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

The regulatory role of protein phosphatases in the chemosensitivity of leukemic cells and pathological processes of keratinocytes

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE
DEBRECEN, 2015

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The Examination takes place at the library of The Department of Physiology, Faculty of Medicine, University of Debrecen at 11: am, 17th February, 2015

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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 13:00, 17th February, 2015

INTRODUCTION

The most common form of the post-translational covalent modification of proteins is the reversible phosphorylation which is catalyzed by protein kinases and phosphatases. In eukaryotic cells, the modification of serine/threonine (Ser/Thr) residues are the most common (more than 90 % of all phosphorylation), which is catalyzed by specific protein kinases and phosphatases and the phosphorylation level of the given protein is balanced by the activities of these enzyme pairs. It has become a widely accepted fact that the regulation of protein kinases and phosphatases has basic roles in changes of the extent of the phosphorylation of substrate proteins. All these findings have turned the attention to the regulatory role of protein phosphatases, mainly focusing on the phospho-Ser/Thr specific protein phosphatase-1 (PP1) and 2A (PP2A) responsible for more than 90 % of the dephosphorylation processes in the cells.

Structure and regulation of PP1 and PP2A

PP2A plays important roles in apoptosis and cell proliferation, numerous signaling pathways, regulation of cell cycle, cytoskeletal arrangement and it is identified as tumor supressors as well. PP2A consists of a dimer molecule composed of a 36 kDa „C" catalytic subunit ($PP2A_c$) and a 65 kDa „A" regulatory subunit (PR65). These two subunit create the core enzyme, which can associate with different variants of „B" subunit forming the trimer PP2A holoenzyme. All subunits (A, B and C) have at least two isoforms, which could be cell- and tissue-specific and these features also contribute to the structural and functional diversities of PP2A.

PP1 can be found in all eukaryotic cells and it plays roles in cell proliferation, apoptosis, protein synthesis, metabolism, cytoskeletal arrangement and regulation of membrane receptors, which indicate the notable importance of PP1. PP1 exists in

holoenzyme forms: its catalytic subunit (PP1c) binds to a great variety of regulatory subunits. Although the regulatory subunits differ from each other by remarkable degree, almost all of them contain a short PP1c binding-motif (K/R-$_{x1}$-V/I-$_{x2}$-F/W), briefly designated as RVxF. In mammals, the five isoforms (PP1cα_1, PP1cα_2, PP1cδ, PP1cγ_1, PP1cγ_2) of the 35-38 kDa PP1c are the products of three genes. The active centre lies at the intersection of the Y-shaped catalytic grooves in the N-terminal region and contains Mn^{2+} and Fe^{2+}as essential metal ions required to the phosphate hydrolyses. The metal ions are coordinated by three histidine-, two asparagine- and one aspartate residues. The metal ions in the active centre activate a structural water molecule in order to facilitate the hydrolysis of the phospho-Ser/Thr side chain of the substrate.

The catalytic activity of PP1 and PP2A enzymes can be inhibited by membrane permeable toxins (microcystin, cantharidine, okadaic acid, calyculin-A, tautomycin) many of them produced by microorganisms. Their advantage is that they are able to selectively inhibit the PP1 and PP2A enzymes in a concentration-dependent manner and inhibit PP2B only in high concentration. These toxins bind to the hydrophobic groove and partially cover the catalytic centre, preventing the binding and the hydrolysis of the phospho-substrate.

Besides the toxins, there are small (14-32 kDa) heat-stable inhibitor proteins like inhibitor-1, inhibitor-2, DARPP-32, CPI-17, PHI-1 and the KEPI, which are also the center of interest regarding the physiological regulation of PP1 activity. The KEPI, like other inhibitor proteins (inhibitor-1, DARPP-32, CPI-17, PHI-1), is phosphorylation-dependent and its inhibitory ability is activated when the Thr73 residue is phosphorylated by PKC or ILK kinases. Since KEPI belongs to the CPI-17 family, their sequence around the inhibitory threonine residue (CPI-17: A^{35}RV**T**VKYDRKE^{45}; KEPI: G^{70}KV**T**VKYDRKE^{80}) shows highly conserved sequence homology. The most important difference is that in contrast to CPI-17 the

KEPI includes an RVxF PP1c binding-motif at the N-terminal region, therefore it is able to bind the PP1c even in its dephosphorylated form.

