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Report

Interaction of cisplatin, paclitaxel and adriamycin with the tumor suppressor PTEN

Thomas Schöndorf,[1] Martina Becker,[1] Uwe-Jochen Göhring,[1] Barbara Wappenschmidt,[2] Hannelore Kolhagen,[1] and Christian M Kurbacher[1]

[1]Department of Gynecology and Obstetics, University of Cologne, 50924 Cologne, Germany. [2]Department of Gynecology and Obstetics, University of Bonn, 53105 Bonn, Germany.

Due to its pivotal role in signal transduction, the universal tumor suppressor PTEN (also termed MMAC or TEP) is one of the putative candidates for involvement in tumorigenesis of several tissues. Although involvement of PTEN in tumorigenesis was shown in different tissues, no data are available concerning PTEN activity in response to antineoplastic agents. Therefore, we assayed the PTEN activity exposed to either blank medium or the commonly used anti-cancer drugs cisplatin, adriamycin or paclitaxel, respectively, in three different concentrations. PTEN activity was determined using the Malachite Green assay basing upon dephosphorylation of phosphatidylinositol-3,4,5-triphosphate (PIP_3) by the PTEN enzyme and subsequent determination of inorganic phosphate released. Although the three different anti-cancer drugs assayed act with different cellular modes, the antineoplastics influenced PTEN activity in a similar manner: at low concentrations tested all three antineoplastics significantly increased PTEN activity. However, increasing drug concentrations exhibited a decline but not a total loss of PTEN activity. The data indicate that PTEN activity is increased following cytotoxic drug exposure and, thereby, exhibits its suppressive function. However, the decrease of PTEN activity in response to increasing drug concentrations suggests an aberration of total functional activity. As far as the regulative checkpoint PTEN is abolished, tumor cells might evade cell death pathways resulting in increased proliferation of cancer cells. This might be a general event in refractory tumor cells surviving chemotherapy. [© 2001 Lippincott Williams & Wilkins.]

Key words: Anti-cancer drugs, Malachite Green assay, PTEN.

Introduction

Since its discovery in 1997,[1,2] PTEN (phosphatase and tensin homologue on chromosome 10) has gained increasing attention in cancer research.[3] PTEN (also termed MMAC1, mutated in multiple advanced carcinomas, or TEP1, transforming growth factor-β-regulated and epithelial cell enriched phosphatase) is a phosphatase with dual specificity.[4] PTEN dephosphorylates and thus inactivates protein kinase B (PKB, also known as Akt),[5] a member of the intracellular signal transduction pathway towards cell survival. Additionally, PTEN dephosphorylates phospholipids, key components of cell growth stimulation and apoptosis prevention.[6] Hence, PTEN acts as a negative regulator of cell proliferation and G_1 progression.[7,8]

Due to its pivotal role in signal transduction, PTEN is one of the putative candidates for involvement in tumorigenesis of several tissues.[9] Due to both its aforementioned functions PTEN is regarded as a universal tumor suppressor.[10] Accordingly, involvement of PTEN in tumorigenesis was shown in different tissues. In glioblastoma, PTEN inhibits PKB.[11] Furthermore, PTEN activity prolongs G_1 cell cycle arrest and reduces tumorigenicity.[12] In murine models, heterozygous mutations of the PTEN gene lead to an increased incidence of lymphomas, gonadostromal tumors, and both colonic and thyroid cancers.[13,14]

In order to improve cancer therapy, new approaches against signal transduction cascades have been developed.[15,16] Due to its crucial role in signal transduction, PTEN might be a promising target for future antineoplastic strategies. Thus, the interaction of commonly used anti-cancer drugs and PTEN activity may be an important clue for the development of PTEN-based therapy. Therefore, we performed a study

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Correspondence to T Schöndorf, Department of Gynecology and Obstetrics, University of Cologne, Kerpener Strasse 34, 50931 Cologne, Germany.
Tel: (+49) 221 478 4993; Fax: (+49) 221 478 4929;
E-mail: thomas.schoendorf@medizin.uni-koeln.de

of the effect of cisplatin, adriamycin and paclitaxel, respectively, on PTEN activity.

Materials and methods

PTEN quantification

Tumor cells (3.2×10^6) of the ovarian cancer cell line SKOV3 were pelleted (2 min, 10 000 *g*), washed in PBS and resuspended in 600 μl of extraction buffer (T-PER tissue protein extraction reagent; Pierce, Rockford, IL; supplemented with 1 mM β-mercaptoethanol, 0.5 mM dithiothreitol and 0.3 U/ml aprotinin). Cells were lysed by ultrasound for 5 min and cell debris was directly removed by centrifugation (10 min, 16 000 *g*).

Protein concentrations in cell lysates were determined in triplicates using standard colorimetric procedures (DC protein assay; BioRad, Munich, Germany). A set of 5–50 μg of total protein was electrophoresed by a discontinuous denaturating 12% polyacrylamide gel electrophoresis. Subsequently, proteins were transferred onto a nitrocellulose membrane in blotting buffer (48 mM Tris, 39 mM glycine and 20% methanol, pH 9.2) for 30 min at 15 V using a semidry blotting unit (TransBlot SD; BioRad).

