SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Regulatory role of poli(ADP-ribosyl)ation and hydrogen-peroxide in osteogenic differentiation and cigarette smoke-induced cell death

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The Examination takes place at 2.306 office at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen at 11 AM, 25th of November, 2014.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1 PM, 25th of November, 2014.

1. INTRODUCTION

1.1. The poly(ADP-ribose) polymerase enzyme family

Poly(ADP-ribose) polymerases (PARPs) occur in most eukaryotes and in some prokaryotes. They catalyze the ADP-ribosylation reactions of acceptor proteins. The PARP family has 17 members; PARP-1 among them is the most well known member. The human PARP-1 gene has been mapped to chromosome 1, the PARP-1 protein contains 1,014 amino acids with a molecular weight of 113 kDa. The enzyme consists of three main domains with distinct functions: DNA binding domain, automodification domain and catalytic domain. PARP-1 plays many roles in DNA repair, maintenance of genomic integrity, cell cycle control, and regulation of transcription and cell death pathways and in many other processes.

1.2. A poly(ADP-ribose) metabolism

Poly-ADP-ribosylation (PARylation) is a reversible posttranslational modification catalyzed by PARPs. Upon activated, PARPs use NAD^+ as a substrate and cleave it to nicotinamide and ADP-ribose, then use the ADP-ribose units for polymer synthesis which are covalently attached to acceptor proteins. The polymers can grow to over 200 units, branching once every 20–50 ADP-ribose units. PAR chains can be attached to glutamate, aspartate or lysine residues of acceptor proteins. The most abundant PARylated protein i*n vivo* is PARP-1 itself. Automodification of PARP-1 leads to the inhibition of its enzymatic activity. Due to the size and negative charge of ADP-ribose units, PARylation influences the physico-chemical properties, stability, activity and interactions of proteins.

Poly(ADP-ribosyl)ation is a reversible, dynamic process, PAR has a short half-life, degradation of polymers starts immediately after synthesis, the enzymes involved in PAR metabolism are strictly regulated. Degradation of polymers are catalyzed by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl hydrolase 3 (ARH3).

1.3. PARP-1 activating stimuli

The central dogma of PARylation held that PARP-1 is activated by DNA damage. UV irradiation, ionizing radiation, oxidative stress and alkylating agents induce PAR synthesis. ROS/RNS including hydrogene-peroxide (H_2O_2), hidroxyl radical (•OH), superoxide ($•O_2$-) and peroxynitrite (ONOO-), directly cause DNA strand breaks and activate PARP-1. An interesting feature of DNA damage-induced PARP activation is that PARP-1 is activated during transcription by DNA double strand breaks created by topoisomerase IIβ. Thus PARP-1 may have a role in transcriptional regulation.

Several lines of evidence reveal that PARP-1 can be activated in the absence of DNA breaks during chromatin remodelling and transcription. Bending of DNA and other non-B-DNA structures (single-stranded regions, cruciform and hairpin structures) can lead to PARP-1 activation. Phosphorylation by protein kinases can also regulate PARP activity. Activation of PARP-1 by acetylation and mono(ADP-ribosyl)ation have also been reported. Certain proteins can influence PARP activity through protein-protein interactions without covalent modification.

1.4. The physiological role of PARylation

1.4.1. DNA repair and genom integrity

PARP-1 has a role in single stranded DNA repair, base excision repair (BER), homologous recombination, non-homologous end-joining (NHEJ) and nucleotide excision repair (NER). Through its DNA binding domain, PARP-1 binds to DNA strand breaks, which is followed by dimerization and automodification. PAR polymers induce chromatin loosening; on the other hand they provide high affinity binding sites for repair enzymes. Auto-poly-ADP-ribosylation inactivates PARP-1, which is then removed from DNA. Degradation of PAR leads to the dissembly of the complex and the next steps of DNA repair can start. For efficient DNA repair the coordinated action of PARP and PARG is necessary.

1.4.2. The role of PARP-1 in the regulation of cell death pathways

During apoptosis PARP-1 is cleaved by cspases to a 24 kDa and 89 kDa fragment. The cleavage inactivates the enzyme, which prevents ATP depletion and necrosis during DNA fragmentation. PARP-1 doesn't have a modulatory role in the early phase of apoptosis, but its cleavage is necessary for the process.

PARP-1 has a regulatory role in regulated necrosis. The overactivation of the enzyme causes NAD^+ depletion which leads to RIP1, TRAF2 and JNK activation and mitochondrial membrane depolarization. AIF is cleavaged and translocates to the nucleus where it forms a complex with cyclofillin-A and H2AX and induces chromatin condensation and DNA degradation.

In parthanatos, the accumulation of PAR polymers induces cell death. PAR can bind to the C-terminal domain of AIF with high affinity, which causes a conformational change in AIF and reduces its affinity to the mitochondrial membrane. The 62 kDa full length AIF translocates to the nucleus.

The role of PARP-1 has been reported in nutrition deficiency-induced autophagy, where DNA damage caused by elevated ROS levels leads to PARP-1 activation. Autophagy and its inhibition are of special interest in tumor cells, as it is a survival mechanism during chemotherapy or radiation therapy.

Regarding the role of PARP-1 in several cell death pathways, it possibly acts as a molecular switch between cell death pathways, and cell fates depend on the severity of DNA damage, the energy state of cells and PARylation.

