Ph D. THESIS

ROLE OF POLY(ADP-RIBOSYL)ATION IN THE REGULATION OF DNA DAMAGE-INDUCED CELL DEATH AND INFLAMMATORY REACTIONS OF THE SKIN

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1. INTRODUCTION

1.1. Poly(ADP-ribose) metabolism

PARP-1 [poly(ADP-ribose)polymerase-1, EC 2.4.2.30] is the best known member of PARP enzyme family. It's molecular weight is 116 kDa. This enzyme is the most abundant nuclear protein, situated in nucleoplasm. During cell division it is located to the centrosome and chromosomes. The protein is expressed in most cell types and conserved among eukaryotes.

PARP-1 is mainly activated by single- and double-strand DNA breakage. It forms homodimers and binds to DNA nicks. Activated PARP uses NAD^+ as a substrate and cleaves it to ADP-ribose and nicotinamide. PARP connects ADP ribose units to glutamate side chains of different acceptor proteins, and forms branching poly(ADP-ribose) (PAR) polymers. Activated PARP is automodified resulting in autoinhibition. Polymers are degraded by poly(ADP-ribose) glycohydrolase (PARG), ADP-ribosyl hydrolase 3 (ARH3) and ADP-ribosyl protein lyase enzymes.

PARP-1 plays a role in maintaining genomic integrity, regulation of replication, gene expression, chromatin structure and differentiation, and in cell death.

1.2. Role of PARP-1 in cell death

Different cell death mechanisms can be distinguished mainly by morphologic characteristics: apoptosis, necrosis and autophagy. PARP activation was first linked to cytotoxic effects of DNA-damaging agents by Berger's group („PARP suicide" model). Their concept was, that PARP overactivation consumes the cellular NAD^+ content. The resynthesis of NAD^+ requires ATP, therefore cellular ATP content may also be depleted. ATP

depletion stalls ATP-dependent processes, NAD$^+$ depletion stalls mitochondrial electron-transport chain, that leads to collapse of the mitochondrial membrane potential. By these events cell dysfunction, eventually cell death occurs. Relation between poly(ADP-ribosyl)ation and necrotic cell death depends on cell type and metabolic status of the cells.

Alternatively, overactivation of PARP-1 leads to overproduction of poly(ADP-ribose) polymer, that induce translocation of the apoptosis inducing factor (AIF) from mitochondria to the nucleus. AIF in the nucleus causes DNA-fragmentation and cell death.

PARP is not required for most forms of apoptosis, but its cleavage is indispensable for the smooth course of apoptosis. PARP plays a role also in autophagy, in a cell type- and stimulus-dependent manner.

1.3. Major stimuli of poly(ADP-ribosy)ation

Fallowing stimuli may cause increased poly(ADP-ribosy)ation: UV radiation, radioactive radiation, topoisomerase inhibitors, oxidative stress and DNS alkylating agents.

Production of reactive oxigen/nitrogen intermediers was noticed in inflammatory processes, in irradiated cell- and tissue cultures, in ischemia-reperfused heart, liver, kidney and brain. These reactive intermediers can damage tissues in different ways: beside DNA-breakage, can inhibit mitochondrial electron-transport chain, cause lipid peroxidation, inhibit ion-channels and oxidize proteins.

Alkylating agents primarily attack nucleophyl groups of DNA. These agents can produce at least 12 kind of alkylated bases or phosphitriesters, most important is product is O^6-alkilguanin. These modifications can lead to defective base-pairing or DNA-breakage, can cause mutations, dysfunctions in

the processes of transcription, replication or recombination. Alkylating agents react with thiol groups of proteins, and so damaging their functions. In the last years many results suggest that MNNG is not only a laboratory test compound, it can be found in smoke and in well-cooked meat.

1.4. Role of protein kinases in regulation of PARP-1

Many kinases have been demonstrated to affect the activity of PARP-1 by protein-protein interactions or by phosphorylation. Phosphorylation can cause activation (ERK 1/2, JNK1, AMPK), or inhibition (PKC, DNA-PK) of PARP-1.

