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PARP-1 inhibition induces a late increase in the level of reactive oxygen species in cells after ionizing radiation

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ABSTRACT

Poly(ADP-ribose) polymerase 1 (PARP1), an enzyme activated by DNA strand breaks, synthesizes polymers of poly(ADP-ribose) (PAR) that modify chromatin and other proteins and play a role in DNA repair. Inhibition of PARP1 activity is considered a potentially important strategy in clinical practice, especially to sensitize tumor cells to chemo- and radio-therapy. Here we examined the influence of inhibition of PARP1 on formation of reactive oxygen species (ROS) and on DNA repair in cells exposed to ionizing radiation (IR). K562 (human myelogenous leukaemia) cells were grown and exposed to 4 or 12 Gy of ionizing radiation in presence or absence of the PARP inhibitor NU1025 (100 μM). Intracellular ROS were assayed using the probe 2,7-dichlorofluorescein with detection by flow cytometry and the rejoining of DNA strand breaks were followed by alkaline single cell gel electrophoresis (comet) assays. In untreated cells a significant increase in PAR formation occurred during the first 5 min after IR, followed by a gradual decrease up to 30 min. Addition of a PARP inhibitor arrested the production of PAR almost completely and decreased the rate of rejoining of DNA strand breaks significantly; however, 3 h after irradiation we observed no difference in the amount of DNA strand breaks between PARP inhibitor-treated and untreated cells. Twelve to 48 h after irradiation, an increase of ROS concentration was observed in irradiated cells and ROS levels in PARP inhibitor-treated cells were significantly higher than in cells without inhibitor. Irradiated cells grown in the presence or absence of PARP inhibitor did not differ in the frequencies of apoptotic and necrotic cells or in the activity of caspases at 24, 48 and 72 h after irradiation. Poly(ADP-ribosylation) and inhibition of PARP1 appeared to modulate DNA strand break rejoining and influence the concentration of ROS in irradiated cells.

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1. Introduction

Poly(ADP-ribose) polymerases (PARPs) contain zinc finger motifs which, with high affinity, bind to DNA strand interruptions and activate the enzyme's catalytic module and synthesis of negatively-charged, branched polymers of poly(ADP-ribose) (PAR) from NAD^+ [1]. The most abundant member of this family, PARP1, has multiple functions in DNA repair, replication and transcription, and cell survival and death (reviewed in [2–4]). Modulation of its activity by stimulation or inhibition has found application in therapy or prevention of several disease states including cardiac infarct [5], diabetes [6], septic shock [7], inflammation [8], neurodegenerative disorders [9], and acute necrotizing pancreatitis [10]. So far, several inhibitors of PARP have been developed, mostly to sensitize tumor cells to chemo- and radio-therapy through inhibition of DNA repair [11]. The exposure of cells to ionizing radiation leads to hydroxy radical-mediated DNA damage including base modifications and single and double strand breaks, which activate PARP1 and other factors including DNA-dependent protein kinase (DNA-PKcs), an important component of DNA double strand break repair systems.

Levels of single strand breaks are increased by inhibitors of PARP1, while inhibitors of DNA-PKcs only affect the repair of double strand breaks and promote killing of tumor cells by ionizing radiation (reviewed in [12]). It has been reported that inhibition of PARP1 also affects repair of double strand breaks in cells exposed to ionizing radiation, which may further contribute to cytotoxicity [13]. On the other hand, inhibition of DNA damage detection or repair using potent PARP inhibitors prevents cells from overproduction of poly(ADP-ribose) following DNA damage, and hence from depletion of their NAD^+ pool with consequent energy deprivation and necrosis (reviewed in [14]).

As described above, PARP is activated through the recognition of DNA damage [1] and therefore genotoxic agents capable of initiating DNA single strand breakage are also of great interest in the context of processes involving PARP1. As well as ROS produced by

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UV and ionizing radiation, those arising from cellular metabolism, including superoxide, peroxynitrite, hydroxyl radicals and hydrogen peroxide, play a role in damage to biomolecules (reviewed in [15]). Intracellular ROS levels are increased in response to many external and internal stimuli including growth factors, cytokines, and environmental stress (reviewed in [15]) and act as signaling factors that regulate cell functions such as growth, differentiation, and viability, modulate signal transduction pathways [16] such as mitogen-activated protein kinase cascades, and influence the activity of transcription factors [17]. Many studies have focused on the processes which lead to the production of intracellular ROS and have shown that the Nox family of NADPH oxidases [18] and mitochondria [19,20] are major sources. Mitochondrial ROS are produced in the respiratory chain where high-energy electrons can react with O_2 to form superoxide ($O_2^{\bullet-}$); up to 2% of the O_2 consumed by healthy mitochondria is converted into superoxide and this level is higher in damaged and aged mitochondria. Since superoxide can react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which is severely damaging for DNA, it might provide a signal for activation of PARP1 [21].

Here, we show that inhibition of PARP1 significantly stimulates the late production of ROS in irradiated K562 (human myelogenous leukaemia) cells, which in turn is correlated with an increased level of DNA strand breaks.