1.4.3. Regulation of gene expression and differentiation

Under normal conditions PARP-1 is bound to the chromatin and can be found in the nucleoli and along chromosomes. Inhibition of PARP-1 modulates gene expression patterns in several cell types. PARP-1 ADP-ribosylates chromatin-bound proteins (e.g. histones), which destabilizes the interaction between chromatin components and DNA. On the other hand, auto-poly-ADP-ribosylation can lead to chromatin loosening.

PARylation regulates splicing. PARP-1 can act as a coregulator and modulates the binding of transcription factors to the chromatin. PARP-1 also modulates DNA metylation thus having a role in epigenetic regulation of gene expression.

1.5. Oxidative stress caused by cigarette smoke

Cigarette smoke is a complex aerosol consisting of thousands of compounds. Smoking-related cell death contributes to COPD, but data regarding the form of cell death and their relative role are somewhat controversial. Smoking can also cause chronic inflammation and decreased protection against airway diseases. Smoking can decrease antiviral immunity, bacterial clearance and complement-mediated phagocytosis and it increases allergen penetration.

The toxicity of cigarette smoke is associated with oxidative stress. The gas phase contains short-lived reactive oxygen species, mostly superoxide and nitrogen.monoxide which can react to form the highly reactive peroxynitrite. The particulate phase is rich in hydroquinones which can contribute to the formation of superoxide, hydrogen-peroxide and hydroxyl radicals, thus smoking can cause a significant oxidative stress. GSH, superoxide decomposing SOD enzymes, hydrogen-peroxide decomposing catalase and enzymes associated with GSH metabolism are thought to have central roles in antioxidant defense. The latter group of enzymes includes glutathione peroxidases (GPX), glutathione reductases (GR), glutamate cysteine ligase (GCL) and glutathione synthase (GS). Disposal of hydrogen

peroxide is closely associated with thiol-containing proteins – namely thioredoxins (TRX) and thioredoxin reductases (TRR).

1.6. Characterization of mesenchymal stem cells (MSC) and osteogenic differentiation

Mesenchymal stem cells are non-hematopoietic multipotent stromal cells that are capable of self-renewal and multilineage differentiation. Differentiation of cells to osteoblasts is a multistep process. The lineage commitment of multipotent MSCs is driven by the selective expression of so-called master transcriptional regulators. Following lineage commitment, osteoprogenitors express alkaline phosphatase, bone sialoprotein, type I collagene and produce an osteogenic extracellular matrix. Finally they express genes involved in mineralization of the extracellular matrix. Oxidative stress and hypoxia were found to modulate osteogenic differentiation. The role of ROS in differentiation is controversial. The majority of studies describe the inhibitory effect of exogenous ROS in osteodifferentiation. However, there is evidence suggesting that endogenous ROS can promote differentiation.

PARylation has been shown to play a role in several differentiation processes. It is necessary for chromatin loosening and regulates the binding of certain transcription factors to the chromatin. The role of PARylation in the transcriptional regulation of osteogenic differentiation has not been investigated. Moreover, osteogenic differentiation is associated with massive cell death. As PARP-1 plays a central role in several cell death pathways, we set out to investigate whether it regulates differentiation-associated cell death.

2. AIMS OF THE STUDY

2.1. The role of PARP-1 in cigarette smoke-induced cell death

Smoking-related diseases are basically caused by oxidative stress and oxidant-antioxidant imbalance. ROS/RNS present in cigarette smoke and produced by the cells as part of the stress response have been demonstrated to damage DNA and other cellular components. However, the regulation of smoke-induced DNA damage sensing and repair is poorly understood. PARP-1 plays a central role in DNA repair and in the regulation of cell death pathways.

In our study we aimed to answer the following questions:

1. Does CSE induce PARP-1 activation in A549 cells?
2. What is the role of PARP-1 in the regulation of CSE induced cell death?

2.2. The role of PARP-1 in osteogenic differentiation-associated cell death

The regulated expression of several genes is necessary for osteogenic differentiation of mesenchymal stem cells and SAOS-2 cells. PARP-1 plays a central role in the maintenance of genom integrity and in the regulation of transcription.

In our study we aimed to answer the following questions:

1. Has oxidative stress any effect on osteogenic differentiation?
2. How does PARP-1 modulate osteogenic differentiation?

3. MATERIALS AND METHODS

3.1. Cell culture

A549 lung epithelial cells were cultured in RPMI 1640 medium, human cMSCs and SAOS-2 cells were cultured in DMEM (1g/L glucose). Cell culture medium was supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured at 37 °C in a 5% CO2 humidified incubator.

3.2. Preparation of cigarette smoke extract (CSE)

One 100 mm cigarette (10mg tar, 0.8 mg nicotine, 10 mg CO, no filter) was combusted with a vacuum pump at a rate of one cigarette per 5 min and the smoke was bubbled through 6 ml of prewarmed serum-free RPMI 1640 medium. The extract was then filtered through a 0.22-mm filter to remove particles and microbiological contaminants. After supplementation with 10% FBS, the extract was defined as 100% CSE. Dilutions were made with culture medium to various concentrations expressed as a percentage (v/v %). CSE was freshly prepared for each experiment.

