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Original Article

Suppression of spindly delays mitotic exit and exacerbates cell death response of cancer cells treated with low doses of paclitaxel

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ABSTRACT

Microtubule-targeting agents (MTAs) are used extensively for the treatment of diverse types of cancer. They block cancer cells in mitosis through the activation of the spindle assembly checkpoint (SAC), the surveillance mechanism that ensures accurate chromosome segregation at the onset of anaphase. However, the cytotoxic activity of MTAs is limited by premature mitotic exit (mitotic slippage) due to SAC silencing. Here we have explored the dual role of the protein Spindly in chromosome attachments and SAC silencing to analyze the consequences of its depletion on the viability of tumor cells treated with clinically relevant doses of paclitaxel. As expected, siRNA-mediated Spindly suppression induced chromosome misalignment and accumulation of cells in mitosis. Remarkably, these cells were more sensitive to low-doses of paclitaxel. Sensitization was due to an increase in the length of mitotic arrest and high frequency of multinucleated cells, both correlated with an exacerbated post-mitotic cell death response as determined by cell fate profiling. Thus, by affecting both SAC silencing and chromosome attachment, Spindly targeting offers a double-edged sword that potentiates tumor cell killing by clinically relevant doses of paclitaxel, providing a rationale for combination chemotherapy against cancer.

Introduction

Attachment between kinetochores and microtubules during prometaphase is monitored by the spindle assembly checkpoint (SAC) that halts the metaphase-to-anaphase transition until all chromosomes are fully attached and properly bioriented at the metaphase plate [1,2]. SAC is activated by the presence of unattached or misattached kinetochores. As a consequence, a diffusible mitotic checkpoint complex (MCC) is formed between the proteins MAD2, BUBR1, BUB3, and CDC20, which promotes the inhibition of the anaphase-promoting complex/cyclosome (APC/C) [3]. APC/C is an E3 ubiquitin ligase that targets securin and cyclin B1 for degradation, thereby promoting sister-chromatid separation and mitotic exit [4]. Once all chromosomes are correctly aligned at the metaphase plate, SAC is switched off in a process called SAC silencing, which in turn relieves the inhibition of APC/C required for the completion of mitosis [1]. Several mechanisms have been proposed to contribute to SAC silencing, including the dissociation of MCC [5,6], and the stripping of SAC proteins from the kinetochore by a dynein motor [7,8].

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Microtubule-targeting agents (MTAs) such as paclitaxel remain amongst the most effective cancer chemotherapeutics used in the clinic [9]. By preventing mitotic spindle assembly, MTAs delay cells in mitosis through chronic activation of SAC [10]. The fate of cells held in mitosis by MTAs is influenced by the duration of the mitotic arrest, and varies greatly between cancer cells [11,12]. Prolonged mitotic arrest can lead to cell death in mitosis (DiM) through the accumulation of an apoptotic signal from the intrinsic pathway [13]. However, some cells can prematurely degrade cyclin B, before the accumulation of the apoptotic signal, and, thus, exit mitosis back to interphase without chromosome segregation, through a process known as mitotic slippage. Depending on the robustness of a still unclear post-mitotic response, these cells may either undergo post-mitotic death (PMD), or enter a new cell cycle [14]. Therefore, having a control over the different cell fates should influence the effectiveness of MTAs in cancer therapy. For instance, delaying mitotic slippage should allow more time for death signals to accumulate thereby shifting cancer cell fates to death.

Spindly protein is a kinetochore-specific regulator of dynein and functions in chromosome alignment and SAC signaling [15]. Knockdown of Spindly was reported to cause unstable kinetochore-microtubule interactions, severe chromosome alignment defects, and extensive prometaphase delay [16,17]. Importantly, kinetochore dynein-mediated removal of Spindly is critical for checkpoint silencing [18,19].