Protein kinase C enzyme family consists of serinr/threonine specific protein kinases, which play role in different signaling pathways of several cell types. Those have important role for example in proliferation, apoptosis, and cytoskeleton-remodeling. There are 12 PKC izoenzymes, which can be categorized in 3 subfamilies by their co-factor requirements. Izoforms are differ in their activation, in distribution among different tissues, and in substrate specificity. Inactive PKCs are located in cytoplasm, by activation they bind to membranes revesibly. For their activation - dependent upon subfamily - phosphatidyl-serine, diacyl-glycerol and Ca^{2+} are required.

It is known, that PKC *in vitro* phosphorylates PARP-1. Phosphorylation inhibits PARP activity and its DNA-binding ability.

1.5. Role of PARP-1 in inflammatory processes

Pathophysiologic role of PARP have been demonstrated in several inflammatory models: streptozotocin-induced diabetes, zymosan-induced vascular disorder, endotoxin-induced septic shock. PARP-1 enhances the activity of 2 trancription factors (NF-κB és az AP-1), which play key role in

expression of inflammatory mediators. Inhibition of PARP decreases granulocyte infiltration and expression of iNOS. PARP-1 reacts with both subunits of NF-κB, but only a part of NF-κB-dependent genes (iNOS, TNFα) are PARP-1-dependent. In other cases, PARP-1 can be replaced by other specific coactivators, probably in cell- and stimulus-dependent manner.

1.6. Possible role of PARP-activation in the pathophysiology of skin

It is demonstrated, that reactive oxigen/nitrogen intermediers play important role in development of several skin diseases (sunburn erythema, contact hypersensitivity, psoriasis). Nitrogen oxide (NO) plays role in physiologic processes in the skin, ranging from regulation of circulation to melanogenesis. However, in excess - combined with superoxide - can produce superoxide, that causes tissue injury in the processes of shock, inflammation and ischaemia-reperfusion. The cast of NO depends on how favourable the physiological status of the cell for production of peroxynitrite. Peroxinitrite causes DNA-breakage, that leads to PARP-activation. Overactivation of PARP results in cell death. In effector phase of contact hypersensitivity, in keratinocytes and in Langerhans cells, an enhanced expression of iNOS was detected. So it is probable, that NO contributes to tissue injury in CHS. Presence of NO is not in every cases negative for the organism. The production if NO in the course of wound healing facilitates the process, moreover slower wound healig was seen on iNOS deficient mice. Peroxynitrite can cause PARP activation in different inflammatory processes of the skin. PARP activation was detected also in CHS. Active PARP – by activating NF-κB - leads to production of inflammatory mediators, inhibition of PARP diminishes inflammation, hereby it is beneficial in non-infectous inflammatory processes, like sunburn erythema and CHS.

2. AIMS

Role of poly(ADP-ribosil)ation in MNNG-induced citotoxicity

1. Is MNNG cytotoxic in thymocytes?

2. How poly(ADP-ribosil)ation influence MNNG-induced citotoxicity?

3. Does second oxidative stress have role in cytotoxic effect of MNNG?

Role of protein kinase C in regulation of MNNG-induced citotoxicity

1. Does protein kinase C have role in regulation of MNNG-induced citotoxicity?

2. Does protein kinase C influence functions of PARP-1 enzyme?

Role of PARP-1 in inflammatory processes of contact hypersensitivity

1. Does PARP-1 activation have role in processes of CHS?

2. Does PARP-1 activation have role in irritant dermatitis?

3. MATERIELS AND METHODS

3.1. Thymocyte preparation

The thymus of 6–8 weeks old mice were used. The animals were sacrificed in CO_2, the chest was opened and thymus was excised. Single cell thymocyte suspension was prepared by pressing the thymus through a wire mesh. Thymocytes were maintained in RPMI-1640 medium, supplemented with 10% FCS at 37 °C, 5% CO_2.