3.3. May-Grünwald-Giemsa staining

Cells were washed with phosphate-buffered saline (PBS), and fixed with prechilled methanol at -20 1C for 20 min. After being washed with distilled water, the cells were incubated for 30 min with freshly diluted May-Grünwald-Giemsa solution and then washed with distilled water. After air drying, coverslips were mounted in Mowiol and viewed with a Zeiss Axiolab microscope.

3.4. Clonogenic survival

A single-cell suspension was prepared at a density of $2.5x10^4$/ml, and the cells were treated with various concentrations of CSE for 30 min at 37 °C. CSE was removed and the cells were further diluted in culture medium and seeded into six-well plates at densities of $1x10^2$ to $2.5x10^3$ cells/well. After 10 days culture, the cells were washed with PBS, fixed with 4% formaldehyde, and then stained with hematoxylin for 10 min. After intensive washing with tap water, the plates were air dried and colonies were counted. Survival was calculated as a percentage, using the equation T/C x 100, where T and C are the numbers of colonies in treated and control (untreated) plates, respectively.

3.5. Cell viability assay (MTT reduction assay)

After treatment, MTT solution was added to each well at a final concentration of 0.5 mg/ml. Following 1 h incubation the medium was aspirated and formazan crystals were dissolved in dimethyl sulfoxide. Optical density was measured at 590 nm using a Multiskan MS plate reader. Relative cell viability was calculated as $A_{treated}/ A_{control}$ x 100, where A is the absorbance.

3.6. Plasma membrane injury (LDH activity)

The assay was performed using the Cytotoxicity Detection Kit (Roche Applied Science, Budaörs, Hungary) according to the manufacturer's instructions. Cytotoxicity was expressed as a percentage of maximum LDH activity.

3.7. Mitochondrial membrane potential

Mitochondrial membrane depolarization was determined with JC-1 staining. Cells were washed twice with HBSS and incubated with 1 µM JC-1 solution in HBSS for 30 min. Red fluorescence was measured with excitation at 530 nm and emission at 590 nm, and green fluorescence was read with excitation at 485 nm and emission at 538 nm, using a Fluoroskan Ascent FL plate reader.

3.8. Electric Cell-substrate Impedance Sensing (ECIS)

Cell proliferation was monitored by electric cell–substrate impedance sensing (ECIS; Applied BioPhysics, Troy, NY, USA). As the cell number increases, the electrode area covered with the cells grows, causing the electrode impedance to rise. Cells were seeded into the wells of 8W10E+ arrays at a density of $3x10^4$ cells/well and exposed to 1.25–10% CSE or left untreated. Changes in cell density were monitored by a timecourse measurement of the resistive portion of the impedance at 4000 Hz. Data were collected with a 120 s time interval and normalized to a 0 s time point.

3.9. Immunofluorescent staining for PAR and AIF

Cells were washed with PBS, then fixed with methanol at -20 °C for 20 min (PAR) or with 4% formaline at RT (AIF). After washing with PBS, aspecific binding sites were blocked with 1% BSA (diluted in PBS Triton X-100), then cells were incubated with primary antibodies for 2 h (dilutions of the antibodies were 1:200 for α-AIF and 1:500 for α-PAR). After washing, cells were incubated with secondary antibodies for 1 h (anti-rabbit IgG-Alexa Fluor 488 conjugate for AIF and biotinylated anti-mouse IgG for PAR). In case of PAR

detection cells were further incubated with Streptavidin-Alexa Fluor 488 conjugate for 30 min. Nuclei were stained with 2 µg/ml DAPI (PAR) or 5 µM propidium-iodide. Pictures were taken with a Zeiss Axiocam digital camera.

3.10. Western blot

Cells were washed with cold PBS and harvested by scraping in cold lysis buffer. After sonication, cell lysates were centrifuged and boiled, and then equal amounts of protein were separated by SDS-PAGE. After electrophoresis, samples were transferred onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked with 5% non-fat dry milk. Membranes were incubated with primary antibody diluted in 1% milk in PBSTw for 2 hours at room temperature, which was followed by incubation with peroxidase-conjugated secondary antibodies. To visualize the antibody reactions, chemiluminescent substrates were added and chemiluminescent signals were detected using a FluorChem FC2 Imager.

3.11. Comet assay

Slides were coated with 1% normal melting point agarose. A single-cell suspension was prepared and the cells were treated with various concentrations of CSE for 30 min at 37 °C. To investigate the efficiency of DNA repair, cells were incubated in cell culture medium for 1 h after CSE exposure. Then the cell suspension was mixed with 0.5% low mwlting point agarose and layered on coated slides. After lysis, electrophoresis was carried out in alkaline buffer, slides were neutralized in 0.4M Tris buffer and nuclei were stained with 10 µg/ml ethidium-bromide. CometScore software was used for quantitative analysis.

3.12. Intracellular superoxide ($O_2^{\cdot-}$) detection

After treatment, cells were washed twice with HBSS, and then incubated for 30 min with 5 µM MitoSOX Red or 20 µM dihydroethidium. Fluorescence was read with excitation at 530 nm and emission at 590 nm using a Fluoroskan Ascent FL plate reader. Mitochondrial superoxide production was also detected by vital staining with MitoSOX Red. Cells were grown on coverslips and exposed to CSE. After washing 2 times with HBSS, cells were incubated with 5 µM MitoSOX Red for 30 min and superoxide induced fluorescence was visualized with a Zeiss Axiolab microscope.