It was previously proved that miosin phosphatase (MP) holoenzyme, containing PP1c and MYPT regulatory subunits, could dephosphorylate and therefore influence the retinoblastoma protein (pRb) phosphorylation level and function in leukemic cells. These findings have raised the question whether MP inhibitory proteins (like CPI-17 or KEPI) have any role in the regulation of phospho-pRb level, thereby in the control of cell proliferation/apoptosis.

Function and structure of retinoblastoma protein (pRb)

During cell proliferation, the cyclin dependent kinases together with their cyclin partners are able to regulate the a G_1/S and G_2/M phases of cell cycle by phosphorylation of distinct proteins. In these processes the pRb is one of the most essential substrate proteins, whose cell cycle-dependent phosphorylation/dephosphorylation at Ser/Thr residues are necessary for cell proliferation. By the binding of E2F, which is animportant partner of pRb, pRb has a crucial role in the regulation of transcription, DNA replication, cell cycle regulation and maintenance of proleferation/apoptosis balance. The binding ability of pRb to other proteins (E2F1, LxCxE-motif containing proteins) depends on its phosphorylated state as well. E2F is bound to hypophosphorylated pRb and released when pRb is hyperphosphorylated. The phosphorylation of pRb is driven by cyclin D/CDK4 complex in the G_1 phase, by cyclin E/CDK2 in the G_1/S phase and by cyclin A/CDK2 in the S phase. The maximal pRb phosphorylation occurs in the G_2/M phase. Phosphorylation of some amino acids has essential role in the liberation and binding of E2F1. The phosphorylation of Thr373 residue induces the N-terminal and small pocket region interaction, while the phosphorylation of Ser608 and Ser612 residues generates the N-terminal and E2F transactivation domain interaction. By the

phosphorylation of Thr821 and Thr826 amino acids, the binding of E2F1 and the LxCxE containing proteins can be inhibited. The dephosphorylation can be catalyzed by both PP1 and PP2A. PP2A contributes indirectly to the dephosphorylation of pRb by the reduction of the activity of cyclin dependent kinases. Despite the fact that all PP1c isoforms can play a role in the dephosphorylation, it may be hypothesized that PP1c isoforms regulate the pRb dephosphorylation in holoenzyme form (like MP for example). In our laboratory it was previously shown that MP could dephosphorylate the pRb at the Thr826 residue. PP1c containing holoenzymes can be regulated by inhibitor toxins or inhibitor proteins, therefore one of our aims is to investigate the possible role of KEPI protein in the control of pRb phosphoylation.

The effect of ultraviolet radiation in malignant skin dieases

Ultraviolet radiation is composed of three wavelengths*:* UVA, UVB and UVC. UV wavelengths of 320-400 nm, called UVA is the closest to the visible spectrum, then comes the UV wavelengths of 280-320 nm, called UVB and finally UV wavelengths of 200-280 nm, called UVC. UVC would be the most dangerous type of ultraviolet radiation but it is almost completely filtered out by the Earth's atmosphere. UVA and UVB have diverse biological effects, since UVA induces apoptosis with liberating reactive oxigen species (ROS) and inducing slight DNA damages, while UVB generates severe DNA damages, pyrimidine-dimers and photo-products.

The melanin, produced by melanocytes in the epidermal layer, is the first defense against the UV rays and protects the skin from the damages by absorbing UV light. Keratinocytes of ectodermal origin represent 90 % of the epidermal cells and produce keratin, therefore they are responsible for the barrier function of the skin, prevent water and heat loss, and also have roles in the defense against infections and UV radiation. Molecules (endothelin, granulocyte-macrophage colony-stimulating

factor (GM-CSF), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF)) produced by keratinocytes are involved in the UV-induced protection as well. These molecules are able to bind to their receptors and activate the MAPK/ERK and PI3/AKT signaling pathways and thereby they play role in the maintenance of apoptosis and in promoting cell survival. Protein phosphatases regulate these signaling pathways as well but only PP2A and PP2B have been studied in details in the UV-induced apoptosis, in the formation of barrier function of epidermis and in the epidermis differentiation. Since PP1 is less investigated in these processes, our aim is to study the role and function of PP1 in UV-induced skin diseases.

