Microtubule inhibition causes epidermal growth factor receptor inactivation in oesophageal cancer cells

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Abstract. Drugs that interfere with microtubule function can prevent cells from mitosis and may cause cell cycle arrest or apoptosis. Various microtubule targeting agents, both stabilizers and inhibitors, are used in a clinical setting to treat cancer. In the current study, we investigated the sensitivity of oesophageal cancer cells to different microtubule targeting agents. The current study demonstrated that different microtubule targeting agents disrupted the microtubule network and inhibited survival of oesophageal cancer cells in a dose-dependent manner. Interestingly, an additional cellular effect with inhibition of tyrosine phosphorylation of the EGFR and subsequent downregulation of EGFR-induced signalling was also observed, suggesting an additional mechanism of action for microtubule destabilising agents. A tyrosine phosphatase inhibitor, sodium orthovanadate, could reverse the EGFR dephosphorylation effects induced by microtubule targeting agents. The EGFR dephosphorylation could be reversed by a tyrosine phosphatase inhibitor, indicating that disruption of the microtubule network may lead to activation of a protein tyrosine phosphatase (PTP) that can regulate EGFR phosphorylation and activation, an effect of potential clinical relevance for combination therapies in patients.

Introduction

The cytoskeleton is comprised of actin filaments, intermediate filaments and microtubules. Microtubules are dynamic structures that are important for a range of cellular functions, such as intracellular trafficking, cell movement and division, where they are involved in chromosome segregation. α- and β-tubulin heterodimers polymerise into a hollow tube denoted microtubule (1,2). The stability of the microtubule is regulated through GTP-hydrolysis of β-tubulin; binding of GTP allows polymerisation, but within the microtubule GTP can be hydrolysed to GDP (1,2). Microtubules constantly polymerise and depolymerise, a process termed dynamic instability. Since microtubules play an essential role in chromosome segregation, drugs that interfere with microtubule function prevent the cell from mitosis. A number of microtubule targeting drugs are used in the clinic to treat human cancers, including oesophageal cancer. Examples are the vinca alkaloids that destabilise microtubules by binding close to the GTP-binding region in β-tubulin (3), and the taxanes which bind to polymerised microtubules and stabilise the GDP-bound form of β-tubulin by forcing them into a configuration resembling the GTP-bound state (4).

Growth factors and their receptors have been shown to be of pivotal significance for the occurrence and development of cancer. The human epidermal growth factor receptor (HER) family is comprised of four members, i.e. EGFR (HER1, ErbB1), HER2 (ErbB2, Neu), HER3 (ErbB3) and HER4 (ErbB4) (5). These receptors are tyrosine kinases that are activated by ligand-induced dimerisation. There are several ligands for the receptors in the HER family, and these have different binding specificities, resulting in formation of homo- or heterodimeric receptor complexes. HER family members are commonly activated in human cancer cells by different mechanisms including autocrine stimulation, mutations or overexpression (6). Dysregulated and improper receptor activation leads to induction of signals that promote proliferation, survival, migration and angiogenesis, events that are all central for tumour development and progression. Over-activated EGFR is recognised as an important mechanism in several types of cancer, including colorectal cancer (7), head and neck cancer (8), and non-small cell lung cancer (9) and has become a target of interest in the treatment of these tumours.

There are data indicating possible interactions between EGFR and the microtubule system. Gao et al demonstrated that histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, associates with the endosomal compartments
and controls EGFR trafficking and degradation (10). This is consistent with data from Deribe et al showing that HDAC6 negatively regulates EGFR endocytosis and degradation by controlling the acetylation status of α-tubulin and subsequently receptor trafficking along microtubules (11).