3.13. Hydrogen-peroxide (H_2O_2) detection

Intracellular hydrogen-peroxide production was measured with Amplex Red reagent. After treatment, cells were washed twice with HBSS and incubated with 50 µM Amplex Ultra

Red reagent and 0.1 U/ml horseradish peroxidase in HBSS for 30 min. Fluorescence was read with excitation at 530 nm and emission at 590 nm using a Fluoroskan Ascent FL plate reader. Hydrogen peroxide concentrations were normalized to protein content.

3.14. Stable knockdown of PARP-1 and PARG in SAOS-2 osteosarcoma cells

PARP-1 and PARG were silenced by lentiviral vectors. pLKO.1-puro plasmids containing PARP-1 or PARG specific short hairpin RNA (TRCN0000007929 and TRCN0000051307 Sigma Mission shRNA constructs) were used. pLKO.1-puro empty vector was used as a negative control. Infection of SAOS-2 cells was carried out by addition of lentivirus with a multiplicity of infection of 10. Following transduction, cells were selected with 5 μg/ml puromycin.

3.15. Isolation of human chorion-derived mesenchymal stem cells (cMSCs)

Placentas from caesarian section were obtained from the Dept. of Obstetrics and Gynecology, University of Debrecen. Placentas were washed with HBSS to remove the remaining blood. After removing the amnion and cord, cotyledon was cut into small pieces and digested with 270 U/ml collagenase type II. Following filtration and ficoll gradient centrifugation, cells were suspended in cell culture medium and cultured under standard cell culture conditions.

3.16. Phenotyping and functional characterization of cMSCs

Phenotyping of cells isolated from human placentas was carried out according to the criteria of ISCT (International Society for Cellular Therapy).

Phenotyping of cMSCs

Determination of surface antigen pattern of cMSCs was carried out by flow cytometry. After trypsinization, cells were washed with HBSS and incubated with (10 μg/ml) primary antibodies (CD34, CD45, CD73, CD90, CD105, vWF, HLA-G). Appropriate isotype controls (IgG1 and IgG2b) were used for each antibody. After washing with HBSS, cells were incubated with secondary antibody (anti mouse IgG-Alexa Fluor 546, 1:500) then fixed with 1% formalin. Fluorescence intensity was measured with BD FACS Calibur flow cytometer, data were analysed with BD Multiset software.

Functional characterization – osteogenic differentiation

The composition of osteogenic differentiation medium was the following: DMEM (1 g/l glucose), 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0,1 µM dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, 10 mM β-glycerol- phosphate, 50 nM vitamin D3. Calcium deposition in the extracellular matrix was detected by Alizarin Red S staining on day 7, 14 and 21 during the differentiation. Cells were washed with PBS, fixed with methanol and stained with Alizarin Red S.

Functional characterization – chondrogenic differentiation

The composition of osteogenic differentiation medium was the following: DMEM (4,5 g/l glucose), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM Na-pyruvate, 10 ng/ml TGF-β1, ITS premix, 0,1 µM dexamethasone, 50 µg/ml ascorbic acid-2-phosphate, 40 µg/ml proline. Cells were digested with trypsin-EDTA, suspended in differentiation medium, seeded into V-buttom plates ($2.5x10^5$ cells/well) and centrifuged at 800 g for 10 min. Cells formed spheres during differentiation, extracellular matrix components were detected by dimethyl-methylene blue (DMMB) staining. Spheres were washed with PBS and fixed in 4% formalin, then paraffin embedded sections were made. After deparaffinization, sections were stained with 0.1 % DMMB for 30 min.

Functional characterization – adipogenic differentiation

The composition of osteogenic differentiation medium was the following: DMEM (4,5 g/l glucose), 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM Na-pyruvate, 1 µM dexamethasone, 200 µM indomethacin, 500 µM IBMX (3-isobutyl-1-metylxanthine), 10 µM insulin. Cells were washed with PBS, fixed with 4% formalin, washed with distilled water and stained with Oil Red O solution.

3.17. Real-time PCR

Relative gene expression was determined with real-time PCR. Total RNA was isolated from cells with Tri reagent. 2 µg RNA was reverse-transcribed to cDNA with High Capacity cDNA reverse transcription kit. TaqMan gene expression assay was carried out with 7500 Fast Real-Time PCR system. GAPDH was used as a housekeeping gene.

3.18. Statistical analysis

For statistical analysis, One-way Anova was performed in case of experiments with cigarette smoke extract. Student's t-test was performed in case of experiments in connection with osteogenic differentiation.

4. RESULTS

4.1. Poly(ADP-ribosyl)ation as a survival mechanism in cigarette smoke-induced cell death

4.1.1. Toxicity of CSE on A549 cells

CSE caused a concentration and time dependent loss of viability in A549 cells. Cell death was also characterized by plasma membrane permeabilization, a sign of necrotic cell death as indicated by increased LDH release. CSE exposure severely impaired the proliferative capacity of cells as determined with ECIS measurements. Only the highest CSE concentrations of CSE triggered the depolarization of the mitochondrial membrane. Even at these high concentrations of CSE, most AIF showed extranuclear localization.

