a rapid cellular evaluation of the damage range, with the outcome depending on the cell type and the intensity of the DNA insult. In neuronal cells, DNA damage triggers cell death by a caspase-independent mechanism involving the mitochondrial apoptosis-inducing factor (AIF) (Yu et al., 2002). PAR synthesis, which might...
generate free polymers through the action of PARG, is required for AIF to translocate from the mitochondria to the nucleus where it induces large-scale DNA fragmentation (Andrabi et al., 2006; Moubarak et al., 2007; Yu et al., 2006). The subsequent PARP1 overactivation leads to massive PAR synthesis, NAD+/ATP depletion, and finally, cell death (Yu et al., 2002). PARP1−/− mice and mice treated with PARP inhibitors are thus protected from many pathophysiological situations where DNA is the target of massive attack by reactive oxygen species, such as acute and chronic inflammatory diseases, ischemia, as well as neurodegenerative disorders (Jagtap and Szabo, 2005). In contrast to PARG+/− mice, transgenic mice overexpressing PARG showed similar protection against cerebral ischemia as observed in PARP1−/− mice (Andrabi et al., 2006), confirming the beneficial effect of decreasing PAR levels. Taken together, these data underscore how the fine-tuning of PARP1 and PARG activity must precisely control the level of PAR and the balance between life and death decisions.

In this study, we have generated a cellular model deficient in all PARG isoforms by the stable and constitutive expression of a shRNA, to gain insight into the function of PARG in the cell response to irradiation. In normal conditions, PARG depletion conferred protection against spontaneous single-strand breaks (SSBs) and telomere aberrations, and showed a spontaneous activation of ATM (ataxia telangiectasia mutated) along with γH2AX phosphorylation. However, PARG-depleted cells were radiosensitive, accumulated radioduced PAR and showed slower rates of repair of SSBs and double-strand breaks (DSBs). In addition, they displayed radioduced centrosome amplification and fragmentation, and alteration of mitotic progression, with the accumulation of aberrant mitotic figures. These aberrant mitotic cells resulted in aneuploidy, polyplody or cell death by mitotic catastrophe.

**Results**

Accumulation of PAR in shRNA-mediated stable knockdown of PARG in HeLa cells

We generated a stable HeLa cell line PARGKD, which constitutively expresses a shRNA directed against PARG from an episomal plasmid (Biard, 2007). The BD650 control line expresses a non-functional shRNA (Biard et al., 2005). The sequence of the PARG shRNA maps to the catalytic domain of PARG, thus preventing the synthesis of all PARG isoforms, which was confirmed by RT-PCR (Fig. 1A). Downregulation of PARG111 was confirmed by western blot using an antibody raised against the N-terminal part of PARG (Fig. 1B) and by a zymogram, which showed that the PARG activity detected at 111 kDa was dramatically decreased in the PARGKD

![Image](https://example.com/Fig1.png)

**Fig. 1.** Efficient shRNA-mediated stable and constitutive knockdown of PARG in HeLa cells leads to accumulation and persistence of PAR. (A) RT-PCR analysis on total RNA extracted from BD650 control (lane 1) or PARGKD (lane 2) cells with primers specific for PARG or, as a negative control, Ogg1 transcripts. (B) Western blot analysis of BD650 control (lane 1) or PARGKD (lane 2) cell lysates probed successively with an anti-PARG N-terminal antibody and an anti-actin antibody to control loading. (C) Zymogram performed by running lysates from BD650 control (lane 1) or PARGKD (lane 2) cells on a 10% SDS-PAGE gel containing 32P-labelled automodified PARP1. The gel was renaturated before autoradiography. (D) Western blot analysis of PARGKD (lanes 1-3) or BD650 control (lanes 4-6) cell lysates successively probed with the 10H monoclonal anti-PAR antibody and with an anti-actin antibody to control loading. Lanes 1 and 4: untreated; lanes 2 and 5: 1 mM H2O2, 10 minutes; lanes 3 and 6: 1 mM H2O2 20 minutes, then incubated at 37°C in fresh medium for the indicated time. (E) Immunodetection of PAR (10H) in BD650 control or PARGKD cell either untreated or irradiated at 6 Gy and fixed at the indicated times. DNA is counterstained with DAPI. Scale bar: 20 μm.
cell extract (Fig. 1C). No PARG activity was detected at sizes corresponding to low molecular mass PARG isoforms, in either the BD650 or PARGKD cell extracts. Altogether, these results validate the efficiency of PARG silencing by this constitutive shRNA approach. The efficiency of PARGKD was unaffected even after long-term culture of the cells for more than 6 months (data not shown).

