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Biochimica et Biophysica Acta 1744 (2005) 245–255
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Paclitaxel resistance in cells with reduced β-tubulin

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Received 1 November 2004; accepted 20 December 2004
Available online 5 January 2005

Abstract

We previously described the isolation of colcemid resistant Chinese hamster ovary cell lines containing α- and β-tubulin mutations that increase microtubule assembly and stability. By analyzing colcemid sensitive revertants from one of the β-tubulin mutants, we now find that loss or inactivation of the mutant allele represents the most common mechanism of reversion. Consistent with this loss, the revertants have 35% less tubulin at steady state, no evidence for the presence of a mutant polypeptide, and a normal extent of tubulin polymerization. In addition to the loss of colcemid resistance, the revertant cells exhibit increased resistance to paclitaxel relative to wild-type cells. This paclitaxel resistance can be suppressed by transfecting the revertant cells with a cDNA for wild-type β-tubulin, indicating that the reduction in tubulin in the revertant cells is responsible for the resistance phenotype. We propose that reducing tubulin levels may represent a novel mechanism of paclitaxel resistance.

Keywords: Colcemid resistance; Gene inactivation; Microtubule assembly; Drug resistance; Tetracycline regulated expression

1. Introduction

Microtubules are essential cytoskeletal structures built from soluble αβ tubulin heterodimers. These heterodimers assemble end-to-end to form linear protofilaments, and side-to-side to form hollow tubes that normally contain 13 protofilaments. Unlike lower eukaryotes that may have only 1 or 2 genes encoding α- and β-tubulin, vertebrate tubulin is encoded by a multigene family, members of which are differentially expressed in all tissues [1,2]. The β-tubulin gene products are very homologous, but differ most radically in their last 15 amino acids. Interestingly, these variable C-terminal sequences are highly conserved across vertebrate species and serve as the basis for classification of 7 distinct β-tubulin isotypes: β1, β2, β3, β4a, β4b, β5, and β6 [3]. Microtubules in most vertebrate cells contain a mixture of 2 or more of these isotypes.

A large number of drugs are known to interfere with microtubule assembly, and they have many important clinical uses, most notably in the treatment of cancer. Some drugs like vinblastine, vincristine, and colcemid bind to soluble tubulin heterodimers and inhibit the assembly of microtubules; while others like paclitaxel, docetaxel, and epothilones bind to polymerized tubulin and inhibit microtubule disassembly [4]. Microtubules are very dynamic structures, and the ability to rapidly assemble and disassemble is critical to their function. Thus, drugs that either stabilize or destabilize microtubules can inhibit assembly dynamics and produce cytotoxicity [5]. Because microtubules represent a unique and effective target in cancer chemotherapy, much effort is being devoted to finding new and better drugs that act by disrupting microtubule function. One of the major limitations in using these drugs, however, is the emergence of drug resistant tumor cells.

Several mechanisms by which cells can acquire resistance to these drugs have been described [6–8]. For example, *P*-glycoprotein mediated multidrug resistance (MDR) limits

Abbreviations: 2D, two-dimensional; CHO, Chinese hamster ovary; GST, glutathione-*S*-transferase; HA, hemagglutinin; MAP, microtubule associated protein; MDR, multidrug resistance; MTB, microtubule buffer; OD, optical density; RT-PCR, reverse transcription-polymerase chain reaction; TTA, tetracycline transactivator; UTR, untranslated region

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doi:10.1016/j.bbamcr.2004.12.003

the intracellular accumulation of a large number of hydrophobic, weakly cationic compounds that include most of the common microtubule active agents [9]. Target-specific mechanisms have also been reported, including mutations in α- and β-tubulin that alter microtubule stability to counteract the action of the drug [10–17], mutations in tubulin that affect drug binding [18–20], increased expression of specific tubulin genes [21,22], and changes in the synthesis or activity of tubulin interacting proteins [23–27].