4.1.2. CSE induces PAR accumulation

To investigate the role of the main PAR-synthesizing enzyme, PARP-1, and the main PAR degrading enzyme, PARG, in the regulation of CSE-induced cell death, we used A549 cell lines in which these enzymes were stably silenced by lentiviral vectors (shPARP-1 and shPARG cells). CSE caused a mild increase in cellular PAR content in control A549 cells carrying empty lentiviruses, as determined by immunofluorescent staining. In the shPARP-1 cell line, however, no PAR signal could be detected. Silencing PARG resulted in stronger PAR signals, which were detectable even at lower (5%) CSE concentrations. The PARP inhibitor PJ34 prevented the formation of the polymer, confirming the specificity of our immunofluorescent staining. CSE-induced PAR formation could also be detected (with higher sensitivity) in Western blot experiments. These data prove that CSE triggers PARP activation and PAR formation and also demonstrate active PAR degradation by PARG in A549 cells.

4.1.3. PARP-1 és PARG regulate CSE-induced cell death

The next question we asked was whether PARP-1 and PARG regulate CSE-induced cell death. Morphological evaluation (May-Grünwald-Giemsa staining) of the cells upon CSE exposure indicated a concentration-dependent detachment of cells from the coverslips. Interestingly the first morphological change observed in CSE-exposed cultures (5%) was cellular and nuclear swelling and lighter cytoplasmic staining, probably indicating necrosis or necroptosis. At the highest CSE concentrations used (20%), most cells detached and the remaining cells displayed a more compact nuclear morphology without nuclear fragmentation. shPARP-1 and especially shPARG cells displayed these morphological alterations and detachment at lower CSE concentrations compared to control cells, suggesting a higher sensitivity of shPARP-1 and shPARG cells compared to the parent cell line. Moreover,

inhibition of PARP activity by PJ34 or silencing of PARP-1 or PARG sensitized cells to CSEinduced loss of viability as determined by MTT reduction assay. Disruption of plasma membrane integrity, a sign of necrotic cell death as assessed by measuring LDH release from cells, was also significantly enhanced in both shPARP-1 and shPARG cells. Interestingly, silencing of PARG resulted in more pronounced sensitization to loss of viability and plasma membrane permeabilization than silencing of PARP-1

To investigate whether the above-described morphological and biochemical parameters of cell death are also accompanied by impaired functionality, the proliferative capacity of CSEexposed cells was also assessed. In clonogenic assays, CSE was found to compromise the proliferative capacity of A549 cells, with silencing of PARP-1 or PARG resulting in sensitization of the cells. In line with the survival assays and morphological assessment of the cells, the PARG status had a higher impact on the clonogenic proliferation of the CSE-exposed cells than the PARP-1 status.

4.1.4. PARP-1 and PARG regulate the repair of CSE-induced DNA damage

Based on the central role of PARylation in DNA single-strand break repair, it seemed plausible to hypothesize that the sensitizing effects of PARP-1 and PARG silencing to cell death and impaired proliferation were due to a DNA repair defect. Indeed CSE-induced DNA single-strand breaks as detected by alkaline comet assay were repaired relatively quickly in control cells but much less efficiently in shPARP-1 and shPARG cells.

4.1.5. CSE induced intracellular ROS production

An important question is the chemical identity of the stimuli that trigger PARylation in CSE exposed cells. On the one hand CSE contains various radicals and reactive nonradical species that can cause DNA breakage and PARP activation. In addition, ROS may also be produced as part of the cellular response to the damaging effects of CSE. Staining of cells with superoxide-sensitive fluorescent dyes such as dihydroethidium and MitoSOX red revealed a time- and concentration-dependent increase in cellular superoxide production in CSE-treated cells. MitoSOX red fluorescence was also visualized by microphotography. Of note, in these experiments CSE was removed from the cells before they were stained with the ROS-sensitive dyes. Therefore increased fluorescence was due to cellular (most likely mitochondrial) superoxide production rather than elicited directly by CSE.

4.1.6. Role of superoxide és hydrogen-peroxide in CSE-induced PARP activation and toxicity

From the ROS species present in CSE and produced by CSE-treated cells, hydrogen peroxide seems to be the key activator of the DNA damage-PARP-1 activation pathway. CSE induced accumulation of PAR was augmented by superoxide dismutase (SOD) and even more so by PEG-modified (cell-permeable) SOD. Catalase (in both native and PEGylated form), however, abolished CSE-induced PAR synthesis. Catalase was also effective in preventing SOD-enhanced PAR synthesis, indicating that the PARP-stimulating effect of SOD was due to increased hydrogen peroxide formation. In agreement with the effects of these treatments on PAR synthesis, the PARP inhibitor PJ34 and SOD (especially PEGylated SOD) sensitized cells to CSE toxicity, whereas catalase provided protection

4.2. The role of hydrogen-peroxide-induced poli(ADP-ribosyl)ation in osteogenic differentiation associated cell death

4.2.1. Phenotyping and functional characterization of cMSCs

Phenotyping of cells isolated from human placentas was carried out according to the criteria of ISCT (International Society for Cellular Therapy). Determination of surface antigen pattern of cMSCs was carried out by flow cytometry. The isolated cMSCs expressed mesenchymal stem cell surface antigens including CD73, CD90 and CD105, and didn't express hematopoietic, endothelial and trophoblast markers (CD45, CD34, vWF és HLA-G negativity).