3.2. Investigation if contact hypersensitivity

Animaly were sensitized by smearing 100 µl 2% (w/v) oxazolone onto the preshaved abdominal wall. Oxazolone was dissolved in acetone:olive oil 1:4 mixture. (The control group was mock sensitized with the vehicle.) One week after sensitization mice were treated with i.p. injection with PJ34 (10 mg/kg) prior to the application of 4x10 µl 0.5% (w/v) oxazolone onto the inner and outer surface of the ear on both sides. After 24 hours, thickness of ears was measured and tissue samples have been taken for laboratory investigations.

3.3. Investigation of irritant dermatitis

PMA (10 µl 0,05% w/v) was smeared onto both sides of the ears of female mice immediately followed by PJ34 treatment as described above. Six hours, thickness of ears was measured and tissue samples have been taken for laboratory investigations.

3.4. Measurement of NO release

NO was determined with the fluorimetric DAF-2 assay. Fluorescence was determined at 485 nm (excitation) and 527 nm (emission). Production of nitric oxide was also determined by the measurement of nitrite and nitrate, the stable endproducts of NO in aqueous solutions. Nitrite + nitrate concentration was measured with the Griess-Ilosvay reaction. The optical density was measured at 550 nm.

3.5. Detection of tyrosine nitration by immunofluorescence

Cells were fixed in ice-cold 95% ethanol and then rehydrated in PBS. Coverslips were blocked in 5% goat serum and were then incubated overnight with polyclonal anti-nitrotyrosine antibody diluted 1:300. Coverslips were incubated with biotinylated goat anti-rabbit IgG diluted 1:200 for 1 h at room temperature. Bound antibody was detected by streptavidine-AlexaFluor 488 conjugate and was visualized by fluorescence microscopy.

3.6. Western blot

Detection of tyrosine nitration, iNOS expression, poly(ADP-ribose) accumulation, determination of PARP phosphorylation (in IP samples) and identification of PKC isoforms was performed by Western blot. Primer antibodies was dissolved in TBST, containing 1% low fat dry milk. Membranes were incubated overnight at 4 °C, followed by incubation with the peroxidase-conjugated secondary antibodies (1h). Detection was done with ECLWestern-blotting detection system.

3.7. Single cell gel electrophoresis (comet assay)

Single stranded DNA strand breaks were assayed by single cell gel electrophoresis (comet assay). Broken DNA unwinds under alkalic conditions and forms cometlike structures after cell lysis and electrophoresis. Cells (10,000/slide) were embedded into low melting point agarose and were plated onto the agarose-covered slides. Cells were lysed, than electrophoresis was performed. Samples were stained with ethidium bromide (10 μg/ml). Comets were analyzed by fluorescent microscopy. A visual score system was set up for analysis.

3.8. PARP activity assay

PARP activity of cell lysates has been determined with an assay based on the incorporation of isotope from ^{3}H-NAD$^+$ into TCA (trichloroacetic acid) precipitable proteins. After 20 min MNNG treatment, medium was replaced by 0.5 ml assay buffer, that contained 0,5 μCi/ml ^{3}H-NAD$^+$. Samples were incubated for 30 min at 37 °C. Cells were then collected and ice-cold TCA 50% (w/v) was added. The pellets were solubilized in 2% (w/v) SDS/0.1N NaOH at 37 °C. Radioactivity was determined using a liquid scintillation counter.

3.9. Detection of mitochondrial depolarization and superoxide production

The mitochondrial membrane potential and mitochondrial superoxide production was quantified by the flow cytometric analysis, of cells stained with 3,3-dihexyloxacarbocyanineiodide [DiOC6(3)] and hydroethidium.

3.10. Caspase activity assay

Six hours after MNNG exposure, thymocytes were lysed. Lysates were combined with assay buffer containing the aminomethylcoumarin (AMC)-conjugated tetrapeptide caspase substrate (DEVD-AMC) in fanal concentration of 50 μM, and incubated for 1 h. Fluorescence of AMC was measured (EX:390 nm, EM: 460 nm).

3.11. Detection of DNA fragmentation (DNA laddering)

Internucleosomal DNA fragmentation was visualized by agarose gel electrophoresis. Agarose (2%) was poured on a horizontal gel support. After solidification of the gel, the top of the gel (above the comb) was removed and replaced with 1% (w/v) agarose. Cells were loaded in sample buffer, after electrophoresis, gel was stained with 2 μg/ml ethidium bromide.

