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Inhibition of protein phosphatase-1 and -2A decreases the chemosensitivity of leukemic cells to chemotherapeutic drugs

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ABSTRACT

The phosphorylation of key proteins balanced by protein kinases and phosphatases are implicated in the regulation of cell cycle and apoptosis of malignant cells and influences anticancer drug actions. The efficacy of daunorubicin (DNR) in suppression of leukemic cell survival was investigated in the presence of tautomycin (TM) and calyculin A (CLA), specific membrane permeable inhibitors of protein phosphatase-1 (PP1) and -2A (PP2A), respectively. CLA (50 nM) or TM (1 μM) suppressed viability of THP-1 and KG-1 myeloid leukemia cell lines to moderate extents; however, they significantly increased survival upon DNR-induced cell death. CLA increased the phosphorylation level of Erk1/2 and PKB/Akt kinases, the retinoblastoma protein (pRb), decreased caspase-3 activation by DNR and increased the phosphorylation level of the inhibitory sites (Thr696 and Thr853) in the myosin phosphatase (MP) target subunit (MYPT1) as well as in a 25 kDa kinase-enhanced phosphatase inhibitor (KEPI)-like protein. TM induced enhanced phosphorylation of pRb only, suggesting that this event may be a common factor upon CLA-induced PP2A and TM-induced PP1 inhibitory influences on cell survival. Silencing PP1 by siRNA in HeLa cells, or overexpression of Flag-KEPI in MCF-7 cells coupled with inducing its phosphorylation by PMA or CLA, resulted in increased phosphorylation of pRb. Our results indicate that PP1 directly dephosphorylates pRb, while PP2A might have an indirect influence via mediating the phosphorylation level of PP1 inhibitory proteins. These data imply the importance of PP1 inhibitory proteins in controlling the phosphorylation state of key proteins and regulating drug sensitivity and apoptosis in leukemic cells.

1. Introduction

Chemotherapy is a common way to treat hematological malignancies to eliminate malignant cells from the body by inducing cell death. Anticancer drugs are known to induce apoptosis by activating proapoptotic regulators and at the same time suppressing survival factors [1]. Several signaling pathways may influence the efficacy of chemotherapeutic drugs and it often includes the modification of the activity of pro- and anti-apoptotic factors by Ser/Thr-specific phosphorylation/dephosphorylation [2,3]. This recognition has directed attention to the regulatory functions of protein kinases and phosphatases as their actual activity ratio determines the phosphorylation state of key proteins in cell survival. It is well established that the elements of certain protein kinase cascades, such as p42/44Erk1/2 and some other MAP-kinases [4,5] as well as Akt/PKB kinase [6], are important determinants of malignant cell apoptosis. They can exert protective influence against the action of anticancer drugs increasing survival, or, on the contrary, the activation of some MAP-kinase types might contribute to the destructive cellular effects.

Protein phosphatase-1 (PP1) and -2A (PP2A), are two major types of the phospho-Ser/Thr-specific protein phosphatases and they also play essential roles in the regulation of cell death or survival [7,8]. It has been shown that induction of cell death by chemotherapeutic agents is often coupled with phosphatase activation [9,10], while on the

Abbreviations: BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CLA, calyculin A; CPI-17, C-kinase phosphatase-1 inhibitory protein of 17 kDa; DMSO, dimethyl sulfoxide; DNR, daunorubicin; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; His-I-2, hexahistidine-tagged recombinant inhibitor-2; KEPI, kinase-enhanced phosphatase inhibitor; LDH, lactate dehydrogenase; MP, myosin phosphatase; MYPT1, myosin phosphatase target subunit; OA, okadaic acid; PBS, phosphate-buffered saline; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ^{32}P-MLC20, ^{32}P-labeled 20 kDa light chain of turkey gizzard myosin; PMSF, phenylmethanesulfonylfluoride; PNUTS, protein phosphatase 1 nuclear targeting subunit; PP1, protein phosphatase-1; PP1c, protein phosphatase-1 catalytic subunit; PP2A, protein phosphatase-2A; pRb, retinoblastoma protein; SDS, Na-dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TBS, Tris-buffered saline; TBST, TBS containing 0.5% Tween-20; TM, tautomycin.

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other hand phosphatase inhibition by cell-permeable inhibitory toxins such as calyculin A (CLA), tautomycin (TM) or okadaic acid (OA) could also decrease cell viability [11–13]. Intriguingly, when the drug and phosphatase inhibitory treatments are coupled, then phosphatase inhibition generally increases the survival of leukemic cells against several drugs implicating the protein phosphatases in the regulation of chemosensitivity of these cells [11,13–15]. However, the molecular mechanisms for the action of protein phosphatases have not been characterized in details. It is believed that PP2A inhibition plays a role in the suppression of apoptosis by diminishing Bax translocation to mitochondria, while PP1 inhibition is thought to be involved in decreasing of CD95/Fas death receptor induced apoptosis [11].