Osteogenic differentiation capacity was characterized by Alizarin Red S staining detecting extracellular calcium deposition. Calcium deposition was detectable after 7 days of differentiation. Adipogenic differentiation capacity was detected by Oil Red O staining. Cells started to accumulate lipid droplets from the third day of differentiation. Chondrogenic differentiation was carried out in V-bottom pates. Cells formed three dimensional structures, small spheres on the first day of differentiation. Sections were stained with DMMB to visualize the presence of extracellular glycosaminoglycans.

4.2.2. Reactive oxygen species (ROS) production is necessary for osteogenic differentiation

Incubation of SAOS-2 cells in differentiation medium resulted in osteogenic differentiation as verified by the expression of osteogenic marker genes (Runx2, osterix, BMP2, osteopontin), extracellular calcium deposition and increased alkaline phosphatase activity. Osteogenic differentiation was accompanied by fluctuations of the intracellular redox balance. Hydrogen-peroxide release slightly, but significantly decreased during the first two

days of differentiation, then increased at the end of the differentiation period. A similar pattern could be observed in lipid peroxidation. The importance of this redox imbalance is indicated by the effect of glutathione and catalase as both antioxidants decreased calcium deposition significantly. Catalase inhibited differentiation, calcium deposition and alkaline phosphatase activity. Moreover, catalase also suppressed the expression of osteogenic marker genes.

4.2.3. PAR accumulation correlates with ROS production during osteogenic differentiation

ROS can activate PARP by causing DNA damage. PAR levels decrease during the first two days of differentiation, but increase afterward. The fluctuation of PAR levels showed a pattern similar to that of the hydrogen-peroxide production, suggesting a causal relationship. Linear regression analysis revealed a correlation between PAR and H_2O_2 production (R=0,78) as well as between PAR and lipid peroxidation (R=0,76). Catalase treatment significantly inhibited and delayed PAR accumulation.

4.2.4. Hydrogen-peroxide production causes cell death during differentiation

Differentiation was accompanied by cell death that started on day 2 and increased continuously until the last day of differentiation. Double staining of cells with Hoechst/propidium-iodide and Annexin V/propidium-iodide revealed that the predominant form of cell death was apoptosis as indicated by chromatin condensation and phosphatidyl-serine externalization. However, necrotic cells were also observable. We could detect cleaved PARP-1 which is a hallmark of apoptosis. The pancaspase inhibitor (z-VAD-fmk) only partially prevented the loss of cell viability and shifted apoptosis towards necrosis. Glutathione and catalase significantly enhanced cell survival in differentiating cells, and both compounds reduced apoptosis as well as necrosis.

4.2.5. Knockdown of PARP-1 and PARG in SAOS-2 cells

Our observations suggested that hydrogen-peroxide production and redox balance can have an important role in osteogenic differentiation. Considering the well-established role of PARylation in cell death pathways, we hypothesized that PARP-1 can regulate osteogenic differentiation associated cell death. To this end we stably silenced PARP-1 and PARG SAOS-2 cells. The mRNA levels of PARP-1 and PARG were determined by real-time qPCR. The level of PARP-1 mRNA was reduced in shPARP-1 cells to approximately 50 % compared to control cells. The effort to achieve more efficient silencing resulted in dramatic cell death, indicating that PARP-1 is essential for the viability in SAOS-2 cells. Silencing of

PARG was more efficient and was better tolerated as cells maintained proliferative capacity. PARP-1 silencing was also confirmed by Western blot analysis. We carried out a functional characterization of the silenced cell lines by detecting PAR accumulation with immunocytochemistry and Western blot. Cells were treated with H_2O_2 for 10 min, and then allowed to recover for 30 min. A reduced PAR synthesis could be observed in shPARP-1 cells exposed to H_2O_2 treatment. shPARG cells showed reduced PAR degrading activity as indicated by the long-term persistence of the polymer.

4.2.6. A PARP-1 and PARG modulates osteogenic differentiationt

Downregulation of PARP-1 and PARG affected PAR accumulation pattern in SAOS-2 cells during osteogenic differentiation. All three cell lines showed mineralization, increased activity of alkaline phosphatase and elevated expression of osteogenic marker genes, but showed different kinetics in changes. In shPARP-1 cells there is a delay and decrease in calcium deposition. ALP activity is reduced in both silenced cell lines compared to the control cells. Both PARP-1 and PARG silencing affected osteogenic marker gene expression.

4.2.7. Poli(ADP-ribosyl)ation regulates cell death in SAOS-2 cells and human mesenchymal stem cells during osteogenic differentiation

Cell death during osteogenic differentiation was also affected by PARP-1 and PARG silencing. shPARP-1 cells had significantly increased viability than control cells, PARP-1 deficiency caused a decrease in differentiation and cell death. PARG silencing resulted in decreased viability and the form of cell death was shifted towards necrosis. The role of H_2O_2-induced PARylation in differentiation-related cell death was also investigated in primary human chorional mesenchymal stem cells. cMSCs also underwent a catalase-inhibitable, H_2O_2-dependent cell death displaying apoptotic and necrotic features. Calcium depositin was reduced in the presence of catalase or 5 μM PJ34 specific PARP-1 inhibitor. In contrast to data obtained with shPARP-1 SAOS-2 cells, treatment of MSCs with PJ34 slightly sensitized cells to differentiation-induced cell death.