Our approach to study drug resistance uses the selection of Chinese hamster ovary (CHO) cell lines that survive treatment with a single lethal dose of a tubulin binding agent [6]. One of the cell lines obtained in this way is Cmd 4, a colcemid resistant and temperature sensitive mutant with a D45Y amino acid substitution in one of its two β1-tubulin gene products [10,13]. To further understand the mechanisms responsible for how cells respond to tubulin binding agents, we selected revertants of this mutant that lost colcemid resistance. In a previous publication we reported that one mechanism of reversion to colcemid sensitivity involved intra-allelic mutations that counteract the microtubule stabilizing effects of the D45Y substitution, while a second mechanism involved a mutation that appears to increase the affinity of tubulin for colcemid [13]. Here we report that the major mechanism of reversion involves the loss or disruption of the D45Y allele such that no mutant tubulin is produced. Unexpectedly, we found that cells that had lost the mutant allele were resistant to the effects of paclitaxel.

2. Materials and methods

2.1. Growth and derivation of cell lines

Cells were grown in alpha modification of minimum essential medium (Sigma-Aldrich, St. Louis, MO) containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) at 37 °C and 5% CO_2. Colcemid resistant mutant Cmd 4 was isolated from wild-type CHO cells following mutagenesis with ethyl methanesulfonate as previously described [10]. Because Cmd 4 is temperature sensitive for growth and has increased sensitivity to paclitaxel [28,29], spontaneous revertants 3H1, 3H3, 3H4, 3L2, and 3H6 were isolated by selecting cells able to grow at the non-permissive temperature (40.5 °C); whereas the remaining revertants (TT2, 3, 5, 6, and 7) were selected using a combination of elevated temperature and 20 nM paclitaxel.

2.2. Transfection

Cmd 4 revertant 3H4 was transfected with pTOP-TTApuro, a plasmid containing a puromycin resistance gene driven by a constitutive SV40 promoter, and a tetracycline transactivator (TTA) gene under the control of a tetracycline regulated promoter [12,30]. Stable TTA clones were isolated in 10 μg/ml puromycin after transfecting with the plasmid DNA using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each TTA clone was tested for expression of the transactivator gene by carrying out a second transient transfection with pTOPHAβ1neo, a plasmid containing a CHO β1-tubulin cDNA (GenBank Accession no. U08342) fused at the 3′ end to a sequence encoding a 9 amino acid hemagglutinin (HA) epitope, and also under the control of a tetracycline regulated promoter. At 24 h post-transfection, each of the TTA clones was screened for efficiency of expression of the HAβ1-tubulin in the presence and absence of tetracycline using immunofluorescence with a rabbit antibody specific for the HA tag (Bethyl Laboratories, Montgomery, TX). The clone giving the highest efficiency of expression with strong suppression by tetracycline was then used to obtain new stable clones expressing HAβ1-tubulin by selection in 2 mg/ml G418 (geneticin, Cellgro Inc., Herndon, VA).

2.3. Drug resistance

Approximately 200 cells from each cell line were seeded into each of 6 replicate wells in 24-well dishes containing growth medium with increasing concentrations of drug and incubated for 7 days until visible colonies appeared. The surviving cells were then stained with 0.5% methylene blue in water, dried, and photographed. To calculate IC_{50} values, the cell-associated dye was solubilized using 200 μl of 1% SDS in 50 mM Tris Cl, pH 6.8 and 100 μl from each well was then transferred to a 96-well dish and the absorbance at 630 nm was read using an Emax microplate reader (Molecular Dynamics, Sunnyvale, CA). Graphs of optical density versus drug concentration were plotted using "pro Fit" software (QuantumSoft, Uetikon am See, Switzerland) and IC_{50} values were calculated as the concentration of drug that inhibited cell growth by 50%.

2.4. Two-dimensional gel electrophoresis

Cells were labeled for 30 min with 20 μCi/ml Tran ^{35}S-label (mixture of methionine and cysteine, 1000 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) in methionine-free minimum essential medium (Sigma-Aldrich) and lysed with hot (100 °C) SDS dissociation buffer [31]. Solubilized proteins were precipitated with 5 volumes of cold acetone. The protein pellets were resolubilized in a urea sample buffer and analyzed by 2D gels as previously described [32,33]. Proteins were visualized by exposing the dried gels to X-Omat film (Eastman Kodak, Rochester, NY).

2.5. Measurement of assembled tubulin

To measure the distribution of tubulin between non-assembled and polymerized pools, cells were grown to