3.12. Cytotoxicity assay (propidium iodide uptake)

MNNG-induced cytotoxicity was measured by propidium iodide uptake. Cells were then stained with 2,5 μg/ml propidium iodide solution for 15 min, washed with PBS once and analyzed by flow cytometry. Cytotoxicity was calculated as $100 \times (T-C)/(100-C)$, where $T = \%$ of the PI positive cells in the samples, the "C" values means the corresponding value of the control samples.

3.13. Cytotoxicity assay (MTT assay)

Cytotoxicity has also been determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Treatments were carried out in Eppendorf tubes and cells were spun down before removal of the medium and addition of dimethylsulfoxide (DMSO).

3.14. Immunoprecipitation

PARP-1 phosphorylation was detected by immunoprecipitation. Cells were lysed, than sonicated. Samples were precleared with sepharose-protein-A slurry. Immunoprecipitation were performed with anti-PARP antibody and sepharose-protein-A, followed by anti-phosphoserine Western blot.

3.15. PKC izoformák lokalizációjának vizsgálata immunfluoreszcenciával

Cells were immobilised on poly-lysine coated coverslips. After ethanol fixation same antibodies were used than in Western blot method. FITC-conjugated secondary antibody was used for immunofluorescent labeling. Nuclei were stained with DAPI. Images were taken with Zeiss LSM 510 META confocal microscope.

3.16. In vitro phosphorylation

Purified PARP-1 enzyme was phosphorylated by purified cPKC mixture (alpha, beta, gamma isoforms) in HEPES assay buffer. Final concentration of PKC mixture was 0.1 µg/ml and was activated by the addition of $CaCl_2$ and phosphatidyl serine–diolein micellas. ATP mixture (5 µl) containing 0.988 mM ATP and 20-fold diluted ^{32}P-ATP (270 CPM/pmol) were added to the samples. Following SDS-polyacrylamide gel electrophoresis, ^{32}P signals were detected by autoradiography.

3.17. Metalloproteinase zymography

Tissue samples were homogenized in TNC buffer. Protein concentrations were determined by Bradford method. Homogenizates were mixed with 2X SDS sample buffer, than 7,5 µg protein was applied to

zymography gels. After running, gels were renatured, and developed overnight at 37 °C. Undigested substrate was stained with Brilliant Blue.

3.18. Peroxidáz aktivitás mérése

Six micrometer thick sections embedded in Cryochrome Blue matrix were used. The reaction was developed for 10 minutes at room temperature. Slides were counterstained with Nuclear Fast Red for 1 min at room temperature. Slides were washed in water, dehydrated and mounted.

3.19. Investigation of cytokine expression with ELISA method

Cytokines (TNFα, IL-1β) and chemokines (MIP-1α, MIP2) were determined from ear lysates by using commercially available (R&D System, Minneapolis, MN) ELISA kits. Protocols of the kits was followed.

3.20. Measurement of myeloperoxidase activity

After homogenizing ears, measurement of myeloperoxidase activity was performed in the presence of 1 mM hidrogen-peroxide, with TMB substrate. MPO activity was detected by photometry, at 650 nm.

3.21. Statistical analysis

All experiments were preformed three times on different days. Student's t-test was applied for statistical analysis and for the determination of significance with $p < 0.05$ considered as significant. For the statistical analysis of the comet assay experiments, Mann and Whitney's U-test was applied.

4. RESULTS AND DISCUSSION

4.1. Investigation of MNNG-induced tyrosine nitration and PARP activation

4.1.1. Investigation of MNNG-induced cell death and PARP-activation

In the case of other cell types, it was known, that MNNG activates DNA-breakage – PARP-activation pathway, so we investigated if MNNG has similar effects on thymocytes. Notable necrotic cell death was caused by MNNG, even at relatively low concentrations, this effect could be blocked by PARP inhibitors or thiol antioxidants.