5. DISCUSSION

5.1. Regulatory role of poli(ADP-ribosyl)ationin CSE-induced cell death

The toxicity of cigarette smoke is associated with oxidative stress. The cell-damaging effects of cigarette smoking, including genotoxic effects, are well documented. However, the fate of the injured cells (repair, survival, apoptosis, necrosis, or autophagy) and signalling pathways involved in the regulation of survival and cell death, are not well characterized.

Here we have shown that CSE is toxic to A549 cells. Injured cells lose proliferative capacity as demonstrated in clonogenic assays. CSE-induced cell death takes place in an unusual form, combining features of both necrosis and apoptosis. The most prominent morphological alteration observed at lower CSE concentrations (1.25–2.5%) was cellular and nuclear swelling resembling a necrotic phenotype. At higher concentrations cells detached and the few adherent cells were more compact and shrunken, and their chromatin stained darker, resembling an apoptotic phenotype. Plasma membrane permeability (also typically considered a sign of necrosis) displayed a different pattern, with LDH release increasing in a CSE concentration-dependent manner. These findings indicate that CSE-induced cell death follows an unusual path and cannot be classified according to classical cell death schemes. Others also reported DNA injury but lack of typical signs of apoptosis (e.g., caspase activation) in CSE-exposed cells. AIF may also emerge as a possible mediator of CSE-induced cell death. Indeed, a small fraction of mitochondrial AIF was found to translocate to the nucleus but the contribution of this minimal AIF translocation to CSE-induced cell death is difficult to judge. Thus we set out to investigate the role of PARP-1 in CSE-induced cell death. Scattered data in the literature indicate that CSE may stimulate PARylation We also found increased PAR levels in A549 cells after CSE exposure. We also proved here by gene silencing that PARP-1 is responsible for CSE-induced PAR synthesis. PARylation is considered a dual-faced regulator of cell death: at mild injury it promotes cell survival by enhancing DNA repair, whereas after severe, necrotic injury, PARylation contributes to cell death by NAD^+/ATP depletion or by triggering a PAR-dependent translocation of AIF and consequent cell death termed parthanatos. In the case of CSE-induced damage we could demonstrate the survival-promoting role of PARylation by PARP-1 and PARG but not the suicidal role of this pathway. As for the latter, CSE-induced damage may not be severe enough, even at the highest concentration, to lead to irreparable damage and overactivation of PARP-1. Thus parthanatos is not an important cell death pathway in CSE-treated A549 cells.

The prosurvival effect of PARylation/dePARylation by PARP-1 and PARG in CSE-exposed cells is demonstrated by impaired DNA repair, clonogenic survival, and viability of cells in which either PARP-1 or PARG was silenced. The similar rather than opposing consequences of PARP-1 and PARG silencing are in keeping with previous reports on the role of these enzymes. These data indicate that the concerted actions of PARP-1 and PARG are required for the efficient repair of CSE-induced DNA breaks and for the survival of CSE-exposed cells.

DNA single-strand breaks (SSBs) are the main activators of PARylation, and SSBs are caused by ROS/RNS species or by DNA alkylation agents. Cigarette smoke contains various DNA-damaging agents. Our data emphasize the importance of ROS, with special regard to superoxide and hydrogen peroxide, in CSE-induced DNA breakage and PARP activation. Using several different ROS-sensitive probes we have shown here that ROS are produced in CSE-treated cells. On the one hand, ROS/RNS are present in the smoke itself, whereas mitochondrial ROS production is part of the cellular stress response to CSE-induced cellular damage. The possible role of mitochondrial superoxide production is also supported by mitochondrial depolarization and by increased fluorescence signals from the mitochondrially targeted, superoxide-sensitive MitoSOX probe. The inhibitory effect of catalase on PAR formation and cytotoxicity indicates that hydrogen peroxide (or most probably hydrogen peroxide-derived hydroxyl radical) is the key activator of DNA damage triggering PARP activation. The higher amount of PAR formed in the presence of SOD and the increased cytotoxicity measured in the presence of SOD indicate that superoxide is either present in the smoke or produced by the CSE-treated cells and its conversion to hydrogen peroxide by SOD fuels the DNA damage-PARP activation pathway. Although the specificity of ROS-sensitive fluorescent probes is not absolute, our data obtained with SOD and catalase provide further support for superoxide and hydrogen peroxide being key mediators of DNA damage and cytotoxicity after CSE exposure.

5.2. The regulatory role of hydrogen-peroxide-induced poli(ADP-ribosyl)ation in osteogenic differentiation associated cell death

Mesenchymal stem cells are promising in regenerative medicine as they are multipotent and capable of multilineage differentiation. SAOS-2 cells represent a good model for the late stages of osteogenic differentiation as they can produce a mineralized extracellular matrix.