PTEN was quantified chemiluminometrically. The membrane was saturated for 30 min at room temperature in phosphate-buffered saline (PBS), supplemented with 3% skim milk powder. After a washing step, the blots were incubated with 1:1000 diluted PTEN-specific polyclonal rabbit IgG antibody (Upstate Biotechnology, Lake Placid, NY) for 30 min at 37°C. Then the blots were washed 4 times in PBS. The primary antibody was detected using a horseradish peroxidase-labeled secondary antibody (dilution 1:1000), incubated at room temperature for 1 h. After an additional washing step, the blots were incubated for 1 min in ECL detection solution (Amersham-Pharmacia Biotech, Freiburg, Germany). The resulting luminescence was documented autoradiographically. PTEN expression was quantified by densitometry using multianalyst software 1.4 (BioRad). Thus, we were able to calculate 25 ng PTEN protein per 40 000 tumor cells (Figure 1).

PTEN activity assay

PTEN activity was determined using the Malachite Green assay kit (Upstate Biotechnology). The test is based upon dephosphorylation of phosphatidylinositol-3,4,5-triphosphate (PIP_3) by the PTEN enzyme and subsequent determination of inorganic phosphate released.[17]

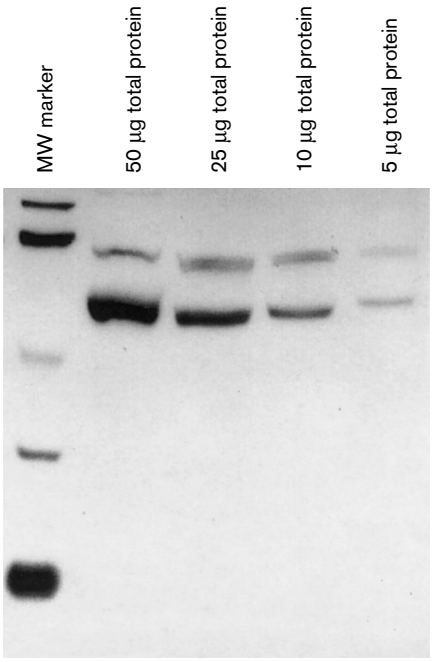

Figure 1. Western Blot to quantify PTEN protein in cell lysates. Both visuable bands represent the PTEN protein.

To prepare phospholipid vesicles (PLV), 300 μg PIP_3 (Biomol, Hamburg, Germany), and 625 μg of both dioleoyl phosphatidylcholine and dioleoyl phosphatidylserine (Avanti Polar Lipids, Alabaster, AL) were added to 1 ml PLV buffer (10 mM HEPES, pH 7.4, 1 mM EGTA and 10% bovine serum albumin). The PTEN reactions were started by adding 5 μl of PTEN enzyme solution to 10 μl PLV in assay buffer (100 mM Tris–HCl, pH 8.0 and 10 mM dithiothreitol) at a total volume of 25 μl. Total enzyme amount ranged from 10 to 50 ng/assay. To each assay point either blank medium or three different anti-cancer drugs in three different concentrations [0.1–10% plasma peak concentration (PPC)] were added (to 10–50 ng PTEN): cisplatin 0.1–10%, PPC=0.038–0.19 ng/μl; adriamycin (ADM) 0.1–10%, PPC=0.005–0.025 ng/μl; paclitaxel (PTX) 0.1–10%, PPC=0.136–0.68 ng/μl. Approximately 25 ng PTEN protein is expressed in 40 000 carcinoma cells. The drug concentrations used *in vitro* are calculated to refer to the clinically achievable tumor cell PPC after i.v. or oral administration of a standard dose.

After a 15-min incubation period the reaction was terminated by adding 100 μl of Malachite Green. Another 15-min incubation was needed to develop the dye. The optical density of each well is proportional to phosphate released, thereby indicating PTEN activity. The absorbance was measured using a

standard microplate reader (model 550; BioRad) with a 655 nm filter. Blank medium without PLV and PTEN served for background detection.

Results

PTEN Western determination revealed 25 ng PTEN per 40 000 tumor cells. Thus, the PTEN activity assays performed reflect its biological activity in 40 000 tumor cells by addition of 25 ng PTEN to the *in vitro* test. Commensurately, 9.5 ng/μl cisplatin, 1.25 ng/μl ADM and 34 ng/μl PTX referred to the clinically achievable cell PPC after administration of a standard therapy.

Figure 2 shows the influence of antineoplastics on PTEN activity; 25 ng PTEN was used. In order to normalize the experiments, PTEN activity of the blank control was set as 100%. Activity assays using 10, 20 and 50 ng PTEN revealed similar results (data therefore not shown). At the lowest concentrations tested (0.1% PPC), all three antineoplastic agents tested produced a significant increase of the PTEN activity at levels ranging between 113 and 142% of the control. Higher cytostatic concentrations, however, exhibited diverse effects: whereas both ADM and PTX at 1% PPC did not influence the PTEN activity significantly, cisplatin at this concentration produced a significant increase of the PTEN activity which, however, did not reach the level seen with 0.1% PPC. Further augmentation of the cisplatin concentration resulted in unchanged PTEN activity versus control, whereas both ADM and PTX at 10% PPC decreased the PTEN activity.