Downregulation of PARG expression led to genotoxic-stress-independent accumulation of PAR in PARGKD cells observed by western blot (Fig. 1D, compare lanes 1 and 4) and immunofluorescence microscopy (Fig. 1E). After treatment with 1 mM H2O2, more PAR accumulated in PARGKD cells than in BD650 control cells. Ninety minutes after H2O2 treatment, the amount of PAR returned to its basal level in BD650 control cells but remained high in the PARGKD cells (Fig. 1D, lanes 3 and 6), confirming the lack of PARG activity. A similar persistence of PAR following H2O2 treatment of PARGKD cells was observed by immunofluorescence microscopy using an anti-PAR antibody, and by measuring PARG activity in permeabilized cells incubated with 32P-labelled NAD+ (data not shown). Accumulation and persistence of PAR was also observed by immunofluorescence microscopy following X-irradiation of PARGKD cells at 6 Gy; whereas PAR returned to basal levels within 60 minutes after X-irradiation in control cells, PARGKD cells still displayed high levels of PAR 90 minutes after irradiation (Fig. 1E), which remained high for up to 3 hours (data not shown).

Altogether, these results indicate that PARG expression is efficiently knocked down in PARGKD cells, resulting in both genotoxic-stress-independent PAR synthesis and DNA-damage-induced accumulation and persistence of PAR.

PARGKD cells display repair defect of radio-induced SSBs and DSBs

The observation of PAR synthesis in untreated PARGKD cells led us to hypothesize that spontaneous DNA breaks might accumulate in the absence of PARG, which could reflect a repair defect. To test this hypothesis, we monitored the level of DNA strand breaks using the alkaline COMET assay, which detects both SSBs and DSBs, and the neutral COMET assay, which detects only DSBs. Alkaline COMET assay (Fig. 2A; supplementary material Fig. S1A) revealed a slight and reproducible, although statistically not significant, smaller number of breaks in undamaged PARGKD cells compared with those in control cells. This probably corresponds to SSBs, because neutral COMET assay showed no spontaneous DSBs in each cell line (Fig. 2B; supplementary material Fig. S1B), and suggests first that PARG-silenced cells could be protected from spontaneous SSBs, and second, that the PAR detected in the absence of genotoxic insult is probably not caused by the accumulation of spontaneous SSBs. In addition, a 6 Gy X-irradiation generated fewer SSBs and DSBs in the PARGKD cells compared with those in control cells. This probably corresponds to SSBs, because neutral COMET assay showed no spontaneous DSBs in each cell line (Fig. 2B; supplementary material Fig. S1B), and suggests first that PARG-silenced cells could be protected from spontaneous SSBs, and second, that the PAR detected in the absence of genotoxic insult is probably not caused by the accumulation of spontaneous SSBs. In addition, a 6 Gy X-irradiation generated fewer SSBs and DSBs in the PARGKD cells compared with the control cells – a reproducible, although not statistically significant, observation. This might be attributed to a quenching effect of the PAR molecules towards free radicals generated by ionization. Time-course evaluation of strand break resealing by alkaline COMET assay revealed an almost complete repair of breaks as early as 30 minutes after irradiation in the BD650 control cells, whereas PARGKD cells required more than 90 minutes to reach a similar level of repair, indicating the presence of a repair defect in these cells (Fig. 2A; supplementary material Fig. S1A). Neutral COMET assay revealed a defect in the repair of radioinduced DSBs in PARGKD cells compared with BD650 control cells (Fig. 2B; supplementary material Fig. S1B). Taken together, these results indicate that PARG is required for efficient repair of both SSBs and DSBs.

We then examined the recruitment of the SSB repair factor XRCC1, which normally accumulates within seconds at damaged sites as a result of its strong affinity for PAR (El-Khamisy et al., 2003; Mortusewicz et al., 2007; Mortusewicz and Leonhardt, 2007; Okano et al., 2003; Pleschke et al., 2000). DNA-damaged sites were introduced locally by UVA laser microirradiation in the presence
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of the photosensitizer Hoechst 33258, leading to both SSBs and DSBs. In the BD650 control cells, PAR synthesis and concomitant recruitment of XRCC1 at the damaged sites were observed as early as 30 seconds after irradiation (Fig. 2C). Thirty minutes after irradiation, PAR was no longer detected, whereas XRCC1 was still observed along the laser path, suggesting ongoing repair. In marked contrast, in PARG KD cells, XRCC1 was less efficiently recruited at the damaged site and was not retained 30 minutes after irradiation, despite massive PAR production (Fig. 2C). Recruitment of GFP-XRCC1 at laser-induced DNA-damage sites in PARG KD cells was similarly affected (data not shown). PAR was rapidly detected all over the cell nucleus, reflecting the detachment of highly automodified PARP1 from the DNA breaks and its delocalization, together with XRCC1, from the damaged site. Taken together, these results suggest that the altered mobilization of XRCC1 at the laser-induced DNA damage sites could explain the repair delay observed in the absence of PARG.

Increased radiosensitivity of PARG KD cells

The sensitivity of PARG KD cells to X-irradiation was assessed by measuring the colony-forming ability of BD650 control and PARG KD (squares) cell lines after treatment with various doses of X-rays. Experiment was performed twice in triplicate. Results are means ± s.e.m. of BD650 control cells 1 hour after irradiation but not 5 or 24 hours later (Fig. 3A). In the PARG KD cells, γH2AX immunostaining persisted for 5 hours and slowly returned to its basal (but high) level 24 hours after irradiation (Fig. 3). The contribution of ATM to the phosphorylation of H2AX was evaluated by immunodetection of Ser1981-phosphorylated ATM in cell extracts, which reflects ATM activation (Bakkenist and Kastan, 2003). Results showed that ATM was already activated in a high proportion of PARG KD cells, even in the absence of irradiation, and that 6 Gy irradiation increased ATM activation more drastically in PARG KD cells than in BD650 control cells (Fig. 3B). Altogether, these results indicate that PARG depletion leads to constitutive and X-ray-induced ATM activation and H2AX phosphorylation, but also triggers a delay in the repair of X-ray-induced DSBs.