Our goal was to shift the fate of cancer cells exposed to clinically relevant doses of paclitaxel towards death. To achieve this goal, we investigated Spindly's dual role in chromosome alignment and in SAC silencing, exploring the possibility of targeting Spindly as a strategy to delay mitotic slippage in paclitaxel-based treatment. We show, for the first time, that targeting cancer cells with a combination of Spindly downregulation and clinically relevant doses of paclitaxel significantly promotes tumor cells death. This significant cell death response is due to an increase in the length and frequency of mitotic arrest and to severe multinucleation of treated tumor cells. The results provide a rationale for a promising future combination chemotherapy.

Materials and methods

Cell lines and culture conditions

HPAEpiC (human pulmonary alveolar epithelial cells), Calu-3 (human lung adenocarcinoma) and A549 (human adenocarcinoma alveolar basal epithelial) cells were grown in DMEM medium with 10% fetal bovine serum (FBS, Biochrom) and 1% non-essential amino acids (Sigma–Aldrich Co., Saint Louis, MO, USA). NCI-H460 (human non-small cell lung cancer) cells were grown in RPMI-1640 culture medium (Lonza, Basel, Switzerland) with 5% FBS. Cells were maintained at 37 °C in a 5% CO_2 humidified incubator and all experiments were performed when exponentially growing cells presented more than 95% viability.

RNA isolation and quantitative real-time PCR

Total RNA was extracted using the PureZOL™ RNA Isolation Reagent (Bio-Rad Laboratories, Inc. Hercules, CA, USA), according to the manufacturer's instructions and quantified through spectrophotometry (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad), following supplier's instructions and was amplified using iQ™ SYBR Green Supermix Kit (Bio-Rad) on iQ Thermal Cycler (Bio-Rad), according to the following program: initial denaturing step at 95.0 °C for 3 min; 40 cycles at 94.0 °C for 20 s; 60.0 °C for 30 s and 72.0 °C for 30 s. The melt curve included temperatures from 65.0 to 95.0 °C, with increments of 0.5 °C for 5 s. Primers, used at final concentration of 10 μM, were as follows: Spindly: forward 5′-CTC AAA GAG GCT GAA GAA GAG-3′ and reverse 5′-TGT TCA TAA CTC TCA GTC ATG G-3′; Actin: forward 5′-AAT CTG GCA CCA CAC CTT CTA -3′ and reverse 5′- ATA GCA CAG CCT GGA TAG CAA-3′. Experiments were performed in triplicate for each data point. Data were acquired with CFX ManagerTM Software (version 1.0, Bio-Rad) and the results were analyzed according ΔCT and normalized against actin expression levels, which was used as control template. A fold value of mRNA level $\geq$ or $\leq$1.5 relatively to that of normal cells was considered as over- or underexpression, respectively.

Gens analyzed in this study

To avoid gene symbol homonymy and species confusion [20], we list here gene symbols and their NCBI ID numbers. Genes under study included Spindly (SPDL1; NCBI ID# 54908), Actin (ACTB; NCBI ID# 60), α-Tubulin (TUBA1B; NCBI ID# 10376), PARP-1 (PARP1; NCBI ID# 142) and Cyclin B1 (CCNB1; NCBI ID# 891).

siRNAs transfection

For siRNAs transfection, cells were seeded in 22 mm poly-L-lysine-coated coverslips in 6-well plates or in 6-well dishes containing complete culture medium and, 24 h later, transfected using INTERFERin siRNA Transfection Reagent (PolyPlus, New York, USA) according to the manufacturer's instructions. The culture medium was replaced 24 h post-transfection with fresh medium. A validated siRNA sequence against Spindly (synthetized by Sigma–Aldrich) or a validated negative control siRNA (AllStars Negative Control siRNA, Qiagen, Germantown MD, USA) was used at a final concentration of 100 nM [18,21].