AIMS

Many studies are concerned with describing and understanding how protein phosphorylations influence the pathological processes in different organs and cells as well as the proliferation/apoptosis balance. Although it is still not known which phosphatases are involved in the dephosphorylation of given proteins, PP1 and PP2A regulate numerous proteins which have roles in cell survival. However, these data are also in conflict with the general view that the inhibition of phosphatases may result in survival or apoptosis depending upon the treatments of the cells. Our laboratory has already shown that the PP1-type myosin phosphatase holeonezyme (MP) regulates the dephosphorylation of retinoblastoma protein (pRb), therefore, PP1 inhibition influences the cell cycle and apoptosis. PP1 can be inhibited by membrane permeable toxins or inhibitor proteins. In this study, we tried to distinguish the different roles of PP1 and PP2A in these cellular processes using PP1 and PP2A specific toxins. We also intended to investigate the role of the lesser known endogenous PP1 inhibitor proteins, termed KEPI, in pRb dephosphorylation. Protein phosphatases play important roles in the regulation of UV-radiation induced DNA damages, as well as in other pathological processes in keratinocytes and it is not known yet in details how PP1 may impact these processes.

Based on the above questions our research objectives have focused on studying the following issues:

1. Investigation of the effect of membrane permeable phosphatase-type specific inhibitor toxins on the apoptosis induced by chemotherapeautic drugs in leukemic cells.
2. Determination of the role of KEPI phosphatase inhibitor protein in the control of the phosphorylation level of pRb.

3. Investigation of the role of PP1 in UVA irradiation-induced pathological alterations in HaCaT cells and in mouse skin

MATERIAL AND METHODS

Cell cultures, treatment and lysis

THP-1 human monocytic leukaemia cells or KG-1 human myeloid leukaemia cells were grown in RPMI-1640, MCF-7 breast cancer cells and HeLa human cervical cancer cells were grown in MEM, tsa201 embryonal kidney cells and HaCaT keratinocyte cells were grown in DMEM supplemented with 10 % heat-inactivated FBS and 2 mM L-glutamine. NHEK normal human epidermal keratinocyte were maintained in Epilife medium complemented with Human Keratinocyte Growth Supplement (HKGS), 100 µg/ml streptomycin, 100 IU/ml penicilin and 50 ng/ml amphotericin B and were cultured on 1% collagen-coated plates. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95 % air.

Prior to treatments, THP-1 and KG-1 cells (~$5x10^6$/ml) were incubated in serum-free medium for 16 h, then cells were treated with 50 nM CLA or 1 µM TM for 1 hour in serum-free media with or without 2 µg/mL DNR for 6 hours. After the treatment, the THP-1 cells were collected by centrifugation (800 *g*, 3 min) and washed with ice-cold PBS and the supernatant was eliminated.

MCF-7 cells were serum starved for 16 hours after FLAG-KEPI transfection then cells were treated with 50 nM CLA (1 hour) or 100 nM PMA (30 min). After the treatment, cells were washed with PBS.

THP-1 and MCF-7 cells were lysed in 100 µL RIPA lysis buffer (50 mm Tris-HCl (pH7,4), 150 mM NaCl, 1 % Triton X-100) supplemented with 0.5% protease inhibitor mix and 0.1 µM microcystine-LR. After a washing step with PBS, cell lysis was accelerated by ultrasonic treatment for 10 min and cell debris was removed by centrifugation (4 °C, 16000 *g*, 10 min). Lysates were boiled at 100°C for 10 min with 5x SDS sample buffer.