PARG KD cells are protected from spontaneous and radiation-induced telomeric aberrations

We next evaluated the consequences of PARG depletion on genome stability by monitoring telomeric aberrations using telomeric FISH analyses in cells either left untreated or 24 hours after a 6 Gy γ-irradiation (Fig. 5). PARG-depleted cells displayed a large drop in cell survival compared with BD650 control cells at low doses of irradiation (4% survival of PARG KD cells compared with 30% survival of BD650 control cells following 3 Gy irradiation), indicating that PARG depletion and the associated repair defect leads to increased radiosensitivity.

Fig. 3. Accumulation and persistence of γH2AX foci in PARG KD irradiated cells. (A) Immunodetection of γH2AX foci in BD650 control (upper panels) and PARG KD (lower panels) cells at the indicated time following 1 Gy X-irradiation. DNA is counterstained with DAPI. Scale bar: 10 μm. (B) Phosphorylation of ATM at Ser1981 after DNA damages in PARG KD cells. Western blot showing phosphorylation of ATM in BD650 control (C) and PARG KD (P) cells 1, 5 and 24 hours after a 6 Gy irradiation. Actin levels were monitored as a loading control.

Fig. 4. Survival curves showing the increased sensitivity of PARG KD cells to ionizing radiation. Cell survival analysis of BD650 control (dots) and PARG KD (squares) cell lines after treatment with various doses of X-rays. Experiment was performed twice in triplicate. Results are means ± s.e.m.
resulting respectively from fusions, deletions or recombinations in G1, were found at similar frequencies in PARG KD and BD650 control cells (Fig. 5C). Interestingly, a 6 Gy irradiation increased the frequency of all types of aberrations in BD650 control cells, whereas PARG KD cells still displayed significant protection against telomere losses (0.9±0.2 and 3.2±0.7 aberrations per metaphase for PARG KD and BD650 control cells, respectively) and sister chromatid fusions (1.2±0.2 and 2.0±0.3 aberrations per metaphase, respectively), leaving other types of aberrations comparable with the irradiated BD650 control cells (Fig. 5). These compelling results indicate that PARG depletion protects telomeres from spontaneous and radiation-induced aberrations occurring in S-G2 phases, but has no apparent effect on telomeres during G1 phase.

Irradiated PARG KD cells display mitotic abnormalities

To evaluate the consequence of PAR and DNA-damage persistence in PARG KD cells on cell cycle progression, FACS analyses were performed on PARG KD and BD650 control cells, 24 hours and 48 hours after 3 or 6 Gy X-irradiation. The cell cycle distribution of both cell lines was comparable in the absence of treatment or after 3 Gy of X-rays (Fig. 6A; and data not shown). Twenty-four hours after a 6 Gy X-irradiation, PARG KD cells accumulated at the G2-M phase of the cell cycle. To determine whether cells accumulated at G2 phase or were blocked in mitosis, we scored the proportion of mitotic cells identified by immunodetection of phosphorylated H3 Serine 10 (Ser10-H3-P) and DAPI staining. Results showed accumulation of mitotic cells in 6 Gy-irradiated PARG KD cells compared to BD650 control cells (13.4% vs. 5.9%) (Fig. 6B). We then scored by immunofluorescence microscopy the number of cells in the different phases of mitosis, by immunodetecting P-Ser10-H3 and staining microtubules with anti-α tubulin, together with DAPI staining. Results revealed that 6 Gy irradiation led to a reduction of the proportion of mitotic cells identified by immunodetection phosphorylated H3 Serine 10 (Ser10-H3-P) and DAPI staining. Results showed accumulation of mitotic cells in 6 Gy-irradiated PARG KD cells compared to BD650 control cells (13.4% vs. 5.9%) (Fig. 6B). We then scored by immunofluorescence microscopy the number of cells in the different phases of mitosis, by immunodetecting P-Ser10-H3 and staining microtubules with anti-α tubulin, together with DAPI staining. Results revealed that 6 Gy irradiation led to a reduction of the proportion of mitotic cells identified by immunodetection phosphorylated H3 Serine 10 (Ser10-H3-P) and DAPI staining. 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which some microtubules emanate to bind to kinetochores (Fig. 7Ab). We therefore monitored the number of multipolar versus bipolar metaphases after 3 Gy (data not shown) and 6 Gy X-irradiation (Fig. 7B). The proportion of multipolar metaphases in PARGKD cells increased dramatically with the irradiation dose 24 hours after irradiation, and this proportion was even higher at 48 hours after irradiation (Fig. 7B). To confirm our observations with live-cell videomicroscopy, we generated HeLa cell lines that constitutively and stably expressed GFP-tagged histone H2B (H2B-GFP) together with either control shRNA (H2B-GFP/BD650) or PARG shRNA (H2B-GFP/PARGKD). Progression into mitosis was followed 24 hours after 6 Gy irradiation for a period of 10 hours. Results displayed in supplementary material Fig. S2, Table S2 and Movies 1-6 showed that the duration of mitosis for unirradiated H2B-GFP/PARGKD cells was increased compared with H2B-GFP/BD650 cells (115 minutes versus 80 minutes, respectively) (supplementary material Fig. S2A), owing to cycles of compaction and decompaction of the metaphase plate. Twenty-four hours after a 6 Gy irradiation, the duration of mitosis for H2B-GFP/PARGKD dramatically increased to more than 400 minutes (compared with 96 minutes for H2B-GFP/BD650 cells) (supplementary material Fig. S2A), with cells arrested at metaphase. All mitoses were abnormal for both cell lines after irradiation (supplementary material Fig. S2B), with a high proportion (more than 65%) of mitoses displaying anaphase bridges (supplementary material Fig. S2D,E and Movie 1). However, in contrast to H2B-GFP/BD650, irradiation of H2B-
GFP/PARG\textsuperscript{KD} cells led to a high proportion of mitoses displaying chromosome misalignment (44%) (supplementary material Fig. S2D,E and Movie 2) or multipolar mitoses (34%) (supplementary material Fig. S2D,E and Movies 3 and 4). In addition, 24% of these aberrant H2B-GFP/PARG\textsuperscript{KD} mitoses ended in cell death (supplementary material Fig. S2C,E and Movie 5; and see below). Taken together, these results indicate that PARG impairment leads to alterations of mitosis in irradiated cells leading to improper or incomplete cell division that favours aneuploidy and/or polyploidy or mitotic cell death. We hypothesized that these alterations could result from both a defective kinetochore function and from the presence of supernumerary spindle poles.