The regulation of cell cycle in malignancy is also an important issue in the uncontrolled growth of cancer cells [16,17]. Retinoblastoma protein (pRb), the product of a tumor suppressor gene, is an essential element of the checkpoint in G1/S transition of cell cycle, and its deletion or mutations are often identified in a variety of different cancers [18]. Hypophosphorylated pRb binds E2F family transcription factors strongly, thereby suppressing transcription of genes necessary for cell cycle progression. Moreover, pRb in association with E2F1 transcription factor can bind to transcriptionally active proapoptotic promoters and this is required to maximal apoptotic effects [19]. The phosphorylation of pRb by distinct cyclin/CDK complexes leads to dissociation of E2F1 allowing progression through G1/S transition and synthesis of the genes in S phase [20]. pRb is phosphorylated at more than 10 Ser/Thr residues, and phosphorylation and communication between several of these sites, such as Thr373, Ser608, Ser612, Thr821 and Thr826, might be implicated in inducing structural changes in pRb which promote possible dissociation of E2F1 [18]. However, among these sites, Thr821 or Thr826 are with special importance since phosphorylation of either residue may result in disruption of the interaction of pRb with interacting partners [20]. While the identity of the protein kinases that phosphorylate the Ser/Thr residues in pRb are well established, the protein phosphatases involved in the dephosphorylation processes are described less in details, although many studies have been carried out to date [20]. These results substantiate that the phospho-Ser/Thr sites in pRb are dephosphorylated by PP1 or PP2A, or both enzymes.

In our previous study, we showed that the C-terminal phosphorylation sites of pRb phosphorylated by cyclinE/CDK2 and cyclinD/CDK4 were preferentially dephosphorylated by PP1, and that myosin phosphatase (MP) in which PP1 catalytic subunit (PP1c) is complexed with myosin phosphatase target subunit 1 (MYPT1) might be one of the PP1 holoenzyme acting on phospho-pRb in THP-1 leukemic cells [13]. In accordance with the above data, increased inhibitory phosphorylation of MP in MYPT1 was correlated with elevated pRb phosphorylation and increased chemosensitivity of THP-1 cells to daunorubicin (DNR) treatment. It was also proven that there is a competition between pRb and MYPT1 for binding of PP1c since both proteins include an RVxF like PP1c-binding motif. With respect to the latter it was demonstrated that PP1c interacted with pRb via a KLRF sequence motif at its regulatory subunit binding site and PP1c was positioned in complex with pRb in a way that it could still exert its catalytic activity on phosphorylated residues [21]. In addition, binding of PP1c to pRb had another important consequence; it blocked interaction of pRb with CDKs; therefore, besides forming an active phosphatase-pRb complex, it might also contribute to the decreased phosphorylation level of pRb through keeping the kinase away from the substrate. The above results, together with previously published data [10,22–24] present compelling lines of evidence that PP1 is the major physiological phosphatase for pRb dephosphorylation. Nevertheless, many reports proved the involvement of PP2A in mediating the phosphorylation level of pRb [25,26] and other pocket proteins [27,28].

In this work, we have attempted to identify how PP1 and/or PP2A may influence the chemosensitivity of leukemic cells against chemotherapeutic drug treatments using cell-permeable phosphatase inhibitory toxins selective for PP2A or PP1. Our present data suggest that PP2A may regulate the phosphorylation level of Erk1/2, Akt and pRb, and it had an influence on caspase-3 activity, too. In contrast, PP1 appears to be involved in the direct dephosphorylation of pRb. It is demonstrated that a 25 kDa kinase-enhanced phosphatase inhibitor (KEPI)-like protein, with known inhibitory potency on both PP1c and MP, may also participate in the inhibition of PP1 in THP-1 cells, thereby increasing the phosphorylation level of pRb. Our results support the conclusion that PP2A influences pRb dephosphorylation indirectly, via regulating the phosphorylation state of PP1c inhibitory or regulatory proteins (KEPI, MYPT1). These data draw attention to the importance of the expression and phosphorylation of PP1 inhibitory proteins in malignant cells and on their role in controlling the phosphorylation state of key proteins in the regulation of cell survival.

2. Materials and methods

2.1. Cell cultures

THP-1 human monocytic leukemia cells, KG-1 human myeloid leukemia cells, MCF-7 breast cancer cells and HeLa human cervical cancer cells were purchased from the European Collection of Cell Cultures (ECACC) and cultured according to the supplier's recommendations at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. In vitro drug sensitivity assay

In vitro drug resistance assays were assessed using a 2-day cell cultures on microtiter plates. THP-1 or KG-1 cells were preincubated initially with 50 nM calyculin A (CLA, Santa Cruz Biotechnology) or 1 μM tautomycin (TM, Merck Millipore) and were washed with PBS after 1 h. Daunorubicin (DNR, Sigma-Aldrich) was dissolved in DMSO, and it was placed out in 50 nL volumes and four concentrations in triplicates on 384-well plates by using a Biomek robot. Each well was loaded with 30 μL cell suspension containing 1000 cells as the final concentrations of DNR were 0.15 μM, 0.61 μM, 1.84 μM and 7.38 μM. After 2 days of incubation, the live and dead cells were differentially stained using fluorescent VitalDye (Biomarker, Hungary). 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) [29]. Fifteen 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. Comparing the two types of control wells, no toxic effect of DMSO could be seen. Mean cell survival was determined from the average of cell survival from the three identical wells.

2.3. Phosphatase activity assay

Prior to treatments, THP-1 cells were incubated in serum-free medium for 16 h. To investigate the influence of the inhibitors on phosphatase activity, cells were treated with 50 nM CLA and 1 μM TM for 1 h in serum-free media, and lysates were prepared for phosphatase assays as described previously [30]. Briefly, cells were washed with phosphate-buffered saline (PBS) followed by 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. Cells were frozen in liquid nitrogen and then thawed and sonicated, and the lysates were clarified by centrifugation at 16,000*g* for 10 min. The phosphatase activity of the supernatants (30-fold final dilution in the assays) was assayed with 1 μM ^{32}P-labeled 20 kDa light chain (^{32}P-MLC20) of turkey gizzard