NHEK and HaCaT cells were serum starved for 16 hours after siPP1c silencing and before TM treatment, then the cells were irradiated by 10 J/cm^2 UVA. In the case of protein phosphatase activity measurements cells were lysed in ice-cold 0.1 M Tris–HCl (pH 7.6), 150 mM NaCl (TBS) containing 0.1 mM EDTA, and then collected in 100 µl ice-cold TBS containing 0.1 mM EDTA supplemented with 0.5% protease inhibitor cocktail and 50 mM 2-mercaptoethanol and assayed right after TM treatment or UV irradiation. After 24 hour incubation cells were either lysed for immunoblotting, microarray analysis or were fixed for immunofluorescent staining.

In vitro drug sensitivity assay

In vitro drug resistance assays were assessed using a 2-day cell cultures on microtiter plates (384-well). Each well was loaded with 30 µL cell suspension containing 1000 cells. THP-1 or KG-1 cells were preincubated initially with 50 nM calyculin A or 1 µM tautomycin and were washed with PBS after one hour. The final concentrations of DNR were 0.15 µM, 0.61 µM, 1.84 µM and 7.38 µM and the cells were incubated for 48 hours. The live and dead cells were differentially stained using fluorescent VitalDye. 15 control wells, that were used to determine the control cell survival, contained cells with only culture medium and 50 nL solvent (DMSO) without drugs, 5 wells contained cells with culture medium alone. The precise number of living cells was determined using a custom built automated laser confocal fluorescent microscope (a modified Perkin-Elmer UltraView LCI) at the Karolinska Institute visualization core facility (KIVIF). The images were captured using the computer program QuantCapture 4.0 whereas the living cells were identified and individually counted using the program QuantCount 3.0. Both programs were developed at the KIVIF using OpenLab Automator programming environment (Improvision). Mean cell survival was determined from the average of cell survival from the three identical wells.

Western blot analysis

Protein samples were loaded onto 10 % SDS-polyacrylamide gel and subjected to SDS-PAGE, then transferred to nitrocellulose membrane, which was blocked by inert proteins. Membranes were probed for proteins of interest using primary antibodies in appropriate dilutions then incubated with HRP-conjugated secondary antibody. The immunoreactive bands were detected by ECL reagents.

Caspase-3 activity measurement

The activity of caspase-3 enzyme was measured by using a fluorometric Caspase-3 Immunoassay/Activity Kit (liberating AMC from DEVD-AMC). After treatments, THP-1 cells (5×10^6) were harvested by centrifugation and washed with ice-cold PBS. Cells were lysed by adding 110 µL chilled lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1 % CHAPS, 1 mM PMSF, 10 µg/mL Pepstatin A, 10 µg/mL Aprotinin, 20 µg/mL Leupeptin). Lysates were centrifuged at 16,000 *g* for 3 min at 4°C. 60 µM substrate in 2x reaction buffer (200 mM HEPES, 20 % sucrose, 0.1 % CHAPS) was added to the supernatant and the samples were incubated for 60 min at 37°C. The fluorescence from appropriately diluted samples was measured at 380 nm excitation and 460 nm emission.

Detection of normal, apoptotic and necrotic cells by differential staining and cell morphology as well as lactate dehydrogenese (LDH) assay

After treatments the cells were washed three times with PBS. The cells were stained with 10 µM Hoechst 33342 (Sigma-Aldrich) in PBS for 15 minutes followed by staining with 5 µM propidium iodide (PI) for 5 min at 37°C in the dark. The cells were seeded on poly-L-lysine coated coverslips. Images were acquired with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany). Cells were

classified as “viable” (Hoechst positive/PI negative, normal nuclear morphology), “apoptotic” (Hoechst positive/PI negative, chromatin condensation, fragmentation, blebbing) or “necrotic” (Hoechst positive/PI positive). The proportion of necrotic cells was also assessed by measuring the release of lactate dehydrogenase (LDH) from damaged cells using Cytoscan colorimetric assay (G-Biosciences) according to the manufacturer’s instructions. Cytotoxicity was expressed as a percentage of maximum LDH activity.