Irradiated PARG\textsuperscript{KD} cells display centrosomal abnormalities
The presence of supernumerary spindle poles in irradiated PARG\textsuperscript{KD} cells prompted us to score the number of centrosomes, which were identified by Cdk1/p34cdc2 (Fig. 8A,B,C) or pericentrin (Fig. 8C) immunostaining. Unirradiated PARG\textsuperscript{KD} cells revealed an increased number of cells with more than two centrosomes (10.7% compared with 2.8% for BD650 control). This proportion raised 24 hours after irradiation with 3 or 6 Gy, and even higher 48 hours after irradiation (52.0% of PARG\textsuperscript{KD} cells compared with 25.3% of BD650 control cells irradiated at 6 Gy). In addition, we observed an increased frequency of cells showing fragmented centrosomal material (Fig. 8Aa,C) observed both with Cdk1 and pericentrin immunostaining, reaching 48.3% in the PARG\textsuperscript{KD} cells but only 17.9% in the BD650 control cells, 24 hours after a 6 Gy irradiation. However, 48 hours after irradiation, the proportion of cells with fragmented centrosomes was similar to the level observed for both unirradiated cell lines. Taken together, these results indicate that depletion of PARG exacerbates the centrosome amplification and fragmentation that has been already described in irradiated cells, in cells deficient in proteins involved in DSB repair or in cells overexpressing PARP3 and treated with an alkylating agent (Augustin et al., 2003; Date et al., 2006; Griffin et al., 2000).

Irradiated PARG\textsuperscript{KD} cells display kinetochore dysfunction
The observation of defective kinetochore function in the absence of PARG prompted us to monitor the functionality of the kinetochore checkpoint. We immunodetected MAD2 checkpoint protein that normally transiently localizes to the kinetochore of misaligned chromosomes and delocalizes following proper alignment (Chen et al., 1996). As expected, in unirradiated and 6 Gy irradiated BD650 control cells, MAD2 transiently colocalized to the unattached kinetochores, but was no longer observed in metaphase when chromosomes were properly aligned (Fig. 9A). By contrast, MAD2 staining could still be detected in metaphase and anaphase kinetochores in unirradiated PARG\textsuperscript{KD} cells, with a more-pronounced staining 24 hours after a 6 Gy irradiation. Quantification of MAD2-positive anaphases revealed that 65% of anaphases from unirradiated PARG\textsuperscript{KD} cells and 85% from irradiated cell displayed residual MAD2 staining at kinetochores. Taken together, these results suggest that depletion of PARG activates the kinetochore checkpoint (SAC) dysfunction. To test this hypothesis, we incubated the two cell lines with nocodazole, which prevents microtubule formation, triggers SAC and blocks mitosis progression in prometaphase. Fig. 9B shows that after 18 hours of treatment with 100 nM nocodazole, the PARG\textsuperscript{KD} cells have overcome the checkpoint, exiting mitosis without correctly partitioning their genome and thus accumulating...
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Fig. 9. Persistence of MAD2 staining in irradiated PARGKD cells and alteration of the mitotic checkpoint after nocodazole treatment. (A) Prometaphase, metaphase and anaphase cells from PARGKD and BD650 control cell lines were stained with DAPI (blue), anti-MAD2 (red) and CREST anti-centromere (green) antibodies 24 hours after mock irradiation or 6 Gy irradiation. Colocalization of MAD2 and CREST signals is shown in the merge column. The percentage of cells with centromeric distribution of MAD2 in anaphases is indicated on the right. Scale bar: 10 μm. (B) PARGKD and BD650 control cell lines were treated with 100 nM nocodazole for 18 hours or mock-treated then stained with DAPI (blue). Scale bar: 30 μm. (C) The accumulation of multinucleated PARGKD cells treated 18 hours with 100 nM nocodazole suggests a mitotic checkpoint alteration. The number of cells counted is reported on the bar chart.

