Berberine slows cell growth in autosomal dominant polycystic kidney disease cells

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ABSTRACT

Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary monogenic disorder characterized by development and enlargement of kidney cysts that lead to loss of renal function. It is caused by mutations in two genes (PKD1 and PKD2) encoding for polycystin-1 and polycystin-2 proteins which regulate different signals including cAMP, mTOR and EGFR pathways. Abnormal activation of these signals following PC1 or PC2 loss of function causes an increased cell proliferation which is a typical hallmark of this disease. Despite the promising findings obtained in animal models with targeted inhibitors able to reduce cystic cell growth, currently, no specific approved therapy for ADPKD is available. Therefore, the research of new more effective molecules could be crucial for the treatment of this severe pathology. In this regard, we have studied the effect of berberine, an isoquinoline quaternary alkaloid, on cell proliferation and apoptosis in human and mouse ADPKD cystic cell lines. Berberine treatment slows cell proliferation of ADPKD cystic cells in a dose-dependent manner and at high doses (100 μg/mL) it induces cell death in cystic cells as well as in normal kidney tubule cells. However, at 10 μg/mL, berberine reduces cell growth in ADPKD cystic cells only enhancing G_0/G_1 phase of cell cycle and inhibiting ERK and p70-S6 kinases. Our results indicate that berberine shows a selected antiproliferative activity in cellular models for ADPKD, suggesting that this molecule and similar natural compounds could open new opportunities for the therapy of ADPKD patients.

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1. Introduction

ADPKD is one of the most common monogenic disorders (1:1000) characterized by the progressive bilateral development and enlargement of kidney cysts that lead to end-stage renal disease (ESRD) [1]. ADPKD causes also hypertension, cardiac valve defects and intracranial aneurysms which are the major cause of death [2]. PKD1 and PKD2 gene mutations account for 85% and 15% of ADPKD cases respectively, and codify for polycystin-1 (PC1), a large membrane protein and polycystin-2 (PC2), a calcium permeable channel, respectively [2]. PC1 functions as adhesion molecule implicated in the maintenance of tubular architecture of the kidney [2,3], while PC2 is a Ca^{2+} permeable channel which may modulate calcium homeostasis [4,5]. They interact each other forming a complex able to regulate calcium channel activity in kidney cells as well as in lymphoblastoid cell lines [5–8]. This complex might regulate a series of

biological features such as cell proliferation and apoptosis by modulating different signals including cAMP, mTOR and EGFR pathways [9–13]. Although several inhibitors of these ways are able to reduce kidney cysts in animal models for ADPKD, results from clinical trials are not completely satisfactory [14–18]. Moreover, there is also evidence of a reduced intracellular Ca^{2+} activity which accelerates cell proliferation in ADPKD cystic cells, resulting in kidney cyst formation, which might be reverted by the treatment with calcium channel activators [19,20]. Consistently, triptolide, a diterpene used in the traditional Chinese medicine, induces intracellular calcium release, leading to cell growth arrest in Pkd1$^{(-/-)}$ mouse cells, and reduces cellular proliferation and cyst formation in a murine model of ADPKD [21].

Here we show that the application of berberine, another product of traditional Chinese medicine, is able to reduce cell proliferation by inhibition of ERK and p70-S6 kinases in ADPKD cystic cells.

2. Material and methods

2.1. Reagents

The material for cell culture and berberine (lyophilized powder) was purchased from Sigma (Italy). Rabbit polyclonal anti-ERK,

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anti-pERK, anti-S6K and anti-pS6K antibodies were obtained from Cell Signaling (Euroclone, Italy). Enhanced chemiluminescent substrates for Western blotting and HRP-conjugated goat anti-rabbit antibodies were purchased from Euroclone (Italy).

2.2. Cell proliferation and cell cycle analysis

Human normal (4/5) and cystic kidney epithelial cells (9.7 and 9.12), derived from proximal tubules of normal and polycystic kidneys [22] as well as mouse Pkd1$^{(-/-)}$ null and Pkd1$^{(+/-)}$ heterozygous kidney cystic cells [23] have been cultured in DMEM/F12 medium supplemented with 10% FBS. Cell proliferation analysis was performed plating 30,000 or 5000 cells/mL in 24- or 96-well plates, respectively. Subsequently, cells were starved for 24 h in DMEM/F12 supplemented with 0.4% BSA and treated for 24 h in DMEM-1% FBS alone or in combination with berberine (1, 10 and 100 μg/mL). Cell proliferation was calculated by direct cell counting after trypan blue staining, using a Burker chamber [24], and by a colorimetric method. The latter consists in the quantitation of formazan, a colored compound produced by the cells through the bio-reduction of tetrazolium salts added in culture medium. The amount of formazan, detectable recording the absorbance at 490 nm with a 96-well plate reader, is directly proportional to the number of living cells (CellTiter cell proliferation assay, Promega).

Cell cycle analysis was carried out by flow cytometry using the FACSCalibur Becton Dickinson Immunocytometry System [25].

The study protocol is in line with the ethical guidelines of the 1975 Declaration of Helsinki.