Viability (MTT) assay

After treatments, methyl-thiazol tetrazolium (MTT) was added to each well and the viable cells convert it to insoluble formazan. The medium was removed and DMSO was added to the wells to dissolve the formazan crystals in the cells and the absorbance at 540 nm was recorded. The optical density is directly proportional to the viable cells.

Phosphatase activity measurement

Phosphatase activity was assayed with ^{32}P-labelled 20 kDa myosin light chain (^{32}P-MLC20) substrate at 30 °C for 5 min in the absence or presence of inhibitor-2. After precipitation of proteins with trichloro-acetic acid and centrifugation the released $^{32}P_i$ was determined from the supernatant.

Transfection of Flag-KEPI and transfection of siRNA

The transfection of MCF-7 cells and the silencing of HeLa and HaCaT cells were carried out by using FLAG-KEPI plasmid and panPP1 siRNA in serum free media. The premix, containing FLAG-KEPI plasmid (2 μg/well) and Gene Juice transfection reagent (6 μL/well) or DharmaFECT 2 transfection reagent (10 μL) and panPP1 siRNA (100 nM final concentration), was added to the cells and incubated in

suspension for 30 min at room temperature. The cells were plated and the same volume of media containing 20 % FBS was added. The next day, we replaced the media with fresh media containing 10 % FBS. Cells were incubated for 48 hours before analysis.

Immunofluorescence microscopy

After different treatments the coverslips were washed by PBS. Paraformaldehyde (3.7 %) was used for fixation for 10 min followed by three washes with PBS. Cells were permeabilized with 0.02 % (v/v) Triton X-100 dissolved in PBS for 10 min at room temperature and washed three times with PBS. After incubation with 1 % (w/v) bovine serum albumin (BSA) containing blocking solution for 1 hour, cells were incubated with primary antibodies (1:100) in 1 % (w/v) BSA/PBS overnight at 4°C. Cells were incubated with Alexa 488-conjugated anti-rabbit antibody at a dilution of 1:200 or Alexa 543-conjugated anti-mouse antibody at a dilution of 1:200 or Texas Red Phalloidin (Molecular Probes) at a dilution of 1:1000 in 0.1 % (w/v) BSA/PBS at room temperature for 1 hour. For visualizing the nuclei and fixing the coverslips we used DAPI containing Slow Fade antifade Kit. Samples were imaged with Zeiss Axioskop HBO50/AC microscope or Leica TCS SP8 Confocal Laser Microscope.

UVA radiation

The control and siPP1c treated HaCaT cell were grown in 96-well plate for MTT analysis or in 6-well plate for Western blot analysis. After 48 hours incubation and prior to UV irradiation, cells were kept in serum-free medium for 16 hours. UVA and UVB irradiation was carried out using Bio-Sun UV irradiation system (Vilbert Lourmat, France). The unit of the Radiation Dosage is J/cm^2.

Mouse skin study and immunohistochemistry

Six-week-old, male Balb/c mice (Charles River) were used for the experiments. All procedures were authorized by the Institutional Ethics Committee (7/2010 DE MÁB). The hair of mice was removed by commercially available depilating cream (Veet). The selected areas were topically treated either with 50 μg vehicle (hydrophylic anionic cream) or with tautomycin (1 μM final concentration in vehicle) for 1 hour before UVA irradiation. For UVA exposure, an irradiation setup equipped with exposure Philips TL-20W/12 lamps (Philips, Eindhoven, the Netherlands) was used and it emitted at wavelengths of 315 – 400 nm. The mice were radiated by 5 J/cm^2 UVA three times a day during 5 days. Animals were killed by cervical dislocation, and then the skin was excised. One-half of the skin samples were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin and processed for hematoxylin and eosin (H&E) staining. Immunohistochemical staining of keratin 1, -10, S100A8 and histone 1b was also carried out incubating the sections with primary antibodies overnight at 4°C. Sections were washed and incubated with anti-mouse or anti-rabbit HRP conjugated secondary antibody and immunosignals were developed by nickel/cobalt enhanced 3,3'-diaminobenzidine (DAB) chromogen substrate without any counterstaining. The other half of the samples was used for Western blot analysis.