Discussion

In this study, we have shown that PARG deficiency protects HeLa cells from spontaneous SSBs and telomere aberrations, whereas irradiation of these PARG-deficient cells leads to the accumulation

as multinucleated cells (Fig. 9C). This result confirmed the SAC deficiency observed in PARGKD cells.

Irradiation of PARGKD cells leads to mitotic catastrophe

Next, we aimed to explain the radiosensitivity of the PARGKD cells and to determine by which mechanism the death occurred. Our first hypothesis was that PARGKD cells would die by AIF-dependent cell death, owing to the massive production of PAR following a 6 Gy irradiation. However, no AIF release from mitochondria could be observed under these conditions (data not shown) indicating that PARGKD cells died by an AIF-independent cell death mechanism. Scoring the proportion of necrotic and apoptotic cells with Annexin V and propidium iodide staining by FACS showed only slight increase of apoptotic cells in irradiated PARGKD cells compared with control cells (data not shown). This was in agreement with the slight increase of PARP1 cleavage observed in PARG KD cells compared with control cells, observed at 48 hours and 72 hours after 6 Gy irradiation (data not shown). This suggested that only a limited number of cells underwent cell death, at each cell division. To examine the possibility that these dying cells resulted from mitotic catastrophe, we looked for the mitotic release of cytochrome c, a hallmark of apoptosis, by immunofluorescence microscopy 48 hours after a 6 Gy irradiation (Fig. 10A). Results showed that irradiated PARGKD cells displayed two types of metaphase cells, one showing multipolar metaphase, with no release of cytochrome c and normal staining of Ser10-H3-P (Fig. 10Af-i). We assumed that these cells generate the polyploid cells frequently observed in irradiated PARGKD cells (Fig. 6A). The second type of metaphases observed showed the release of cytochrome c that is found associated with chromatin, probably because of the high affinity of cytochrome c for DNA (Kleinschmidt and Zahn, 1959). These metaphase cells showed non-aligned chromosomes stained by DAPI and Ser10-H3-P (Fig. 10Ak-n), and probably died by mitotic catastrophe, leading to the characteristic formation of large nuclear bodies stained with DAPI (Fig. 10Ao) (Castedo et al., 2004). Scoring the proportion of cells dying by either interphasic apoptosis or mitotic catastrophe after irradiation revealed that irradiated PARGKD cells essentially died by mitotic catastrophe (Fig. 10B,C). This hypothesis was confirmed by live-cell videomicroscopy using the H2B-GFP/BD650 and H2B-GFP/PARGKD cell lines. Following mitotic progression for 10 hours, 24 hours after a 6 Gy irradiation, we observed that 24% of mitotic H2B-GFP/PARGKD cells died by mitotic catastrophe (supplementary material Fig. S2C,E and Movie 5). Taken together, these results suggest that the increased radiosensitivity of PARGKD cells is likely to be a consequence of mitotic catastrophe.
of PAR, a delay in the repair of DNA strand breaks, centrosome amplification and mitotic defects, generating polyploid cells or resulting in cell death by mitotic catastrophe.

PARG depletion leads to accumulation and persistence of PAR. High levels of PAR were detected within PARG KD cells in the absence of exogenously introduced DNA damage; however, alkaline COMET assays revealed rather fewer SSBs in these cells than in control cells, suggesting that PAR accumulation is not solely due to spontaneous SSBs. Therefore, these PAR molecules could result from PARP1 activation triggered by any of the recently described DNA-damage-independent mechanisms of PARP1 activation observed during gene expression regulation or cell signaling, such as binding to particular DNA structures or chromatin states or post-translational modifications (for reviews, see Cohen-Armon, 2007; Kraus, 2008). Alternatively, these PAR molecules could reflect the accumulation of the reaction product of any of the other active PARP family members. A comparable genotoxic-stress-independent accumulation of PAR has been reported in PARG−/− mice (Koh et al., 2004) and in an Arabidopsis mutant that affects the catalytic activity of the PARG homologue, tej (Panda et al., 2002). Although delayed, the disappearance of PAR observed after irradiation could result either from residual PARG molecules or from the activity of the recently identified 39 kDa protein ARH3, which has PAR-degrading activity (Oka et al., 2006).