Inhibition of PARP decreased necrotic cell death, but simultanously increased apoptosis. At low MNNG concentration, elevated caspase activity and internucleosomal DNA fragmentation was measured, in contrast at higher MNNG concentrations both parameters were diminished. Pretreatment with PJ34 PARP inhibitor was counterbalanced the effect of MNNG. PARP activation was also caused by MNNG, that could be inhibited by thiol antioxidants and PARP inhibitor as well.

4.1.2. Investigation of DNA-breakage and mitochondrial parameters in MNNG-treated thymocytes

Applied antioxidants provided provided protection against MNNG-induced DNA-breakage, whereas DNA-damage wasn't influenced PARP inhibitor.

By our previous results, peroxynitrite - or hydrogen-peroxide - induced necrotic cell death is followed by mitochondrial alterations, like mitochondrial membrane depolarisation, secondary superoxide production and structural damage of mitochondria. It is also proven in thymocytes, that these alterations are caused by PARP-activation. As an effect of MNNG-treatment, similar

mitochondrial alterations was seen, like earlier in oxidative stressed thymocytes.

4.1.3. Investigation of the effects of NO, released from MNNG

It was proved by several observations, that reactive intermediers may be produced during the decomposition of MNNG. MNNG contains a nitroso group, so it is pissible that nitrogen oxide may be produced during decomposition of MNNG, like from organic nitroso compounds. Concentration-dependent nitrite-/nitrate-production was found in aqueous solition of MNNG, that indicates NO release from MNNG. Results were confirmed with NO-specific fluorogen probe DAF-2. By these data, it was supposed, that MNNG releases NO, which can produce peroxynitrite by combinating with superoxide in MNNG-treated thymocytes.

Peroxynitrite can nitrate tyrosine and triptophane residues of proteins and guanine bases of DNA. Strong immunopositivity was shown for nitrotyrosine in MNNG-treated thymocytes, which was dose- and time-dependent. Generated peroxynitrite - at least in part - can be responsible for cytotoxic effect of MNNG.

4.1.4. Effect of depletion of intracellular and extracellular GSH to MNNG-induced cytotoxicity

In our experiments, significant protection was provided against cytotoxic effect of MNNG by depletion of intracellular glutathione, that indicates that GSH/GST system serves bioactivation of MNNG in observed cells. Extracellular thiols are probably detoxify MNNG extracellularly. This is confirmed by our results, that was seen when thiols were wased out prior to MNNG-treatment.

4.1.5. Investigation of the role of superoxide, nitrogen oxide and peroxynitrite in cytotoxic effect of MNNG

By our results, it is possible, that PARP-activtion plays role in cytotoxic effect of both peroxynitrite and MNNG. So we examined if peroxynitrite plays role in cytotoxic effect of MNNG. Protective effect of thiol antioxidants against MNNG-induced DNA-breakage and cytotoxicity agrees with our hypothesis. However, GSH and NAC could also scavenge alkyl radicals in addition to reactive oxygen or nitrogen intermediates (ROI and RNI, respectively). Therefore, we have also investigated the effects of ROI/RNI specific inhibitors. MNNG-induced DNA strand breaks and cytotoxicity were not inhibited by cellpermeable superoxide dismutase, catalase, cPTIO (NO scavenger), L-NMMA (NOS inhibitor) and FP15 (peroxynitrite decomposition catalysts. These data indicate that neither NO nor superoxide or their reaction product peroxynitrite are involved in MNNG-induced cytotoxicity. NO, reactive oxygen species and peroxynitrite are formed in MNNG-treated cells but do not contribute to MNNG-induced DNA-damage and cell death in thymocytes.

4.2. Role of protein kinase C in regulation of cytotoxic effect of MNNG

It is known, that PARP can be a substrate of many kinases (AMPK, DNA-PK, JNK, PKC). PKC is able phosphorylate PARP-1 *in vitro*.

Expression of PKC isoformes (α, β_1, β_2, δ, ϵ, ζ, θ, μ) was shown with Western blot. *In vitro* phorsphorylation of PARP-1 by PKC was also confirmed.