Oesophageal carcinoma is the seventh most common cause of cancer-related death in the Western world (12). The standard treatment for localised oesophageal carcinoma includes a combination of radiation and chemotherapy, sometimes followed by surgery. Preoperative chemoradiotherapy or chemotherapy (13) has been demonstrated to give a significant survival benefit. However, patients with advanced metastatic disease that are treated with palliative chemotherapy have a poor prognosis with a median survival time of less than one year. The 5-year survival rate of all diagnosed patients is only around 15%. Thus, there is an urgent need to improve current therapies. In oesophageal carcinoma patients, EGFR has been reported to be commonly overexpressed (14) and the overexpression is correlated to lymph node metastasis, vascular invasion and shorter survival (15-17). The EGF and TGF ligands function as mitogens for oesophageal tumour cells (18) and activation of EGFR signalling has been implicated in metastasis via modulation of cell adhesion, angiogenesis, invasion and migration.

In the current study we have investigated the possible interaction of anti-microtubule drugs and the EGFR signalling system in human oesophageal cancer cells. Treatment with the drugs led to inhibition of proliferation of the cells. Additionally, microtubule destabilising agents were also shown to inhibit EGFR phosphorylation. These effects could be inhibited by simultaneous addition of the protein tyrosine phosphatase inhibitor sodium orthovanadate, suggesting that disruption of the microtubule network leads to release or activation of a tyrosine phosphatase. This study shows that microtubule targeting drugs have other effects beyond interfering with the mitotic spindle.

Materials and methods

Cell culturing and counting. The human ESCC (oesophageal squamous cell carcinoma) cell lines Kyse30, Kyse70, Kyse140, Kyse150, Kyse180, Kyse410, Kyse450, Kyse510 and Kyse520 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were cultivated in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 units of penicillin and 50 µg streptomycin/ml (Sigma-Aldrich, St. Louis, MO, USA). Cells were split twice a week by incubation in 13-20 µl/cm² 2% Triton X-100. Coverslips were blocked with 10 mM glycine at room temperature for 1 h, incubated with primary mouse anti-α-tubulin antibody (Sigma-Aldrich), followed by incubation with a secondary polyclonal goat anti-mouse antibody labelled with FITC (Dako, Glostrup, Denmark). The nuclei were stained with DAPI. Coverslips were mounted on object slides using Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, USA). Microtubule staining was visualised using a Zeiss immunofluorescence microscope at x40 magnification.

Immunoprecipitation and western blot analysis. Subconfluent Kyse70 and Kyse140 cells were treated with different concentrations (0.5, 5 and 10 µM) of PPT, vincristine or docetaxel for 24 and 48 h in starvation medium containing 0.1% FBS. Subsequently, cells were stimulated with 100 ng/ml EGF (Chemicon, Temecula, CA, USA) or IGF-1 (R&D Systems, Minneapolis, MN, USA) for 5 min and washed with ice-cold phosphate-buffered saline before lysis. Cell lysates were prepared according to Lennartsson et al (21). Briefly, total protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Total cell lysate (TCL) were submitted to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoprecipitation, antibodies against IGF-1Rβ were added to each lysate at a concentration of 1 µg/ml. Protein A-Sepharose was added in order to collect immunocomplexes. After washing of the beads, samples were boiled in reducing sample buffer and subjected to SDS-PAGE. Separated proteins were electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), membranes blocked using 5% BSA, and then incubated with primary antibody overnight at 4°C. Antibodies used were anti-EGFR, anti-IGF-1Rβ and anti-Akt1/2 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphotyrosine mouse monoclonal antibodies PY99 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphotyrosine mouse monoclonal antibodies PY99 (Santa Cruz Biotechnology), phospho-specific anti-Erk and phospho-specific anti-Akt antibodies (Cell Signalling Technology, Beverly, MA, USA), anti-PTP1B mouse antibodies (BD Biosciences, San Jose, CA, USA), anti-β-actin mouse monoclonal antibodies (Sigma-Aldrich) and anti-Erk2 rabbit serum (Ludwig Institute for Cancer Research, Uppsala, Sweden). Anti-PTP e rabbit serum was from Dr A. Elson (The Weizmann Institute of Science, Israel). EGF was from Chemicon and IGF-1 from R&D Systems. After washing, membranes were...
incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (Amersham Biosciences, Uppsala, Sweden) and proteins visualised using ECL western blotting detection systems (Roche Applied Science, Indianapolis, IN, USA) on a cooled charge-coupled device (CCD) camera (Bio-Rad Life Science, Hercules, CA, USA). The images were analysed using the software Quantity One.