Protective effect of PAR spontaneously produced in PARG-depleted cells

Previous studies have proposed that the absence of PAR is detrimental to the cells, because Par−/− trophoblast cells showed decreased proliferation and increased cell death (Koh et al., 2004). In our shRNA approach, which efficiently silenced PAR expression in HeLa cells, the basal and constitutive PAR synthesis did not dramatically hamper cell behaviour. We rather observed a beneficial effect, at least for genome integrity and telomere stability. We cannot rule out the idea that a residual amount of PAR might be sufficient for this protection. Alternatively, this protection might occur only in tumour cells (HeLa) and not in...
untransformed cells (trophoblast cells or ES cells), but this remains to be determined.

The spontaneous γH2AX staining detected in unirradiated PARGKD cells correlates with the observed constitutive ATM activation. It is, however, unlikely that it reflects the presence of spontaneous DSBs, because this would certainly have repercussions on cell viability, which is not the case. ATM activation has been proposed to result from changes in chromatin structure, independently of DNA damage (Bakkenist and Kastan, 2003; Soutoglou and Misteli, 2008), a phenomenon that probably occurs in PARGKD cells, supported by the established role of PAR in the modulation of chromatin superstructure. In addition, a functional interplay between PARP1 activity and ATM activity has been reported (Aguilar-Quesada et al., 2007; Haince et al., 2007), supporting the correlation between PAR accumulation and ATM activation observed in PARG-depleted cells.

Most of the spontaneous and radioinduced telomere aberrations found at lower frequency in PARGKD cells result from the lack (sister chromatid fusions), improper (telomere doublers) or unstable (telomere losses) formation of protected telomere structures during or after replication (Pennarun et al., 2008). Interestingly, these types of aberrations are observed at higher frequency in ATM-deficient cells (Pennarun et al., 2008). The constitutive activation of ATM observed in PARGKD cells could thus contribute to the observed protection of telomeres from aberrations. It is also conceivable that one of the PARPs reported to localize at telomeres, such as PARP1, PARP2 or tankyrase 1 (TNKS1), could be directly implicated in telomere protection. Indeed, we have previously proposed that poly(ADP-ribosylation) of the telomeric factor TRF2 by PARP1 or PARP2, leading to its detachment from the telomeric DNA, could favour the maintenance of telomere integrity (Dantzer et al., 2004; Gomez et al., 2006).

Depletion of PARG leads to SSB and DSB repair delay

The delay in the repair of SSBs and DSBs generated by X-irradiation, observed in the absence of PARG, is consistent with previous observations of a reduced rate of repair of oxidative lesions in PARG-depleted A549 cells (Fisher et al., 2007). XRCC1 was shown to relocalize in more distinct nuclear foci introduced by H2O2 treatment, and these foci persisted longer (Fisher et al., 2007). By contrast, using laser microirradiation to locally introduce DNA lesions, we observed that XRCC1 was less efficiently mobilized at these damaged sites, and was then rapidly delocalized throughout the nucleus. Since XRCC1 interacts noncovalently, but with high affinity, to PAR through its BRCT1 domain (Pleschke et al., 2000), it is likely that the genotoxic-stress-independent PAR produced in the PARGKD cells binds to XRCC1, thus preventing its further mobilisation to the laser-induced DNA breaks. Since PARG is not present to tightly regulate the poly(ADP-ribosylation) status of PARP1 at the site of massive DNA damage (Keil et al., 2006), as introduced by the laser microirradiation, XRCC1 might get rapidly delocalized together with the highly automated PARP1 molecules. This premature delocalization of XRCC1 from the damage site could account for the repair defect observed in irradiated PARGKD cells.

PARP depletion increases radioinduced mitotic aberrations

Mitotic aberration is a common response to X-irradiation, but PARGKD cells displayed a higher proportion of aberrant mitoses than irradiated control cells. In addition, a high proportion of cells displayed supernumerary spindles, an observation that correlated with the increased proportion of aneuploid and polyploid cells in irradiated PARG-depleted cells.

Tankyrase 1, vPARP and PARP2 were found to be associated with the mitotic spindle (Chang et al., 2005; Kickhoefer et al., 1999; Schreiber et al., 2004) and PAR was detected in the spindle in normal conditions. Tankyrase 1 activity was shown to be required for the formation and maintenance of bipolarity of the mitotic spindle but also for the separation of telomeres during anaphase (Chang et al., 2005; Dynek and Smith, 2004). In Xenopus egg extracts, PAR hydrolysis by the addition of PARG rapidly led to misalignment of chromosomes and disruption of bipolar spindle structure (Chang et al., 2004). These reports clearly define PAR as a molecule that directly controls spindle function. In PARGKD cells, we could detect PAR at the spindle at similar levels to those in control cells (data not shown). In addition, formation of the spindle was apparently not altered in the absence of PARG, even after irradiation.

PARGKD cells showed a defective spindle assembly checkpoint: whereas these cells normally activate the kinetochore checkpoint, they escape this checkpoint prematurely, as shown by the persistence of MAD2 at kinetochores in metaphases and anaphases. It is, however, unlikely that the majority of the kinetochores remained unattached at anaphase.