4.2.1. Effect of PKC to MNNG-induced PARP-activation

As an effect of MNNG-treatment, notable PARP-activation was found in thymocytes, that could be inhibited by PARP-inhibitor. That agrees with our previous results. Inhibition of PARP was also seen as an effect of PKC activator PMA pretreatment. Accordantly, significant decrease of ADP-ribose polymers was also seen in PMA-treated cells. PARP-inhibiting effect of PMA was counterbalanced with PKC inhibitors.

4.2.2. Effect of PKC activation to MNNG-induced cytotoxicity

PKC-activators and inhibitors are influence cytotoxicity as well. Inhibition of PARP-activity by PMA-treatment – like PARP inhibitors – protects against necrotic cell death, while shifting the cell death pathways towards the apoptotic route. Effect of PMA could be decreased by used PKC inhibitors (GF109203X, Gö6976). Similar effects have been obtained with PMA stimulation in splenocytes representing a mature lymphoid population, but not in Jurkat T lymphoid cell line or adherent cell lines (A549 lung epithelial cells, HaCaT keratinocytes), indicating that our findings can be extended to more mature lymphoid cells but not to transformed cell lines.

4.2.3. Effect of PKC-activation to MNNG-induced DNA-breakage

Neither PMA nor PKC inhibitors had any effect on MNNG-induced DNA breakage. These results confirm our surmice, that PKC influences PARP activation itself, not the processes that lead to PARP-activation.

4.2.4. Investigation of phosphorylation of PARP-1 through PKC

Our findings, demonstrating that in a cell-based system PARP-1 is phosphorylated by PKC, and its activity is thereby reduced, and this reduction

can confer cytoprotection.   Our results can be viewed in the context of a multitude of published reports in the area of PKC, PARP-1 and cell death. It has been documented in multiple prior studies that cell death induced by various stimuli including oxidants or pro-inflammatory cytokines has been shown to be suppressed by PKC activating phorbol esters. In addition, overexpression of PKC suppresses cell death and PKC inhibitors potentiate cell death in various systems. PKC has also been shown to mediate resistance to myocardial infarction, a pathophysiological condition in which myocardial necrosis is mediated in part by PARP overactivation.

It may be interesting to note here that PKC has also been shown to activate PARP-1 in fibroblasts and human monocytes. Although several possible explanations could be given for this seeming contradiction with our current data, we propose an alternative explanation. PMA may cause a low level of PARP-1 activation leading to PARP-1 automodification which is known to inhibit the activity of the enzyme. In PMA-pretreated cells, MNNG-induced massive DNA breakage can thus only trigger a weak PARP activation which is not sufficient to induce necrosis. Whether or not this may be the case requires further investigation.

4.2.5. *Effect of indirect stimulation of PKC pathway to PARP-activity*

Indirect stimulation of the PKC signaling pathway, e.g. in activated thymocytes or splenocytes may also lead to PARP inhibition as indicated by our finding that stimulation of cells caused an inhibition of MNNG-induced PARP activation. Moreover, immunofluorescent staining for conventional PKC isoforms revealed that PKC β_1 but not other isoforms (α, $\beta2$, δ, ϵ, ζ, θ, μ) translocates into the nucleus following concanavalin A (conA) stimulation.

These data raise the possibility that PKC β_1 may mediate inhibition of PARP-1 in activated thymocytes and splenocytes.

Our study provides direct evidence for the regulation of cellular PARP-1 activity by PKC in intact thymocytes. In addition, the current study is the first to implicate the modulatory role of PKC activators and inhibitors in the process of MNNG-induced cell necrosis. Based on the data presented in the current report, we propose that PKC, (at least in part) via modulation of endogenous cellular PARP-1 activity, plays a significant role in modulating cell death in thymocytes.

4.3. *The role of PARP-1 in inflammatory processes of contact hypersensitivity*

Contact hypersensitivity (CHS) is a form of delayed type of hypersensitivity. The CHS reaction can be divided in two phases: sensitization and elicitation. The elicitation phase is dominated by the production of proinflammatory cytokines and cellular infiltration by lymphocytes and granulocytes. Infiltration is accompanied by strong oxidative stress due to the formation of superoxide, nitrogen-monoxide, peroxynitrite and further reactive species. Inhibition of PARP activity or knocking out the PARP-1 gene has been shown to suppress inflammatory reactions such as colitis, arthritis and uveitis. The aim of the current study was to investigate whether PARP activation contributes to CHS.

