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CK2 inhibition induces apoptosis via the ER stress response

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ABSTRACT

Protein kinase CK2 is a ubiquitously expressed serine/threonine kinase consisting of two catalytic α/α′ and two regulatory β subunits. Expression of CK2 is highly elevated in tumor cells where it protects cells from apoptosis. Accordingly inhibition of CK2 is known to induce programmed cell death, making it a promising target for cancer therapy. In the present study we investigated apoptosis induction by the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) in prostate tumor cells. In contrast to PC-3 cells LNCaP cells respond to CK2 inhibition with apoptosis. Most interestingly we found the mitochondrial pathway induced in LNCaP as well as in PC-3 cells as monitored by down-regulation of bcl-2 and subsequent cytochrome c release. In both cell lines activation of caspase 9 was not detected. Instead, an activation of the endoplasmic reticulum (ER) stress response in LNCaP cells after treatment with the CK2 inhibitor TBB was found. We show that this ER stress response led to an up-regulation of the death receptor DR5 and subsequent apoptosis in LNCaP cells.

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer in elderly men in the western world [1]. Development and progression of PCa are dependent on androgen receptor (AR) signaling. Therefore, androgen ablation by orchiectomy, by treatment with LHRH-analoga or anti-androgens is the most common therapy. Hormone ablation results in apoptosis of most prostate cancer cells. Moreover, chemical castration very often results in the selection of hormone refractory cells. Advanced hormone refractory prostate cancer cells are most frequently characterized by activation of anti-apoptotic signaling pathways [2]. The identification of new mechanisms or factors able to induce apoptosis in PCa cells might therefore be a useful approach to treat advanced prostate cancer.

In PCa cells inhibition of protein kinase CK2, a ubiquitously expressed serine/threonine kinase, was shown to induce programmed cell death [3]. Protein kinase CK2 is composed of two regulatory β-subunits and two catalytic α- or α′-subunits [4]. It is involved in a broad spectrum of cellular processes like the regulation of cell cycle and proliferation as well as suppression of apoptosis. Activity of the kinase is elevated in highly proliferating normal as well as in tumor cells. Compared to normal tissue CK2 activity was found to be elevated 3–5-fold in PCa [5]. Consequently CK2 has recently been suggested as a prognostic marker for prostate cancer [6]. There is experimental evidence that targeting CK2 induces cell death *in vivo* [7–9], making it a promising target for cancer therapy. A phase I trial of a CK2 inhibitor (CX-4945) started in patients suffering from several advanced solid tumors including prostate cancer. An additional phase I study using a CK2 peptide-based inhibitor on patients with cervical malignancies showed a clinical benefit of CK2 inhibition in these patients [10]. In the light of using CK2 inhibitors as anti-tumor drugs it is of particular interest to characterize the involved signaling pathways. CK2 inhibition has an impact on extrinsic, receptor mediated as well as on intrinsic mitochondrial apoptosis pathways in several prostate cancer cell lines [11,12]. CK2 inhibitors apigenin and 4,5,6,7-tetrabromobenzotriazole (TBB) enhanced induction of apoptosis by death receptor ligands (TRAIL, FasL and TNFα) in ALVA-41 prostate cancer cells. In contrast CK2α overexpression rescued PC-3 prostate cancer cells from receptor mediated apoptosis [12]. Moreover, CK2 was shown to be involved in the regulation of the mitochondrial apoptotic pathway [13]. In LNCaP and PC-3 cells inhibition of CK2 resulted in the generation of reactive oxygen species (ROS) that may be responsible for subsequent mitochondrial cytochrome c release leading to the activation of the intrinsic apoptotic pathway [14–17].

We previously reported that inhibition of CK2 by apigenin or by TBB resulted in apoptosis of LNCaP but not of PC-3 prostate cancer cells [15,18]. In order to elucidate the differences in apoptosis signaling in the two cell lines, we focused on TBB, the more specific

Abbreviations: AR, androgen receptor; CHOP, C/EBP-homologous protein; ER, endoplasmic reticulum; PARP, Poly-ADP-ribose polymerase; PCa, prostate cancer; TBB, 4,5,6,7-tetrabromobenzotriazole.

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compound for CK2 inhibition [19]. A first obvious discrepancy between LNCaP and PC-3 cells is the lack of p53 in the latter cell line. Knocking down p53 in LNCaP cells revealed that p53 is not necessary for apoptosis induction mediated by CK2 inhibition with TBB although the tumor suppressor protein was stabilized after TBB treatment [15].

In the present study we investigated the activation of the intrinsic mitochondrial apoptosis pathway after CK2 inhibition in prostate tumor cells. Most interestingly we found the mitochondrial pathway induced in LNCaP as well as in PC-3 cells but no activation of caspase 9 in both cell lines. Instead we found an activation of the endoplasmic reticulum (ER) stress response after TBB treatment in LNCaP cells only. We presume that this ER stress response may lead to activation of caspase 8 and subsequent apoptosis in LNCaP cells.