Incubation of SAOS-2 cells in differentiation medium resulted in osteogenic differentiation as verified by the expression of osteogenic marker genes (Runx2, osterix, BMP2, osteopontin), extracellular calcium deposition and increased alkaline phosphatase activity. We could detect hydrogen-peroxide production, which is essential for differentiation, as catalase decreased extracellular calcium deposition and the activity of alkaline phosphatase. Catalase also decreased or delayed the expression of osteogenic marker genes. The role of ROS in osteogenic differentiation is somewhat controversial. he majority of studies describes the inhibitory effect of exogenous ROS in osteodifferentiation. However, there is evidence suggesting that ROS can promote differentiation. BMP2-induced osteogenic differentiation of preosteoblasts was demonstrated to be mediated by NOX4-derived superoxide anion. Superoxide was also found to act as a procalcifying cell signal in osteoblastic differentiation of vascular smooth muscle cells. Continuously generated hydrogen-peroxide stimulates the proliferation and osteoblastic differentiation of human periodontal ligament fibroblasts. Increased ROS levels during osteogenic differentiation might be associated with NADPH oxydases, mainly NOX4 and NOX4. It was also demonstrated that ROS has a regulatory role in chondrogenic and adipogenic differentiation pathways.

We could detect PAR accumulation during osteogenic differentiation of SAOS-2 cells and a correlation was found between PAR levels and H_2O_2 production. Data obtained with catalase treatment proves that PARP activity was induced by hydrogen-peroxide.

Osteogenic differentiation of SAOS-2 and MSC cells is associated with cell death. The most prominent form of cell death is apoptosis. Glutathione and catalase increased cell viability, suggesting that cell death was induced by hydrogen-peroxide. Based on the close linkeage between H_2O_2 production and PARylation we set out to investigate the effects of PARP-1 and PARG silencing on osteogenic differentiation. Effective PARP-1 silencing resulted in a dramatic loss of cell viability in SAOS-2 cells, thus only partial (50%) silencing could be established.PARG silencing did not affect cell proliferation. PARP-1 and PARG silencing modulated extracellular calcium deposition, alkaline phosphatise activity and altered teh expression of osteogen marker genes. The role of PARP-1 is demonstrated in the regulation of several transcription factors, but its role in modulating Runx2 and other osteogenic transcription factors has not been investigated.

Based on the central role of PARylation in cell death pathways, we investigated if it influences differentiation associated cell death. PARG silencing decreased cell viability and shifted the form of cell death toward necrosis. As PARP-1 has a pro-survival effect in mild oxidative stress and mediate cell death in severe stress, we hypothesized that PARP-1

deficiency would cause a decrease in cell viability. Our data show that PARP-1 silencing prevented cell death in SAOS-2 cells, but PARP inhibition resulted in a decreased viability in MSCs, which suggests that the regulatory network is complex and not only PARP-1 activity but protein-protein interactions may modulate the cell death pathway.

The role of ROS in osteogenic differentiation was also confirmed in primary MSCs. We demonstrated that mineralization depended on hydrogen-peroxide production as indicated by the inhibitory effect of catalase. It was also shown that ROS are key mediators of differentiation associated cell death.

6. CONCLUSIONS

Regulatory role of poly(ADP-ribosyl)ation in cigarette smoke-induced cell death

1. Cigarette smoke has toxic effects on A549 cells.
2. A PARP-1 and PARG silencing sensitizes cells to the toxic effects of CSE.
3. PARP-1 and PARG silencing causes decreased efficiency of DNA repair.
4. CSE treatment induces ROS production in A549 cells
5. Intracellular ROS is a key mediator in CSE-induced cytotoxicity.

Hidrogén-peroxid által indukált poli-ADP-riboziláció szabályozó szerepe az osteogén differenciáció során bekövetkező sejthalálban

1. We isolated human placenta-derived stem cell that are capable of multilineage differentiation
2. Hidrogen-peroxide causes cell death during osteogenic differentiation.
3. Differentiation associated ROS production leads to PARP-1 activity.
4. PARylation modulates osteogenic differentiation associated cell death.

7. LIST OF PUBLICATIONS

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List of publications related to the dissertation

1. Robaszkiewicz, A., Erdélyi, K., **Kovács, K.**, Kovács, I., Bai, P., Rajnavölgyi, É., Virág, L.: Hydrogen peroxide-induced poly(ADP-ribosyl)ation regulates osteogenic differentiation-associated cell death.
   Free Radic. Biol. Med. 53 (8), 1552-1564, 2012.
   DOI: http://dx.doi.org/10.1016/j.freeradbiomed.2012.08.567
   IF:5.271

2. **Kovács, K.**, Erdélyi, K., Hegedűs, C., Lakatos, P., Regdon, Z., Bai, P., Haskó, G., Szabó, É., Virág, L.: Poly(ADP-ribosyl)ation is a survival mechanism in cigarette smoke-induced and hydrogen peroxide-mediated cell death.
   Free Radic. Biol. Med. 53 (9), 1680-1688, 2012.
   DOI: http://dx.doi.org/10.1016/j.freeradbiomed.2012.08.579
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List of other publications

3. El-Hamoly, T., Hegedűs, C., Lakatos, P., **Kovács, K.**, Bai, P., El-Ghazaly, M.A., El-Denshary, E.S., Szabó, É., Virág, L.: Activation of poly(ADP-ribose) polymerase-1 delays wound healing by regulating keratinocyte migration and production of inflammatory mediators.
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IF:4.824 (2013)

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J. Invest. Dermatol. 130 (11), 2629-2637, 2010.
DOI: http://dx.doi.org/10.1038/jid.2010.190
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Total IF of journals (all publications): 27,346
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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