RNA and microarray processing and data analysis

Total RNA was extracted from control HaCaT and siPP1 HaCaT cells using Trizol reagent. Global expression pattern was analyzed on Affymetrix GeneChip Rat Gene 1.0 ST arrays. Ambion WT Expression Kit (Life Technologies) and GeneChip WT Terminal Labeling and Control Kit (Affymetrix) were used for amplifying and labeling 250 ng of total RNA samples. RNA labeling and hybridization was processed by UD-GenoMed Medical Genomic Technologies Ltd (Debrecen, Hungary).

RESULTS AND DISCUSSION

Role of PP1 and PP2A in the regulation of THP-1 and KG-1 cell viability

The effects of the membrane permeable PP2A-specific inhibitor calyculin-A (CLA) have been studied on leukemic cell survival earlier and it has been established that when CLA was given together with chemotherapeutic drugs (cytosine arabinoside, etoposide, daunorubicin) it counterbalanced drug induced cell death. We studied the molecular background of these findings and we also wanted to investigate the role of the PP1-selective tautomycin (TM) in daunorubcin (DNR)-induced apoptosis. In our experiments both CLA and TM decreased slightly the viability in THP-1 and KG-1 leukemic cells, respectively, but when the cells were preincubated with CLA or TM before DNR treatment the cell death inducing efficacy of DNR was profoundly attenuated. We observed activation of caspase-3 as an indicator of apoptotic cell death upon DNR treatment, which was attenuated in the presence of CLA, but was not changed upon combination of TM with DNR. It is known from the literature that DNR induces dephosphorylation and degradation of pRb, while CLA treatment preserves the phosphorylation state of the pRb thereby decreasing its degradation and leading to increased cell survival. Our findings presume that PP1 and PP2A inhibition enhances pRb phosphorylation level, which attenuates the arrest of cells by DNR allowing G1→S transition. We examined other survival proteins (Erk1/2, Akt) as well and it turned out that only CLA increased their phosphorylation level (Erk1/2$^{pT202/pY204}$ and Akt^{pT473}) but TM pretrearment was without any effect implicating PP2A in the dephosphorylation of these proteins. Regarding the above findings, we assume that PP1 and PP2A influence the DNR-induced apoptosis in different pathways and the chemosensitivity of leukemic cells, and the regulation of pRb phosphorylation level by these enzymes appear to be a common pathway in their actions.

The effect of KEPI inhibitor protein on retinoblastoma protein phosphorylation

The Thr826 site in pRb is dephosphorylated by the isoforms of PP1c, but it is presumed that these isoforms are present in holoenzyme forms in which PP1c is bound to a regulatory subunits. The effect of the regulatory subunits on pRb dephosphorylation can be different: for examples the PNUTS regulatory subunit decreases while the MYPT1 regulatory subunit of MP increases the PP1 activity against phospho-pRb. Since the activity of MP can be regulated by inhibitory phosphorylation sites (Thr696 and Thr853) in MYPT1 and by phosphorylation-dependent inhibitor proteins (CPI-17, KEPI), we examined how CLA and TM influence these phosphorylation processes. CLA treatment increased the phosphorylation of the inhibitory sites in MYPT1 and also in KEPI (Thr73) in THP-1 cells. In contrast, TM treatment did not influence the phosphorylation level of the above mentioned residues (MYPT1^{pT696}, MYPT1^{pT853}, KEPI^{pT73}). Our present data imply that PP2A is involved in the dephosphorylation the PP1c inhibitory sites on both MYPT1 and KEPI, while PP1c neither „autodephosphorylate" P-MYPT1 nor influences the phosphorylation level of KEPI. We wanted to determine the role of PP1 in pRb^{pT826} dephosphorylation by PP1c silencing in HeLa cells. The partial knock-down of PP1c resulted in a rise in pRb^{pT826} phosphorylation level confirming the essential role of PP1 in pRb dephosphorylation.