2.3. Apoptosis

Apoptosis was analyzed by caspase-3 assay, Hoechst method and DNA fragmentation. Caspase-3 activity was evaluated using EnzChek® caspase-3 Assay Kit (Invitrogen). After treatment with 1% FBS alone or in combination with 10 and 100 μg/mL of berberine for 24 h, cells were harvested, lysed and centrifuged according to the manufacturer's instructions. 50 μL aliquots of supernatant were incubated with 50 μL of 2× substrate working solution containing 5 mM Z-DEVD-R110. Fluorescence was measured every minute for 90 min at 520 nm by a fluorimeter (VICTOR3 1420 Multilabel Counter, PerkinElmer). Values were normalized with the protein content by the Bradford method.

Apoptotic nuclei were detected by Hoechst method in cells cultured in 0.4% BSA alone or in combination with 10 and 100 μg/mL of berberine for 24 h. The presence of apoptotic cells was determined by fluorescence after cell staining with Hoechst 33258 (10 μg/mL). Images were acquired through a Zeiss Axiovert 200 fluorescence microscope, equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ).

DNA fragmentation was evaluated after DNA isolation by phenol/chloroform extraction. Ladder DNA was separated by electrophoresis in a 1.5% agarose gel and visualized through an UV transilluminator Bio RAD (Italy) after staining with ethidium bromide.

2.4. Western blotting

Berberine treated and untreated cells were lysed in a 1% Triton X-100 solution and processed for immunoblots as already described [6]. Blocked membranes were probed overnight at 4 °C with the primary antibody and then for 2 h with the secondary antibody. Finally, proteins were visualized using the chemiluminescence system. Band intensity was detected by X-ray film scanning with a imaging densitometer (BIO-RAD, Italy). Quantitative phosphorylation was calculated as ratio between phosphorylated and un-phosphorylated protein [10].

2.5. Statistical analysis

Analysis of data was performed using Student's *t* test (unpaired analysis). Differences are considered significant at a value of $p < 0.05$. All data are reported as mean ± SD (standard deviation) of at least three independent experiments in duplicate.

3. Results

3.1. Berberine slows cell proliferation by cell accumulation in G_0/G_1 phase of cell cycle

To verify if berberine may inhibit cell proliferation of ADPKD cells, we have treated normal (4/5) and cystic (9.7 and 9.12) cells with different doses of berberine (1, 10 and 100 μg/mL). The treatment with 10 μg/mL of berberine causes a significant reduction of cell proliferation in 9.7 and 9.12 cells, but not in normal 4/5 cells (Fig. 1A). At 100 μg/mL the effect of this molecule is stronger but also affects control cells (Fig. 1A). At this dose, the reduction in cell number is associated with an overall increase of dead cells, not observed at lower doses (Fig. 1A). The decrease in cell proliferation is still maintained after 48 and 72 h of culture with 10 μg/mL of berberine in 9.7 and 9.12 cystic cells only. As observed for 24 h of culture, berberine at 100 μg/mL affects also 4/5 control cells (Fig. 1B and C) and induces cell death in all cell types (data not shown). Consistently, the reduction of cell growth after 10 μg/mL of berberine is also shown in mouse Pkd1$^{(-/-)}$ null cells as well as in Pkd1$^{(+/-)}$ heterozygous cells which are both PKD1 haploinsufficient (Fig. 1D). In these cells, the treatment with 100 μg/mL of berberine still induces cell death, although not as strong as in human cells (Fig. 1D and A, respectively). By a colorimetric method, the reduction of cell growth after berberine treatment (10 and 100 μg/mL) in both human and mouse cystic cells has been also confirmed (Fig. 2A and B). No significant changes after treatment with 1 μg/mL of berberine are observed (Fig. 1A, D and Fig. 2B).

The effect of berberine on cell proliferation has been also evaluated by cell cycle analysis in human normal and ADPKD cells. The treatment with 1 μg/mL of berberine does not modify the cell cycle (Fig. 2C), but at the dose of 10 μg/mL a significant increase of G_0/G_1 phase in 9.7 and 9.12 cystic cells compared with untreated cells is observed (Fig. 2C). Conversely, an higher dosage of berberine (100 μg/mL) causes a strong rise of G_0/G_1 phase in all cell lines, with the appearance of an apoptotic pre-G_1 peak in 4/5 control cells (arrow in Fig. 2C).

In the light of these findings, 10 μg/mL of berberine seems to be the optimal dose capable to reduce cell growth in ADPKD cells without inducing cell death.

3.2. Berberine decreases cell proliferation through the reduction of ERK and p70-S6 kinase activity

Since berberine inhibits the metastatic potential of melanoma cells through a decrease in ERK activity [26], we have investigated whether the berberine-induced reduction of cell proliferation in ADPKD cystic cells occurs through the inhibition of ERK kinase. As expected, in normal conditions (1% FBS), ERK activity is higher in cystic than in control cells (Fig. 3A). After treatment with 10 μg/mL of berberine a statistically significant reduction in ERK phosphorylation in 9.7 and 9.12 cystic cells only is observed (Fig. 3A). Moreover, berberine is able to reduce the activity of p70-S6 kinase, a downstream effector of mTOR in 9.7 and 9.12 cystic cells (Fig. 3B). Data are also confirmed in mouse Pkd cystic cells (data not shown).

These findings suggest that this molecule may reduce cell proliferation in ADPKD cystic cells by inhibiting the activity of ERK and p70-S6 kinases.