**siRNA interference.** siRNA was employed to knockdown PTPε and PTP1B expression. Anti-PTPε siRNA targets sequence: GCGAACAGGUACAUUCAUA; anti-PTP1B siRNA targets sequence: GGAGAAAGGUUCGUUAAAA; non-targeting siRNA was used as a control (target sequence CGTACGCGGAATACTTCGA). To downregulate PTPIB and PTPε expression, different concentrations of siRNA for anti-PTPIB and anti-PTPε were incubated with Kyse70 and Kyse140 cells for 48 h. Levels of knockdown were tested after measuring protein levels by immunoblotting. Meanwhile, different concentrations of microtubule targeting drugs were added to the cell cultures for 24 h.

**Sodium orthovanadate treatment.** SubconfluentKyse70 and Kyse140 cells were treated with 10 µM PPT, vincristine or docetaxel for 24 h. Before cell lysis, sodium orthovanadate (Na3VO4) was added in the medium at a concentration of 1 mM for 1 h, followed by stimulation with 100 ng/ml EGF for 5 min. Total cell lysates were used for immunoblotting analysis.

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Figure 1. The effect of microtubule interfering drugs on microtubule network in oesophageal carcinoma cells. Kyse70 and Kyse140 cells were grown on coverslips and treated with different concentrations of microtubule targeting drugs PPT, docetaxel or vincristine. Cells were fixed with 2% formaldehyde and microtubules (green) analysed by fluorescence microscopy. The figure is a representative of three repeats.

Figure 2. Effects of microtubule targeting agents on proliferation in (A) Kyse70 and (B) Kyse140 cells. ESCC cells were treated with PPT, docetaxel or vincristine at indicated concentrations for 24 and 48 h, and cell survival analysed using the resazurin assay. Kyse70 and Kyse140 cells were seeded into 96-well plates at a density of 5,000 cells/well. The experiments were done in triplicates and error bars represent ± SD.
Results

Podophyllotoxin (PPT), vincristine and docetaxel interact with the microtubule network and affect survival of ESCC cells. After 24-h treatment, the microtubule destabilising drugs PPT (5 µM) and vincristine (5 µM) disrupted the microtubule network while the microtubule stabilising drug docetaxel (5 µM) stabilised the network (Fig. 1) in oesophageal cancer cells. After 24-h drug treatment, we did not observe nuclear pyknosis and massive apoptotic bodies in either Kyse70 or Kyse140 cells under microscope following DAPI staining. Treatment with PPT, docetaxel and vincristine in Kyse70 and Kyse140 cells resulted in time-dependent plateau-shaped dose-response curves in a proliferation assay, consistent with tubulin interaction and cell cycle arrest. Maximal inhibition (>80%) of Kyse70 (Fig. 2A) was achieved at low concentrations (0.1 µM PPT, 0.1 µM docetaxel and 0.05 µM vincristine at 48 h), while Kyse140 cells appeared slightly less sensitive (Fig. 2B).

Microtubule destabilising drugs decrease EGFR phosphorylation and downstream signalling in ESCC cells. It has been reported that EGFR is commonly overexpressed in oesophageal carcinoma (14). Variable but detectable levels of EGFR were observed in all 9 oesophageal carcinoma cell lines used in this study (Fig. 3). The possible interactions between EGFR and microtubules were investigated by treatment of Kyse70 and Kyse140 cells with increasing concentrations of microtubule targeting drugs for 24 or 48 h, followed by 5 min EGF stimulation. The concentrations used in this study are comparable to or slightly higher than the clinically achievable peak plasma concentrations in patients (22,23). The microtubule destabilising agents PPT (Fig. 4A) and vincristine (Fig. 4B) inhibited EGFR tyrosine phosphorylation after 24 h treatment at all concentration where we did not observe the inhibition of EGFR expression. However, the microtubule stabilising agent docetaxel had a minor inhibitory effect on EGFR phosphorylation after 24 h treatment (Fig. 4C). After 48 h of drug treatment with PPT, vincristine and docetaxel, a substantial inhibition of EGFR expression and phosphorylation could be observed, indicating a possible indirect effect due to the cytotoxicity of microtubule targeting drugs (Fig. 4). Dephosphorylation was observed already after 24 h of treatment whereas EGFR downregulation was most pronounced after 48 h, indicating that inhibition of phosphorylation and receptor downregulation are two distinct events.