We expressed Flag-KEPI in MCF-7 cells (which does not express endogenous KEPI). PKC catalyzed phosphorylation of KEPI at the Thr73 inhibitory site was probed via challenging the cells with the PKC activator PMA. PMA treatment resulted in a rise of phosphor-KEPI level and induced its translocation from the cytosol to the nucleus in MCF-7 cells. We also observed an increase in the phosphorylation level of pRb^{pThr826}. These data present further lines of evidence that PP1 inhibition has a regulatory role in the phosphorylation of pRb.

We also increased the inhibitory activity of KEPI by inducing its phosphorylation via CLA treatment of KEPI-transfected MCF-7 cells. It was proven that PP2A had a major role in the regulation of the KEPI Thr73 phosphorylation site, since PP2A-specific inhibition by CLA increased the level of phosphorylation at this site ($KEPI^{pT73}$) in FLAG-KEPI transfected MCF-7 cells. In parallel, CLA treatment increased the phosphorylation level of pRb^{pT826} in non-transfected MCF-7 cells as well. This might be due to both MYPT1 and KEPI phosphorylation, however, the contribution of KEPI phosphorylation to the increased pRb^{pT826} phosphorylation level is substantiated by the facts chat pRb^{pT826} is higher in KEPi transfected cells compared to non-transfected ones.

The above data might implicate that PP1 directly dephosphorylate pRb^{pT826} and the PP1 inhibitor KEPI takes part in this process inducing high pRb^{pT826} phosphorylation level by PP1 inhibition. PP2A regulates the dephosphorylation of pRb presumably in an indirect manner by the dephosphorylation of MYPT1 regulatory subunit of MP at the inhibitory sites and the KEPI inhibitor protein.

The role of PP1c and UVA radiation in HaCaT keratinocytes

We can say generally that the phosphorylation/dephosphorylation state of proteins has crucial role in the maintenance of the homeostasis in human keratinocytes as well as in UV-induced pathological conditions. Among the P-Ser/Thr specific protein phosphatases, the roles of PP2A and PP2B have been characterized in UV-induced apoptosis, epidermis barrier functions and differentiation, however, the role of PP1 is less known in maintaining the homeostasis of keratinocytes.

HaCaT subcellular fractions were analyzed by Western blot, immunofluorescent microscopy and protein phosphatase activity assay and we found that PP1cα/γ isoform was detected mainly in cytoplasm and less in the nucleus but PP1cδ isoform was localized to both the nucleus and the cytoplasm.

UVA irradiation resulted in a decrease of phosphatase (PP1 and PP2A) activity and viability of HaCaT cells. Based on our viability and phosphatase activity assay and according to the daily UVA dose (~10-18 J/cm^2), known from the literature, we selected 10 J/cm^2 dose of UVA irradiation for the further experiments. To study the role of PP1 isoforms we silenced PP1c in HaCaT cells then we examined cell viability and phosphatase activity. Western blot analysis and a decrease in phosphatase activity proved the success of PP1c silencing. In contrast, UVA radiation had no effect on the expression of PP1 isoforms, therefore the decreased phosphatase activity was not the consequence of the reduced expression. UVA irradiation is assumed to be coupled with generation of reactive oxygen species (ROS), which could induce the inactivation of PP1 and PP2A by oxidizing amino acids and metal ions at the catalytic centers. The decrease of cell viability after PP1c silencing confirms the essential role of PP1 in the maintenance of homeostasis of HaCaT cells.

We carried out microarray analysis to examine the role of PP1 isoforms and UVA radiation on the regulation of gene expression. After PP1c silencing and UVA radiation we observed changes in the expression of genes coding for free radical scavengering, regulating cell cycle and apoptosis, DNA repairing and cell morphology. We analyzed the change in the expression of genes coding for keratin 1 (KRT1) and keratin 10 (KRT10), S100A8 as well as histone 1b, since these are referred as melanoma-associated proteins. The changes observed at the gene expression levels were confirmed on protein levels as well. We detected an increased protein level of keratin 1, keratin 10 and S100A8, and a decreased protein level of histone 1b by Western blot analysis after PP1c silencing and UVA irradiation

The role of PP1c and UVA radiation in Balb/c mice

We studied the role of PP1with using TM, a PP1 inhibitory toxin on the skin of Balb/c mice and irradiated the animals with a total cumulative dose of 531 J/cm^2