At least two PARPs, PARP1 and PARP2, could be important for kinetochore function and mitotic progression of damaged cells. PARP1 and PARP2 accumulate transiently at centromeres (during S-G2 for PARP1 and pro-metaphase for PARP2) and interact with the constitutive centromeric proteins CENPA, CENPB, the kinetochore protein Bub3 and the mitotic kinase Aurora B (AURKB) (Monaco et al., 2005; Saxena et al., 2002a; Saxena et al., 2002b). These centromeric proteins were found to be poly(ADP-ribosyl)ated after X-irradiation or oxidative damage, and poly(ADP-ribosyl)ated Aurora B is no longer able to phosphorylate H3 Ser10 (Monaco et al., 2005). In addition, treatment of PARP2−/− cells with a monofunctional alkylating agent leads to G2-M arrest, polyploidy and increased cell death (Menissier de Murcia et al., 2003). These results suggest that PARP1 and PARP2 are required for centromere and/or kinetochore function when DNA integrity is challenged. However, in PARGKD cells, Aurora B kinase activity does not seem to be impaired, because phosphorylation of H3 Ser 10 was observed to be efficient.

PARG-depleted cells are radiosensitive and die by mitotic catastrophe

The observed radiosensitivity of PARGKD cells is in agreement with previous observations in Caenorhabditis elegans in which expression of the two PARG homologues Pme-3 and Pme-4 has been knocked down (St-Laurent et al., 2007), and with the observed radiosensitivity of PARGΔ2/Δ3-deficient mice (Cortes et al., 2004). Excessive amount of PAR is known to be cytotoxic, because BioPORTER-mediated delivery of PAR induced AIF-dependent cell death (Andrabi et al., 2006). However, no AIF activation and translocation could be detected in irradiated control or PARGKD cells (data not shown). This suggests that massive PAR production is not sufficient per se to activate the AIF-dependent cell-death pathway. However, we cannot completely exclude the notion that the HeLa cells used in this study are impaired in the AIF-dependent cell death pathway, because treatment with 100 μM MNNG was able to trigger AIF-dependent cell death in only a small but similar proportion of cells (1%; data not shown) for both BD650 control and PARGKD cell lines.
Our results show that irradiated PARGKD cells die mostly during mitosis. Mitotic catastrophe is a cell death pathway arising in many tumour cells when damaged DNA enters mitosis, or when bipolar spindle assembly is prevented by centrosome amplification and the establishment of supernumerary spindle poles (Dodson et al., 2007). Both situations could synergize to increase the proportion of mitotic catastrophe observed in irradiated PARGKD cells. The centrosome was proposed to have a direct role in the DNA-damage response as a checkpoint regulator or effector, with centrosomal amplification being the endpoint leading to mitotic catastrophe to eliminate the damaged cell (Löffler et al., 2006). The latter hypothesis is probable, explaining the outcome of irradiated PARG-depleted cells as shown in this study.

Whereas the significance of centrosome fragmentation is still under debate (Dodson et al., 2004; Hut et al., 2003), centrosome amplification could result from the uncoupling of centrosome duplication from DNA duplication, a process observed in PARP−/− mouse fibroblasts (Kanai et al., 2003). Alternatively, defective cytokinesis can also lead to unequal partition of spindle poles. PAR and several PARPs were detected at centrosomes, such as PARP1, PARP3 and tankyrase 1 (Augustin et al., 2003; Kanai et al., 2003; Smith and de Lange, 1999). In addition, treatment of cells with PARP inhibitors leads to centrosomal amplification, indicating that PAR is involved in the regulation of centrosome duplication (Kanai et al., 2003). Our results suggest that PAR levels must be tightly regulated, because uncontrolled PAR synthesis can also deregulate centrosome duplication. However, whether PAR acts at the centrosome itself, or centrosome amplification is an endpoint consequence of deregulated nuclear PAR synthesis remains to be determined. ATM and ATR activities have been reported to be involved in centrosome amplification following irradiation (Bourke et al., 2007). Thus, the increased ATM activity in irradiated PARGKD cells is consistent with the observed centrosome amplification.

Taken together, our results suggest that the absence of PARG is beneficial for undamaged cells, but detrimental to irradiated cells, and this radiosensitivity is the consequence of repair defects, centrosome amplification and mitotic spindle checkpoint defects, leading to either polyplody or cell death by mitotic catastrophe. Thus, a fine tuning of PAR synthesis and degradation is essential to regulate the fate of the damaged cell, because prevention of either PAR synthesis in PARP- or tankyrase-1-deficient cells or of PAR degradation in PARG-depleted cells can both lead to cell death, but probably via different mechanisms. Whether the increased radiosensitivity observed in the absence of PARG is cell-type dependent remains to be determined, but our results suggest that PARG could be a novel potential therapeutic target for radiotherapy.