2. Materials and methods

2.1. Chemicals

The PARP inhibitors 8-hydroxy-2-methylquinazolin-4-[3H]one (NU1025) (Sigma–Aldrich), 5-iodo-6-amino-benzopyrone (5-I) and 1,5-isoquinolinediol (1,5-IQD) (Calbiochem) (kindly provided by R. Hancock, Laval University Cancer Research Center, Québec, Canada) were dissolved in DMSO at 50 mM, and aliquots were stored in the dark at −20 °C. DAPI (4,6-diamidino-2-phenylindole) was obtained from Serva and 2′,7′-dichlorofluorescein diacetate (DCFH-DH) from Sigma–Aldrich. All other reagents were of the highest purity commercially available.

2.2. Cell culture and irradiation

Cells of the human myelogenous leukaemia line K562 were cultured in RPMI 1640 supplemented with L-glutamine (Sigma–Aldrich), 15% fetal bovine serum (FBS; Gibco), and 0.04% gentamycin at 37 °C in a humidified atmosphere and 5% CO_2. Cells growing exponentially in microplate wells were incubated with or without a PARP inhibitor (100 μM) for 15 min, irradiated at room temperature with 4 Gy or 12 Gy X-rays (6 MV) generated by a therapeutic accelerator (Clinac 600), and then incubated further for indicated time with or without PARP inhibitor in standard culture conditions. Parallel control samples were handled identically but not irradiated.

2.3. Alkaline comet assays

The kinetics of rejoining of DNA strand breaks were followed using the alkaline version of the single cell gel electrophoresis (comet) assay according to international recommendations [22]. Briefly, $\sim 4 \times 10^4$ cells were mixed with 2 volumes of low-melting agarose and immediately transferred onto slides on ice. After the agarose had polymerized, slides were incubated in lysis solution at 4 °C for 60 min, and in alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min. After electrophoresis at 0.75 V/cm and 300 mA for 20 min at 4 °C, slides were washed twice with neutralizing solution (0.5 M Tris, pH 7.4), fixed with 96% ethanol, and stained with ethidium bromide. The tail moment was calculated for at least 100 nuclei per slide using comet scoring software (Comet Score).

2.4. Assays of ROS

Cellular ROS content was determined using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), which rapidly penetrates cell membranes and becomes deacetylated by intracellular esterases; this non-fluorescent DCFH is then trapped in the cytosol and, upon oxidation to DCF, serves as a sensitive cytosolic marker for oxidative stress [23]. Cells were collected and incubated in the dark with DCFH-DA (30 μM) for 30 min at 37 °C, washed, resuspended in PBS, and kept for 15 min on ice in the dark. Fluorescence was determined by flow cytometry (Becton Dickinson FACS Canto) using the FITC configuration (488 nm laser line, LP mirror 503, BP filter 530/30) and expressed in arbitrary units. To examine the influence of mitochondrial complex I on the ROS level, rotenone (Sigma Aldrich) dissolved in DMSO, a mitochondrial complex 1 inhibitor, was added to the culture medium at a final concentration of 50 nM and ROS level was assessed 30 min later.

2.5. Immunocytochemical quantification of PAR

Cells were cytospun onto glass slides at 4 °C and fixed in methanol:acetone (1:1) at -20 °C. After blocking non-specific sites in 5% nonfat milk:PBS:0.05% Tween-20 at room temperature for 1 h, and incubation with a rabbit polyclonal antibody (LP96-10-04, 1:50 dilution, overnight), ADP-ribose polymers were visualized with Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen, 1:100 dilution, 1 h at 37 °C). Nuclei were stained with 1% DAPI. Images were acquired with a 40×10 objective. The fluorescence intensity of ∼100 cells per experimental point was measured using custom-made software and confirmed by Image J (available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, NIH). Mean values were expressed as percentage of control.

2.6. Apoptosis and necrosis assays

Viability of cells was assessed by flow cytometry using an annexin V-FITC Apoptosis Kit (Invitrogen). Cells were collected, washed with PBS, suspended in annexin V-staining buffer, and propidium iodide (PI) and annexin V-FITC were added 10 min before assays. This assay discriminates between intact cells (PI−/FITC−), early apoptotic cells (PI−/FITC+), and late apoptotic or necrotic cells (PI+/FITC+). Apoptotic cells were considered to be those not stained by PI.

2.7. Caspase activity assays

The activities of caspases were determined in cell lysates using the caspases 1, 3, and 8 colorimetric assay kit (Biomol) with the chromogenic peptidyl substrates YVAD-pNA, DEVD-pNA and IETD-pNA for caspases 1, 3, and 8 respectively, and LEHD-pNA for caspase 9. Absorbance was measured on a NanoDrop spectrophotometer at 405 nm after 2 h of incubation at 37 °C and was standardized using the free colorimetric substrates. Caspase activities were calculated as picomoles of substrate cleaved by 1 mg of protein in 1 min [24]. The concentration of protein in cell lysates was measured by UV absorption at 280 nm [25].

2.8. Determination of the cellular ATP level

The level of adenosine 5′-triphosphate was assessed with the ATP Bioluminescent Cell Assay Kit (Sigma–Aldrich) according to the protocol provided by the manufacturer. Briefly, 100 μl of Somatic Cell ATP Releasing Agent was added to wells of a 96-well

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