To investigate if decreased phospho-EGFR also resulted in decreased signalling downstream of EGFR, cells were treated with microtubule targeting drugs for 24 h thus avoiding EGFR degradation. Docetaxel, PPT and vincristine downregulated phosphorylation as well as protein levels of Akt (Fig. 5) in both Kyse70 and Kyse140 cells. Moreover, the microtubule destabilising agents PPT and vincristine inhibited phosphorylation of Erk but did not cause downregulation of total Erk protein.
levels. Docetaxel had no effect on the activation of Erk in either cell line.

**Microtubule targeting drugs demonstrated no inhibition of phosphorylation or expression of IGF-1R in ESCC cells.** IGF-1R, which is becoming an important target in the treatment of cancer, has been found to be significantly overexpressed in oesophageal squamous cell carcinoma tissue compared with adjacent normal tissue (24). We found that IGF-1R was expressed in all nine ESCC cell lines (Fig. 3). To test whether microtubule targeting drugs can affect IGF-1R, we treated cells with increasing concentrations of microtubule targeting drugs (0.5, 5 and 10 µM) for 24 or 48 h, followed by 5 min of IGF-1 stimulation. We were not able to observe any effect either on ligand-induced IGF-1R phosphorylation or on IGF-1R expression (Fig. 6). Thus, it appears that disruption of the microtubule system selectively inhibits EGFR function over that of IGF-1R, indicating that EGFR downregulation is not caused by general drug cytotoxicity.

**Reduced EGFR phosphorylation induced by microtubule destabilising agents can be reversed by treatment with a tyrosine phosphatase inhibitor.** EGFR dephosphorylation caused by microtubule destabilising agents was not correlated with protein downregulation for all drugs and cell lines. In addition, the EGFR dephosphorylation occurred before substantial receptor downregulation could be seen, suggesting that these effects are independent of each other (Fig. 4). To further explore the mechanism of EGFR dephosphorylation we treated cells for 24 h, to avoid major EGFR downregulation, with microtubule targeting drugs in the presence or absence of the tyrosine phosphatase inhibitor sodium orthovanadate (Na₃VO₄). As can be seen in Fig. 7, sodium orthovanadate treatment to a large extent abolished EGFR dephosphorylation. Thus, one possibility is that disruption of the microtubule network releases or activates a tyrosine phosphatase that can dephosphorylate EGFR but not IGF-1R.

**PTP1B or PTPε downregulation can not reverse EGFR dephosphorylation induced by microtubule disrupting agents.** The tyrosine phosphatase PTPε co-localises with microtubules in cells and its binding to tubulin can inhibit its activity; conversely disrupting microtubule structures increased PTPε activity (25). PTP1B has been reported to interact with endocytosed EGFR and promote its dephosphorylation, and this complex is disrupted by sodium othovanadate (26,27). To further explore which protein tyrosine phosphatase is involved in EGFR dephosphorylation induced by microtubule disruption, RNAi was employed to downregulate PTP1B and PTPε expression in Kyse70 and Kyse140 cells as shown in Fig. 8A and B. Neither PTP1B nor PTPε downregulation could reverse the effect of the microtubule disrupting drugs on EGFR dephosphorylation (Fig. 8C and D). Furthermore, we tested if PTPε downregulation could affect the EGFR dephosphorylation induced by microtubule targeting agents in the A431 cell line which is originated from epidermoid carcinoma and known to overexpress EGFR. PPT dephosphorylated EGFR in both A431 moc (cryPTε negative) and A431 (cryPTε overexpressed) cells (Fig. 8E), proving also in other cells than ESCC cells that PTPε downregulation cannot reverse EGFR dephosphorylation induced by microtubule targeting agents.
Discussion