Materials and Methods

Cell lines

siRNA design and cloning into pEBVisRNA vectors and establishment of stable knockdown and control HE-La clones were carried out as previously described (Biard, 2007; Biard et al., 2005). The RNAi sequence for PARG (NM_003631) stretched nucleotides 2325-2343. HE-La cells expressing constitutively H2B-GFP were described elsewhere (Kanda et al., 1998). Establishment of stable PARG knockdown and control H2B-GFP expressing HE-La clones was carried out as previously described (Biard, 2007; Biard et al., 2005), leading to H2B-GFP/PARGKD and H2B-GFP/BDE650 cell lines, respectively. All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco/BRL, Invitrogen), an Alexa Fluor 488 goat anti-human IgG (1:600, Molecular Probes, Eugene, OR), or anti-pericentrin (1:1000, Babco), and mouse monoclonals anti-poly(ADP-ribose) c-erbB (IgG3, 1:1000, Alexis, Lausen), anti-Ser10-H3- (P:1:2000, Upstate) or anti-pericentrin (1:1000, Babco), and mouse monoclonals anti-poly(ADP-ribose) 10H (IgG3c, 1:1000, anti-β-actin (IgG1, 1:2000, Upstate), anti-actin (IgG1, 1:2000, Upstate), anti-α-tubulin (DM 1A) (IgG1k, 1:600, Sigma, anti-MAD2 (1D7D10) (IgG1, 1:500, Santa Cruz Biotechnology), anti-MAD1 (1D6) (IgG1, 1:1000, Santa Cruz Biotechnology), an anti-γ-tubulin (IgG2, 1:800, Pharningen) or a human polyclonal CREST antibody (hAb, 1:800, kindly donated by K. H. Andy Choo, Royal Children’s Hospital, Parkville, Australia). After washing, cells were incubated for 2 hours at room temperature with the appropriate secondary antibodies: an Alexa Fluor 488 or 568 goat anti-mouse IgG or IgG1 or IgG2 or IgG3 (1:2000, Molecular Probes, Invitrogen), an Alexa Fluor 488 goat anti-human IgG (1:600, Molecular Probes, Invitrogen), an Alexa Fluor 488 or 568 goat anti-rabbit IgG. After three washes with PBS, 0.1% Tween (v:v), DNA was counterstained with DAPI. Immunofluorescence microscopy was performed using a Leica DMRA2 equipped with an Orca-ER CCD camera (Hamamatsu) and the capture software OpenLab 4.1 (Improvision). 3D deconvolution analysis and imaging of image stacks was performed using Velocity 4.0 (Improvision) when indicated in the figure legend. Merging images was done using Photoshop CS3 (Adobe).

Identification and scoring of mitotic figures

Cells were stained with anti-Ser10-H3-P, anti-α-tubulin antibodies and DAPI and observed by microscopy. Classification of prometaphase and abnormal metaphase was difficult to make due to the defect in chromosome congression phenotype observed in PARGKD cells. These two mitotic stages were therefore combined in the various scoring and noted as prometaphase/metaphase.

Single cell gel electrophoresis (COMET) assay

Cells were trypsinized and resuspended in low melting point agarose at 0.5% (103 cells/ml) and dropped onto 1% agarose in PBS coated slides. The alkaline COMET assay was used to detect both SSBs and DSBs. Slides were irradiated as described in figure legend, and immediately processed according to Trucco et al. (Trucco et al., 1998). To detect DSBs, a neutral COMET assay was performed: the slides, following irradiation and DNA damage recovery, were treated in a non-denaturating lysis solution (25 mM EDTA (pH 9.5), 34 mM sodium lauryl sarcosinate, 87 mM SDS) for 2 hours at RT, then washed 5 minutes in water. Electrophoresis was performed in 1× TBE (pH 8.4) at 16 mA, 2.5 V/cm for 4 minutes. After dehydration in 100% ethanol at −20°C for 10 minutes, the slides were dried. The migrated DNA was stained with ethidium bromide and visualized using a Leica DMRA2 fluorescence microscope with the 20× lens. For each time point, several fields as those represented in the supplementary material Fig. S1 were captured by the Hamamatsu Orca-ER CCD camera and used for qualitative assessment of DNA damage using the visCOMET software (Impul).
were analyzed on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software. Ten thousand cells gated as single cells were analyzed.

Colony-forming assay

Cells cultivated on 150-mm culture dishes were irradiated at various doses as indicated above, trypsinized and counted. After appropriate dilution, 3 x 10^4 cells were seeded on 6-cm culture dishes in triplicate and left to grow for 13 days. The number of colonies for each dish was counted using ImageJ (NIH, Bethesda) after crystal violet staining.

Fluorescent in situ hybridization (FISH)

Metaphase spreads and analysis of telomere aberrations were performed as previously described (Pennart et al., 2008). Numbers of chromosomes and metaphases analyzed in FISH experiments is shown in supplementary material Table S1.

Live videomicroscopy

Cells were grown on glass coverslips mounted in a Lucind Chamber (LIS). Live microscopy was carried out using an inverted microscope (Olympus IX81) placed in an incubator chamber (LIS) maintained at 37°C, and coupled with a CoolSNAP HQ camera (Princeton Instruments) controlled by Metamorph software (Universal Imaging). Fluorescent images were taken on 10-15 fields using a 20 x objective every 2 minutes for 10 hours.

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