Increased cell cycle arrest with ectopic Op18-Q18E mutation compared with ectopic wild-type Op18/Stathmin

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Op18/Stathmin is a catastroph-promoting protein of microtubules (MTs). In esophageal adenocarcinoma tissue, a mutation where Gln18 changed to Glu was discovered that impairs the regulation of Op18. By flow cytometry analysis of DNA and MTs we have seen the mutation in leukemia K562 cell line had an increased frequency of arrest in G2/M phase than wild-type of Op18. This would suggest Op18-Q18E is more an obstackle for cancer formation rather than a benefit.

Introduction

The eukaryotic cell contains an intra organizing system of threads stretching outwards from the cell center to the cell surface. These threads are a part of the cytoskeleton and consists of heterodimers of α -tubulin and β -tubulin. Together they can polymerize into long threads, which is called *microtubules* (MTs). Along the MT runs energy-driven proteins, transporting substances along the way. MT directs signal and cell growth to various parts of the cell. Regulaton of MT is therefore an important quest to maintain homeostasis and can be done by changing the activity of polymerization of the tubulin building blocks. Oncoprotein 18/Stathmin (Op18) is a protein found in a wide range of animals (Bo Segerman, 2003). The protein regulates MT stability, meaning increses the ability for MT to depolymerize. The regulation is done through phosphorylation of four Ser residues (S16, S25, S38, S63). Each phosphorylated site lowers its activity (Gavet *et al*, 1998; Horwitz *et al*, 1997).

In esophageal adenocarcinoma tissue (Larsson *et al*, 1999) identified a point mutation in op18 where Gln18 had changed to a Glu. The Op18-Q18E

Table 1: DNA histogram through flow cytometry. Numbers represent the amount of cells in G2/M phase compared to G1 phase. Cells were induced in 0.5 μ M Cd(II)-containing media and harvested 0h or 24h after induction. 24h samples were either cultivated in 0.5 μ M Taxol or not.

	Vector-Co	Op18-wt	Op18-tetraA	Op18-Q18E
0h	24	23	24	24
24h	23	35	52	39
24h+Taxol	58	63	52	66

mutation is unable to phosphorylate Ser16, Ser25 has a reduced ability to be phosphorylated while Ser38 is almost unaffected (Misek *et al*, 2002). We examined the activity of the Q18E mutation in Op18 and found that cell cycle arrest were more expressed in Op18-Q18E than in normal Op18-wt.

Results

A few years ago a point mutation in Op18 was discovered in esophageal adenocarcinoma tissue. At residue 18 a Gln had changed to Glu (Larsson *et al*, 1999), causing an interference in phosphorylation of Ser16 and Ser25 (Larsson *et al*, 1999).

We examined the activity of this mutation by transfecting K562 cells as described in "Matherials and methods". The resulting DNA histogram in Table 1 reveals an increased fraction of Op18-wt cells that are in G2/M phase after 24h of induction compared with Vector-Co. An almost complete cell cycle arrest occured in Op18-tetraA after 24h (compared with 24h + Taxol). Interestingly Op18-Q18E places itself in between the wild-type Op18 and Op18-tetraA. This does not a contradict that Op18-Q18E could have a different activity than normal Op18-wt.

MT are formed by polymerization of α - and β -tubulin heterodimers. MT analysis in Table 2 shows that the amount of non-polymerized tubulin was greatly increased in ectopic Op18 cells compared with normal cells (Vector-Co). This is not contradicting with the theory of Op18 as a MT-destabilizing agent. The change in polymerization of Op18-tetraA were lowest (except Vector-Co) and Op18-Q18E had a slightly higher change of polymerization than Op18-wt.

Western Blot analysis showed ectopic Op18 were expressed in all samples except in Vector-Co (my unpublished data). Also, all samples (including Vector-Co) expressed endogenous Op18.

Table 2: MT flow cytometry analysis. Numbers represent median-value of percent polymerized tubulin in K562 cell line after 0h and 8h of Cd(II)-induction. The polymerization status was calculated as described in (Holmfeldt *et al*, 2001). The change is the difference in percent units of the two polymerization states.