Microtubules are involved in various cellular functions, including cell adhesion, movement, replication and division. Microtubule inhibition can interfere with chromosome segregation during mitosis and disrupt cell signalling, hence promoting cell cycle arrest and cell death (28,29). In the present study, we have investigated the cytotoxic effects of the microtubule targeting drugs docetaxel, vincristine, and PPT in oesophageal carcinoma cell lines. As expected, microtubule targeting drugs disrupted the microtubule network and inhibited cell survival in oesophageal carcinoma cells. Surprisingly, we also found that disruption of the microtubule network was associated with dephosphorylation of EGFR and subsequent reduced activation of Akt and Erk. Co-treatment with a tyrosine phosphatase inhibitor diminished this effect, suggesting that disruption of the microtubule network leads to exposure of EGFR to an active tyrosine phosphatase. Neither 24 nor 48 h of drug treatment had any effect on IGF-1R phosphorylation or stability, suggesting some degree of receptor selectivity and that the EGFR downregulation is not due to general toxicity.

In both Kyse70 and Kyse140 cells, phosphorylation of Akt was inhibited by docetaxel, vincristine and PPT. However, the degradation of total Akt protein after drug treatment may partially explain the dephosphorylation of Akt. Compared to Akt, Erk protein levels were not affected by drug treatment, while phosphorylation of Erk was inhibited by vincristine and PPT but not docetaxel in both Kyse70 and Kyse140 cells. This suggests that Akt protein levels may be more easily affected by a general drug response and that microtubule destabilisa-
tion, but not stabilisation, affects downstream signalling of EGFR. The observed dephosphorylation of EGFR could be reversed by a tyrosine phosphatase inhibitor, suggesting that a tyrosine phosphatase is activated following microtubule disruption. It is possible that microtubule destabilising agents activate a phosphatase that is selective for EGFR over IGF-1R in ESCC cells. Protein tyrosine phosphatases (PTPs) strictly control receptor tyrosine kinase (RTK) phosphorylation and downstream signalling. Several PTPs have been reported to dephosphorylate tyrosine residues of EGFR and regulate signalling, including T-cell PTP (TCPTP), Src homology phosphotyrosine phosphate 1 and 2 (SHIP 1 and 2), PTP1B, PTPN9, density-enhanced phosphatase-1 (DEP-1), RPTPα and RPTPξ (30-35). So far only PTPβ and PTP1B have been reported to interact with the microtubule system (25-27). However, using siRNA to downregulate PTP1B and PTPβ expression in ESCC cells, we found no evidence supporting that PTP1B or PTPβ downregulation could influence the effect of the microtubule disrupting drugs on EGFR dephosphorylation (Fig. 8C and D). Elucidating which PTP(s) is important for regulation of EGFR phosphorylation in ESCC cells following disruption of the microtubule network is subject for future studies.

The additional mechanism of action of tubulin inhibitors on EGFR signalling suggested in the present work may have clinical impact on the selection of drug combinations for the treatment of oesophageal cancer as well as other cancer types. Several clinical studies involving EGFR targeted therapies in oesophageal cancer have been performed, including the antibody cetuximab as well as the tyrosine kinase inhibitors erlotinib (Tarceva®) and gefitinib (Iressa®). Although not yet a standard of care, the results from these studies suggest that treatment with EGFR targeted therapies, alone or in combination with chemotherapy and/or radiotherapy, is feasible with promising clinical activity (36,37). Ongoing and future clinical trials involving EGFR targeted therapies and anti-tubulin acting chemotherapy in combination is recommended to consider the potential interactions between these treatments, both with respect to clinical efficacy of the treatment and to the selection of appropriate biomarkers.

We demonstrated that microtubule targeting drugs inhibited the survival of oesophageal cancer cells involving a reduction of tyrosine phosphorylation and activation of EGFR, and that this effect is reversible by inhibition of tyrosine phosphatases using sodium orthovanadate. Thus, we propose that PTP1B is involved in the survival of oesophageal cancer cells involving a phosphatase that is selective for EGFR over IGF-1R. These findings may have clinical impact on the selection of chemotherapeutic drug combinations for the treatment of oesophageal cancer as well as other cancer types.

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