Discussion

The recently discovered PTEN gene modulates cell cycle progression via inhibition of the Akt/PKB signal transduction pathway.[8,9] Heterozygous mutation of the gene results in an increase of tumor incidence. Complete loss of PTEN leads to death during the embryonic period. Both findings provide evidence of the essential role of PTEN in cellular development.[8] Consistent with these reports, mutations of the PTEN gene are found in several tumors of different origins, including gliomas or cancers of the endometrium or prostate, respectively.[10] Therefore, PTEN is considered a tumor suppressor. The loss of functional PTEN activity is thought to be a general event in progression of refractory tumor cells.

Due to its pivotal role in tumor biology, we have been interested in the influence of commonly used antineoplastics on PTEN activity. To this end, we performed *in vitro* studies on PTEN activity. First, we determined the PTEN content of the ovarian cancer cell line SKOV3. Subsequently, we perfomed PTEN activity studies at concentrations resembling the *in vivo* situation in a tumor cell. The Malachite Green test is proportional to the amount of inorganic phosphate, thereby indicating total PTEN activity. The PLV construction must be fast, but, nevertheless, the assay was reproducible and reliable and could be incorporated into laboratory routine easily.

Three different anti-cancer drugs commonly used with different modes of action were investigated, cisplatin, ADM and PTX. Cisplatin inhibits DNA replication by DNA alkylation, ADM is a topoisomerase II poison inhibiting DNA repair and PTX interferes

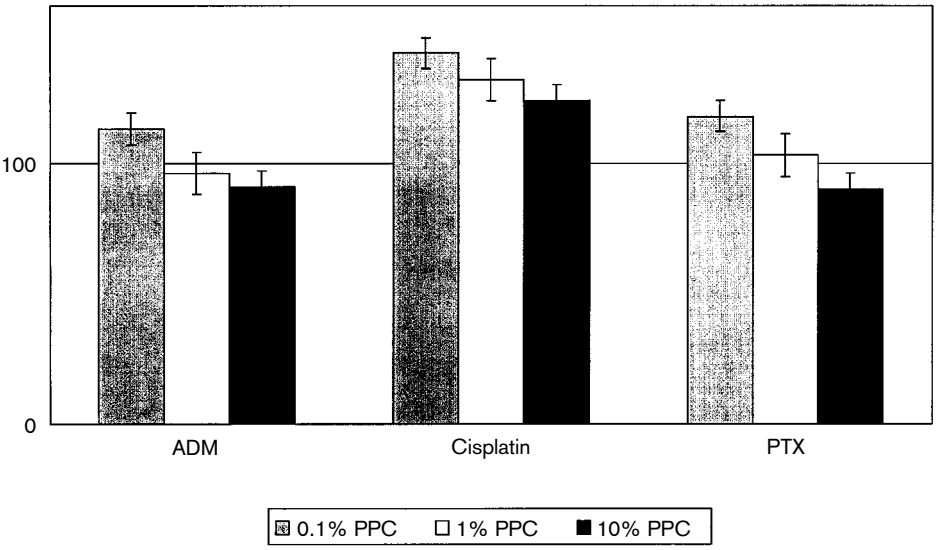

Figure 2. Influence of anti-cancer drugs on PTEN activity *in vitro*.

with microtubuli reassembly, thereby inhibiting cell division.[18,19] Normally, these effects lead to apoptosis. However, some cells might survive antineoplastic therapy. As a result, the tumor becomes refractory.

At low dosage, all three antineoplastics could increase PTEN activity. This indicates that PTEN activity augments in the presence of cytotoxic drugs. In viable cells, PTEN is able to arrest the cell cycle at the G_1 checkpoint or, as a consequence of irreparable DNA damage, start the apoptotic cell death pathway.[20,21] Thereby, PTEN exhibits a protective function. This effect might follow cytotoxic exposure. However, increasing drug concentrations decrease PTEN activity. Moreover, both ADM and PTX reduced PTEN activity over 20% lower than the blank control. This reduction of activity observed in our studies using higher drug levels suggests an aberration of total functional activity. As far as the regulative checkpoint PTEN is abolished, tumor cells might evade cell death pathways and, consequently, increase proliferation of cancer cells. This might be a general event in refractory tumor cells surviving chemotherapy. Our data resemble the clinical and experimental experience of plateauing dose–response curves which may be a result of both direct cytotoxic action and negative influence of factors regulating intracellular hemostasis.[22]

Although our data have been acquired in *in vitro* tests, our findings might be a first step in a general explanation of the role of an aberrant PTEN-mediated signal transduction pathway in tumor therapy. We suggest that tumor cells surviving chemotherapy might become refractory due to inhibtion of the tumor suppressor PTEN. This affect is initiated by the anticancer drug itself. Further ongoing studies should give more insight into the role of PTEN in cancer development and progression. PTEN is a promising target for new therapeutic approaches. The assay presented here may be routinely performed to create an individually designed PTEN-mediated therapy regimen.

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