4.3.1. Role of PARP-inhibition in neutrophyl infiltration

PARP inhibition by PJ34 prevented edema indicating a lower level of inflammation. Reactive oxygen and nitrogen intermediates (ROI and RNI, respectively) are produced in large quantity in inflammatory conditions including CHS. ROI and RNI can stimulate the DNA breakage-PARP-1 activation pathway that has been shown to mediate endothelial dysfunction in other experimental settings such as in diabetic blood vessels. As most ROI/RNI are likely produced by the infiltrating granulocytes, inhibition of granulocyte migration from the circulation to the site of inflammation may result in suppressed ROI/RNI production. Indeed our results indicate that PARP inhibition markedly inhibits the extravasation of granulocytes as indicated by peroxidase staining and MPO activity measurements of ear lysates.

4.3.2. Cytokine expression and MMP-activation in CHS

PARP1 - as the coactivator of NFκB - regulates the expression of cytokines in different inflammatory models. Chemokines/cytokines such as MIP-1α, MIP-2, TNFα and IL-1β were greatly induced in CHS. Chemokine induction was prevented by treatment of the mice with PJ34.

Matrix metalloproteinases (MMPs) are now considered as inflammatory mediators as indicated by the anti-inflammatory effects of MMP inhibitors as demonstrated in various models of inflammation including CHS. When secreted and activated, MMP9 plays an important role in the facilitation of the movement of the infiltrating immune cells through the protein mesh of the ECM. The *de novo* expression of the MMPs can be stimulated by different chemokines, such as TNFα or Il-1β, and MMP9 can be also be synthesized by a number of cell types in the inflamed skin. The MMP activity is

counterbalanced by a family of tissue inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). In samples from sensitized mice increase of expression and activity of MMP was found, which was decreased as an effect of PJ-34 treatment. I case of TIMP2, contradictory results was seen. As MMP expression is under the control of NF-κB, inhibition of MMP9 expression by PJ34 may be explained by the same mechanism as outlined above for inflammatory cytokines and chemokines.

In summary, our data demonstrate that PARP activity mediates inflammation in CHS. PARP acts as a pleiotropic modulator of the inflammatory reaction by enhancing leukocyte migration, production of inflammatory cytokines and chemokines and the activation of MMPs.

4.3.3. Role of PARP-activation in irritant dermatitis

Epicutaneously applied haptens may also induce non-antigen-specific irritant dermatitis. In order to see whether PARP inhibition also suppresses irritant dermatitis we have determined the effect of PJ34 on PMA-induced ear swelling. PMA induced a less severe inflammation as compared to oxazolone and the PARP inhibitor significantly reduced ear swelling and inhibited cellular infiltration, indicating that it targets general inflammatory pathways and the specific immune response as well.

5. CONCLUSIONS

Investigation of the role of poly(ADP-ribosyl)ation in MNNG-induced cytotoxicity

1.   MNNG is cytotoxic to thymocytes.
2.   A PARP enzimnek átkapcsoló szerepe van az MNNG által kiváltott sejthalálban.
3.   Thiol antioxidants provide protection against MNNG-induced cytotoxicity, DNA-breakage and PARP-activation.
4.   NO is released during the decomposition of MNNG
5.   NO is combined with superoxide to peroxynitrite, which may cause tyrosine-nitration
6.   Neither NO and superoxide, nor peroxynitrite play role in cytotoxic effect of MNNG.

Investigation of the role of protein kinase C in regulation of MNNG-induced cytotoxiciy

1.   PKC phosphorylates PARP-1 *in vitro* and *in vivo*
2.   Protein kinase C inhibits PARP-1 in mouse thymocytes.
3.   Activation of protein kinase C decreases cytotoxic effect of MNNG by inhibition of PARP-1.

Investigation of the role of PARP-1 in inflamatory proceses of contact hypersensitivity.