UVA-radiation, which approximates the minimal erythemal dose (510 J/cm^2). We also found that TM treatment similarly to UVA irradiation, or combination of TM and UVA, resulted in epidermal hyperplasia without inflammatory symptoms. The other half of the skin samples was used for the detection of the changes of expression in the levels of the above mentioned proteins (keratin 1, keratin 10, S100A8, histone 1b) to establish whether they were altered in the same manner as in HaCaT keratinocytes. The protein level of keratin 1, keratin 10, S100A8 increased in TM-treated skin samples compared to control skin and showed a significant increase after UV-irradiation or when the challenges were combined (TM plus UVA-irradiation). The protein level of histone 1b was decreased similarly but not significantly as in case found with HaCaT keratinocytes. In summary, our results in HaCaT keratinocytes and Balb/c mice imply that PP1 enzymes have an important role in the maintenance of normal skin homeostasis. These results indicate that protein phosphatases might be novel target for prevention and treatment of skin diseases.

SUMMARY

1. In THP-1 and KG-1 leukemic cells the cell-death inducing efficacy of daunorubicin (DNR), a chemotherapeutic agent often applied to treat leukemia, decreased profoundly during specific inhibition of PP2A by calyculin-A (CLA) or PP1 by tautomycin (TM) suggesting that phosphatase inhibition was coupled with decreased chemosensitivity to DNR. PP1 and PP2A inhibition enhanced the phosphorylation level of pRb which might help proceeding through G1→S transition in cell cycle suppressed by DNR.
2. Silencing PP1 catalytic subunit (PP1c) in HeLa cells as well as expression of a PP1 inhibitor KEPI protein together with inducing its phosphorylation in MCF-7 cells suggested that direct dephosphorylation of pRb occurred by PP1 while PP2A mediates pRb phosphorylation level via regulation of the dephosphorylation of PP1 inhibitory proteins.
3. The combined effect of PP1c silencing and UVA-irradiation affected significantly the expression of 19 genes of which keratin 1, keratin 10, S100A8 and histone 1b are known as melanoma marker proteins. The changes in the expression of these genes on the protein levels were observed in HaCaT keratinocytes and mouse skin after PP1 inhibition (PP1c silencing or TM treatment), UVA-irradiation and their combined effect. PP1 enzymes have an important role in the maintenance of normal skin homeostasis. These results indicate that protein phosphatases might be novel target for prevention and treatment of skin diseases.

Register number: DEENKÉTK/422/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Dóra Dedinszki
Neptun ID: QSB320
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10034522

List of publications related to the dissertation

1. **Dedinszki, D.**, Sipos, A., Kiss, A., Bátori, R., Kónya, Z., Virág, L., Erdődi, F., Lontay, B.: Protein phosphatase-1 is involved in the maintenance of normal homeostasis and in UVA irradiation-induced pathological alterations in HaCaT cells and in mouse skin.
Biochim. Biophys. Acta-Mol. Basis Dis. 1852 (1), 22-33, 2015.
DOI: http://dx.doi.org/10.1016/j.bbadis.2014.11.005
IF:5.089 (2013)

2. **Dedinszki, D.**, Kiss, A., Márkász, L., Márton, A., Tóth, E., Székely, L., Erdődi, F.: Inhibition of protein phosphatase-1 and -2A decreases the chemosensitivity of leukemic cells to chemotherapeutic drugs.
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IF:4.471 (2013)

List of other publications

3. Bécsi, B., **Dedinszki, D.**, Gyémánt, G., Máthé, C., Vasas, G., Lontay, B., Erdődi, F.: Identification of protein phosphatase interacting proteins from normal and UVA-irradiated HaCaT cell lysates by surface plasmon resonance based binding technique using biotin-microcystin-LR as phosphatase capturing molecule.
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DOI: http://dx.doi.org/10.1016/j.jphotobiol.2014.06.004
IF:2.803 (2013)

Total IF of journals (all publications): 12,363
Total IF of journals (publications related to the dissertation): 9,56

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.

09 December, 2014