Cell extracts and Western blotting

Total cell protein extracts were prepared and Western blot analysis carried out as previously described [21]. The primary antibodies used were: rabbit anti-Spindly (1:3000, gift from Dr. R. Gassmann, IBMC/i3S, Portugal); rabbit anti-cyclin B1 (1:500, C8831, Sigma–Aldrich); mouse anti-PARP-1 (1:2000, H-250, sc-7150, Santa Cruz Biotechnology); rabbit anti-α-tubulin (1:2000, Abcam) and mouse anti-α-tubulin (1:5000, T568 Clone B-5-1-2, Sigma–Aldrich). Horseradish peroxidase (HRP)-conjugated secondary antibodies were diluted at 1:1500 (anti-mouse, Vector) or at 1:1000 (anti-rabbit, Sigma–Aldrich). The protein signal intensity quantification was performed using ImageJ 1.4v software (http://rsb.info.nih.gov/ij/) and normalized against α-tubulin expression levels.

Indirect immunofluorescence

Cells grown on coverslips were fixed in fresh 2% (w/v) paraformaldehyde (Sigma–Aldrich) in phosphate-buffered saline (PBS) for 12 min, rinsed three times in PBS, and permeabilized with 0.5% Triton X-100 (Sigma–Aldrich) in PBS for 7 min. Alternatively, to visualize spindle microtubules, cells were immediately fixed in −20 °C cold methanol (Sigma–Aldrich) for 10 min and rehydrated twice for 5 min in PBS. Then, cells were blocked with 10% FBS in PBST (0.05% Tween-20 in PBS) for 30 min at room temperature, followed by 1 h incubation with primary antibodies diluted in 5% FBS in PBST. The following primary antibodies were used: human anti-CREST (1:4000, gift from E. Bronze-da-Rocha, University of Porto, Portugal); mouse anti-α-tubulin (1:2500, T568 Clone B-5-1-2, Sigma–Aldrich) and rabbit anti-Spindly (1:3000, gift from Dr. R. Gassmann, IBMC/i3S, Portugal). After washing in PBST, cells were incubated for 1 h with Alexa Fluor 488 and 568 conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA), diluted at 1:1500. DNA was stained with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) diluted in Vectashield mounting medium (Vector, H-1000, Burlingame, CA, USA).

Mitotic index determination

Mitotic index, the percentage of mitotic cells over the total cell population, was determined by cell-rounding under phase-contrast microscopy in 48 h control- or Spindly siRNA-treated cells. Alternatively, mitotic index was determined under fluorescence microscope after DNA staining with DAPI. Paclitaxel (Sigma–Aldrich) was used at concentrations ranging from 0 to 100 nM. This clinically relevant dose range of paclitaxel were adapted from previous studies on breast cancer cell lines [22] and on lung cancer cell lines [23]. Nocodazole (Sigma–Aldrich) was used as a positive control at 1 μM. For each condition, more than 2000 cells were counted from random microscope fields.

Cell viability assay

Cell viability was determined with MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (Sigma–Aldrich). A total of 5×10^4 control- and Spindly siRNA-treated cells were seeded in 96-well plate, allowed to attach for 6 h and treated with paclitaxel (0–100 nM). Forty-eight hours later, cells were placed in fresh FBS-free medium and 20 μl MTT reagent (5 mg/ml in PBS) was added and incubated at 37 °C and 5% CO_2 for 4 h. The purple formazan crystals were solubilized with a detergent solution (89% (v/v) 2-Propanol, 10% (v/v) Triton X-100, 1% (v/v) HCl 3.7%), for 2 h. Optical density was measured at 570 nm in a microplate reader (Biotek Synergy 2, Winooski, VT, USA) and retrieved through the Gen5 software (version 1.07.5, Biotek, Winooski, VT, USA). Cell viability was calculated relative to control siRNA-treated cells.

Colony forming assay

A total of 500 cells from 24 h control- or Spindly siRNA-transfected cell cultures were seeded in six-well plates, allowed to attach for 6 h, and treated with 4 nM of paclitaxel. Forty-eight hours later, paclitaxel was removed by washing cells twice with PBS, then fresh medium was added and cells were allowed to grow for 10 days.