1.   PARP plays role in the processes of edema-formation, neutrophyl-infiltration, cytokine expression and MMP activation during CHS.

2. PARP influences swelling and neutrophyl infiltration also in irritant dermatitis. PARP targets general inflammatory pathways and the specific immune response as well.

6. PUBLICATIONS

Publications the Ph.D. thesis is based on:

Csaba Hegedűs, Petra Lakatos, Gábor Oláh, Balázs Tóth, István Kovács, Szabolcs Gergely, Éva Szabó, Tamás Bíró, Csaba Szabó, László Virág (2008): Protein kinase C protects from DNA damage-induced necrotic cell death by inhibiting poly(ADP-ribose) polymerase-1 *FEBS Letters FEBS Lett.* **582(12)**:1672-1678.

Bai P*, **Hegedűs Cs***, Erdélyi K, Szabó E, Bakondi E, Gergely Sz, Szabó Cs, Virág L (2007): Protein tyrosine nitration and poly(ADP-ribose) polymerase activation in N-methyl-N-nitro-N-nitrosoguanidine-treated thymocytes: implication for cytotoxicity. *Toxicol Lett.* 170 (2007) 203–213 IF.: 2.43 ***These authors contributed equally to the work.**

Bai P*, **Hegedűs Cs***, Szabó É*, Gyüre L, Bakondi E, Brunyánszki A, Gergely S, Szabó C and Virág L (2008): Poly(ADP-ribose) polymerase mediates inflammation in a mouse model of contact hypersensitivity. *J. Invest. Dermatol.* 2008 Jul 17. PMID: 18633442 (in press) ***These authors contributed equally to the work.**

Conferences (lectures):

Bai P., Erdélyi K., Bakondi E., **Hegedűs Cs**., Gergely P., Szabó Cs., Virág L.: Possible role of peroxynitrite in N-methyl-N'-nitro-N-nitrosoguanidine

(MNNG) induced citotoxicity (Signal Transduction Conferense of Hungarian Biocamical Society, Sopron, 2004)

Bai, P., Erdélyi, K., Bakondi, E., **Hegedűs, C.,** Gergely, P., Szabó, C., Virág, L.: Potential role of peroxynitrite in the poly(ADP-ribose) polymerase-mediated cytotoxicity caused by the alkylating agent N-methyl-N-nitro-N-nitrosoguanidine in thymocytes. (Poly[ADP-ribosyl]ation in Health and Disease, FEBS Advanced Course, Debrecen, 2003)

Other lectures:

Hegedűs Csaba, Lakatos Petra, Kovács Katalin, Kovács István, Kónya Krisztina, Pazurik István, Patonay Tamás, Gergely Pál, Virág László: Identification of cytoprotective and antioxidant components by HTS (high throughput screening) technique (38th Membrane Transport Conference, Sümeg, Hungary, 2008)

Posters:

Hegedűs Csaba, Lakatos Petra, Bai Péter, Kovács István, Virág László: Role of protein kinase C (PKC) and poly(ADP-ribose) polymerase 1 in regulation of MNNG-induced citotoxicity (Conference of Hungarian Biochemical Society (HBS), Szeged, Hungary, 2008)

Éva Szabó, Péter Bai, **Csaba Hegedűs** and László Virág (2008): Poly(ADP-ribose) polymerase mediates inflammation in a mouse model of contact hypersensitivity (Common Conference of German-Hungarian Dermatological Societies, Budapest, Hungary, 2008)

Hegedus C., Gergely S., Bai P., Erdelyi K., Virag L.: Modulating effect of protein kinase C (PKC) on N-methyl-N'-nitro-N-nitrosoguanine (MNNG) -

induced poly(ADP-ribose) polymerase (PARP) -dependent citotoxicity (Conference of HBS, Pécs, Hungary 2006)

Hegedus C., Gergely S., Bai P., Erdelyi K., Virag L.: Role of protein kinase C (PKC) and poly(ADP-ribose) polymerase 1 in regulation of MNNG induced citotoxicity (Cell-analytical Conference, Budapest, Hungary 2006)

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Other posters

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