2. Materials and methods

2.1. Cell culture

LNCaP cells are androgen-sensitive prostate cancer cells, which were established from a lymph node metastasis. The PC-3 cell line is androgen-insensitive and was established from a bone metastasis. HeLa cells were cultured to obtain positive control extracts for apoptosis induction. Cells were maintained at 37 °C in RPMI 1640 and DMEM, respectively, supplemented with 10% fetal calf serum (FCS) in an atmosphere enriched with 5% CO_2. The CK2 inhibitor TBB (Calbiochem, Merck KGaA, Darmstadt) was dissolved in dimethyl sulfoxide (DMSO) to a 10 mM stock solution. Doxorubicin was dissolved in H_2O to a stock solution (10 mg/ml). Cells were treated with the CK2 inhibitor or doxorubicin at the indicated final concentrations for different times or the same volume of vehicle as control. Transfection of cells was performed by using the Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2. Extraction of proteins

For harvesting cells were scraped off the plate with a rubber policeman and sedimented together with floating cells by centrifugation (7 min, 4 °C, 400×*g*). Cells were washed with cold phosphate buffered saline (PBS) and lysed with the double volume of RIPA buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% sodium desoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate) supplemented with the protease inhibitor cocktail complete™ according to the instructions of the manufacturer (Roche Diagnostics, Mannheim, Germany). After lysis cell debris was removed by centrifugation. The protein content was determined according to a modified Bradford method with the BioRad reagent dye (BioRad, München, Germany). Protein extracts were immediately used for Western Blot analysis.

2.3. Cytochrome c release

The cytochrome c release from mitochondria into the cytosol was analyzed according to a protocol of Dr. O. Rössler (Medical Biochemistry and Molecular Biology, Homburg). Briefly, cells were seeded on 6 cm plates and allowed to attach over night. After TBB treatment they were washed with cold PBS and scraped off the plate in 1 ml sucrose buffer (210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, pH 8.0, 0.2 mM EGTA, 5 mM succinate, 0.15% BSA) and pelleted by centrifugation (3 min, 400×*g*, 4 °C). The cell pellet was resuspended in 50 µl sucrose buffer and cell membranes were permeabilized by the addition of digitonin (stock: 20 mg/ml) for 1–2 min on ice. The amount of digitonin for the disruption of the cell membrane but not of the mitochondrial membrane was optimized in several experiments for each cell line. After centrifugation the supernatant was removed (cytosol extract) and the pellet was extracted again with 50 µl PBS/0.5% Triton X-100 for 5–10 min on ice. Cytosolic and Triton X-100 extracts were further analyzed by Western Blot.

2.4. Western Blot analysis

Proteins were separated by SDS polyacrylamide gel electrophoresis according to the procedure of Laemmli [20]. For Western Blot analysis proteins were transferred to a PVDF membrane by tank blotting with 20 mM Tris/HCl, pH 8.7, 150 mM glycine as transfer buffer. Membranes were blocked in PBS with 0.1% Tween20 (PBS-T) and 5% dry milk for 1 h at room temperature. The membrane was incubated with the primary antibody (usually in a dilution of 1:1000, unless otherwise stated) in PBS-T with 1% dry milk for another hour. Cytochrome c release was analyzed with mouse monoclonal antibody 7H8 (Santa Cruz, Heidelberg, Germany). Caspase 3 was identified with a monoclonal rabbit antibody (clone 8G10) from Cell Signaling Technology (distributed by New England Biolabs, Frankfurt am Main, Germany). Bcl-2 was detected with the monoclonal antibody Ab-1 (Merck KGaA, Darmstadt, Germany) and survivin was detected with the mouse monoclonal antibody D-8 (Santa Cruz, Heidelberg, Germany). The monoclonal antibodies for detection of Caspase 9 (10-1-87), HSP27 (G3.1) and HSP70 (SM5066) were purchased from assay designs (Ann Arbor, USA distributed by Biomol, Hamburg, Germany) and Acris Antibodies GmbH (Herford, Germany) respectively. HSP90α/β and CHOP/GADD153 were detected with the antibodies H-114 and B-3, respectively, obtained from Santa Cruz (Heidelberg, Germany). For detection of FLAG-tagged protein we used a monoclonal mouse antibody (clone M2, Sigma-Aldrich, München, Germany). As marker for loading equal amounts of protein we used either an α-tubulin antibody (DM1A, Sigma-Aldrich, München, Germany) or a GAPDH antibody (FL-335, Santa Cruz, Heidelberg, Germany). The membrane was washed with PBS-T three times before incubating with the peroxidase coupled secondary antibody in a dilution of 1:30 000 in PBS-T with 1% dry milk. Signals were developed and visualized by the Lumilight system of Roche Diagnostics (Mannheim, Germany).

2.5. Transfection

CHOP cDNA was subcloned from pFUW-FLAG-hCHOP (kindly provided by Prof. G. Thiel, Medical Biochemistry and Molecular Biology, Homburg) into the *EcoRI*/*BamHI* restriction sites of p3XFLAG-CMV™7.1 (Sigma Aldrich, München, Germany) to obtain p3XFLAG-CMV™7.1-CHOP. For transient transfection with the CHOP expression vector, PC-3 cells (2×10^5/well) were seeded into a 6 well plate and allowed to adhere over night. Cells were transfected with 1 µg p3XFLAG-CMV™7.1-CHOP or p3XFLAG-CMV™7.1 per well using Effectene® Transfection Reagent (Qiagen, Hilden, Germany). Two days after transfection cells were harvested, lysed and analyzed by a caspase 3/7 assay and Western Blot.

2.6. Reporter gene assay

Prostate cancer cells were seeded into a 6 well plate (2×10^5cells/well) and allowed to attach over night. The reporter gene construct pGL3-DR5p (kindly provided by Prof. Wang, Penn State College of Medicine, Hershey, Pennsylvania, USA) [21] and pcDNA3.1/Hygro/lacZ (invitrogen GmbH, Darmstadt, Germany) encoding β-galactosidase were cotransfected (0.5 µg of each DNA) using Effectene® Transfection Reagent (Qiagen, Hilden, Germany). After 30 h the cells were treated with TBB for another 42 h. The luciferase activity was measured by using the luciferase assay system from Promega GmbH (Mannheim, Germany). β-Galactosidase staining was performed as described in Ref. [22].