	Vector-Co	Op18-wt	Op18-tetraA	Op18-Q18E
0h	58	38	34	37
8h	49	8	9	5
change	9	30	25	32

Discussion

Op18/Stathmin destabilizes MT both through catastrophe promoting activity and tubulin sequestering (Howell *et al*, 1999). Wild-type Op18 is regulated through phosphorylation of four serine residues. When these sites are phosphorylated the MT destabilization activity of Op18 decreases (Gavet *et al*, 1998; Horwitz *et al*, 1997). If the phosphorylation of one or more of these sites would be inhibited a lower degree of polymerized MT is expected.

In Table 2, we saw that the change in activity of Op18-Q18E does not differ much from wild-type Op18. However, the decrease in polymerized tubulin (i.e MT formation) is not contradicted by the destabilizing activity of Op18.

In our experiment, DNA histogram revealed K562 leukemia cells were found stuck in the G2/M-phase more frequently than both the Vector control group and the ectopic Op18-wt (Table 1). But also less frequent than the Op18-tetraA group. It has been demonstrated that the Op18-Q18E mutation inhibits the phosphorylation of Ser16 and Ser25 (Misek *et al*, 2002), making the microtubules less stable (Table 2). Together with our results this would suggest the mutation is more of an obstackle for cell proliferation than a benefit. It is therefore possible that Op18-Q18E has no direct involvment in cancer formation, but rather arise as a by-product of the higher frequency of mutation in cancer cells, at least *in vitro*.

However, overexpression of Op18 has been found in several cancer strains of different origin (listed in Misek *et al*, 2002), and *in vivo* studies have indeed shown that eptotic Op18 expression can cause tumor growth (Misek *et al*, 2002) (though the mice had reduced immune defence). It may therefore be that the mutation can have other, currently unknown, effects on cellcycle regulation and/or apoptosis that may outweight the slightly decreased proliferation ability that is caused by the more fragile microtubules.

We expressed ectopic Op18 in cell lines that already were cancer cells. One could spekulate whether the results of ectopic expression of Op18 (with or without mutation) in cancer tissue can be applied to normal tissue with expression of ectopic Op18. I would think not.

Matherials and methods

DNA construct, transfection and cell cultivation We used the vector pMEP4 (given by the Martin Gullberg group in Umeå university) as a carrier for cDNA. Four premade DNA-mixture were (provided by the Martin Gullberg group) containing 14 μ g recombinant pMEP4, 5 μ g CMV-EBNA1 and 25 μ g pBlueScript (explained in (Marklund *et al*, 1994)) in a total volume of 50 μ l PBSA. Recombinant pMEP4 contained no cDNA as a vector control (Vector-Co), cDNA for wild-type Op18 with an unique amino acid sequence added as a "flag-epitope" (Op18-wt-F) recognized by a certain anti-body. cDNA for Op18 where all sites of phosphorylation have been replaced with Ala (Op18-tetraA-F), cDNA for Op18 where Gln on position 18 was replaced with Glu (Op18-Q18E).

Cells were cultivates as described in (Marklund *et al*, 1994). In brief; 20×10^6 , K562 leukemia cells were transfected and cultivated over-night (37°C, 5% CO₂) in a Leighton-tube with 0.8 ml 25 μ M EDTA-containing special media (described in Marklund *et al*, 1994). Then cells were recultivated in small tissue culture flask containing 0.5 mg/ml Hygromycin B in 30 ml special media during 5 days. Then the hMTIIa promotor were induced in 0.5 μ M CdCl₂. Cells were harvested after 0h of Cd(II) induction for MT and DNA flow cytometry, 8h for MT flow cytometry and protein for Western Blot, 24h for DNA flow cytometry + DNA flow cytometry of cells cultivated in 0.5 μ M Taxol.

Antibodies and reagents MT were stained with $anti-\alpha$ -tubulin antibody (SIGMA) and green flourescence stained with Rabbit-anti-mouse antibody (FITC) from Dako. Hygromycin B came from Roche. DNA was stained with propidium iodide.

MT polymerazion analysis, DNA flow cytometry and Western bloting Protein separation and western blotting were handled by the kind members of the Martin Gullberg group. In brief, separation was done on a 10% to 20% gradient SDS-PAGE and western blotted with a special Flag-epitope antibody and anti-Op18.

MT-polymerization analysis was performed as in (Holmfeldt *et al*, 2001), but with no degree of freedom. MT-stabilization- and MT-fixation buffers used were provided by the Martin Gullberg group. DNA flow cytometry was performed as in (Marklund *et al*, 1996). Flourescence microscopy was also prepared with MT-staining, but results were not "interesting enough" to be included in this article (since I did not know what to look for).

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