CELL CYCLE DEPENDENT CHANGES IN PACLITAXEL CYTOTOXICITY AND PROTEIN O-GLYCOSYLATION

Doctoral (PhD) thesis

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INTRODUCTION

1.Paclitaxel

Eukaryotic cell division is a tightly controlled, complex process which phisiologically results in two, genetically identical daughter cells. Synchronization methods are the most frequently used tools for studying different cell cycle dependent mechanisms. During synchronization, cell cycle progression is blocked at a specific step to gain phase-homologous populations. After resolving the block, the cells continue their cell cycle in a synchronized way. Afterwards, cell cycle dependent parameters can be measured by collecting samples at certain time points. One disadvantage of the technique is the fact that 100% synchronization is difficult to produce, since blocked cells become apoptotic after a relatively short time. Thus, the timing and the duration of synchronization needs to be optimized to each cell population and to each cell cycle phase desired to study, to gain the highest synchronization degree with the lowest presence of apoptotic cells.

One of the most common synchronization agents is the natural chemotherapeutic drug, paclitacel (taxol). Paclitaxel acts by binding to microtubules, and then it induces polymerization and inhibits depolymerization of tubulin bundles.(Kampan et al. 2015) Thus, microtubules formed in the presence of paclitaxel are extremely stable and therefore dysfunctional. The disruption of normal microtubule dynamics results in cell cycle arrest during mitosis, and without resolving the block, in cell death.(Rowinsky EK 1995; Parness & Horwitz 1981)

Paclitaxel is approved by the Food and Drug Administration for the treatment of ovarian, breast, and lung cancer, as well as Kaposi's sarcoma. It is used off-label to treat gastroesophageal, endometrial, cervical, prostate, and head and neck cancers, in addition to sarcoma, lymphoma, and leukemia.(Weaver 2014) It is mostly administered by 3- and 24-hour infusions at dose levels of 135 mg/m^2 or 175 mg/m^2, in 21- or 7-day cycles. Taxanes are widely used in combination with radiotherapy or other chemotherapeutic agents.(Dyer et al. 2013; Kumar et al. 2015; Markman et al. 2013)

However, paclitaxel had been shown to affect cell viability in other cell cycle phases,(Janssen et al. 2013; Zasadil et al. 2014) its cytotoxic activity is most effective during M phase.(Liebmann et al. 1993) As low as 5 nM of paclitaxel is enough to arrest and to subsequently kill tumor cells, but the effectiveness is highly dependent on the length of the treatment and the cell type.(Liebmann et al. 1993) Namely, the treatment has to accommodate

the cell cycle dynamics. It has been shown that synchronizing ovarium cancer cell lines reverses paclitaxel resistance.(Wang et al. 2013) On the other hand, cell-cycle arrest and synchronization was also found to significantly contribute to cell sensitization by paclitaxel to radiotherapy,(Supiot et al. 2005; Wenz et al. 1999) since cells show the highest sensitivity to radiotherapy in G_2 and M phases as well.(Tishler et al. 1992)

2. The role of O-glycosylation in cell cylcle control

Cell cycle is the result of precisely controlled processes involving transcriptional, translational, and posttranslational regulations as well protein degradation. Each step of cell cycle is influenced by hundreds of regulators. Alterations to the regulatory mechanisms due to either intrinsic (mutations) or extrinsic (environmental changes, chemicals, etc.) factors may have serious consequences; pathological changes of the cell cycle could lead to uncontrolled proliferation, but disturbance in the cell cycle regulation has also been associated with Alzheimer's disease, diabetes or autoimmune diseases.(Wang & Wang 2015; Slawson et al. 2010; Balomenos & Martínez-A 2000)

The importance of O-glycosylation in cell division became evident in the last few years.(Slawson & Hart 2011) O-glycosylation (O-GlcNAc) is a posttranslational modification affecting serine (Ser) and threonine (Thr) amino acids. During the process, the addition of O-linked β-N-acetylglucosamine (O-GlcNAc) happens to the hydroxyl groups of Ser and Thr amino acids of cytoplasmic, nuclear, and mitochondrial proteins. The number of known O-GlcNAcylated proteins is rapidly increasing thanks to advances in mass spectrometry techniques – over 3000 O-GlcNAc proteins has been identified so far, including regulators of gene expression, translation, protein degradation, signal transduction, and cell cycle regulation etc.(Zachara et al. 2015)

An important feature of O-GlcNAcylation is its strong relationship with the cell's metabolic state ("nutrient sensor"). The substrate of the modification is the uridine-diphosphate-N-acetylglucosamine (UDP-GlcNac) which is at the same time the end-product of the hexosamine biosynthetic pathway (HBP) that is linked to both nucleotide, glucose, amino acid and free fatty acid metabolism. Approximately 2-4% of glucose influx is directed toward the this pathway.(Goldberg et al. 2006)

It has been shown that manipulation of the O-GlcNAc balance leads to disturbed cell cycle progression.(Slawson et al. 2005; Wang et al. 2012; Dehennaut, Hanoulle, et al. 2008) An optimal rate of the modification is crucial for the proper histon phosphorylation,(Delporte et al. 2014) mitotic spindle assembly,(Tan et al. 2013) and physiological expression of c-myc

protein.(Itkonen et al. 2013) Also, nucleoporins represent one of the most heavily O-GlcNAcylated proteins (J. A. Hanover et al. 1987) and O-GlcNAc has been shown to strongly affect nuclear transport mechanisms.

O-GlcNAc levels were found to either increase (Wang et al. 2010; Dehennaut, Hanoulle, et al. 2008; Yang et al. 2012; Lefebvre et al. 2004) or decrease during mitosis.(Fong et al. 2012; Sakabe & Hart 2010) However, the role of O-glycosylation in cell cycle regulation is undeniable, the exact mechanisms are still unknown and publications regarding the global O-GlcNAc changes during cell cycle are contradictory.

AIMS

1. Our first goal was to design a cytotoxic treatment protocol specifically optimized to a cell line based on its cell cycle characteristics, according to the following steps:
    a. Characterization of the Sp2 cell line's cell cycle, including not only the establishment of doubling time but the length of the single phases as well.
    b. Optimization of timed, sequential paclitaxel treatments in accordance with the cell cycle kinetics of the Sp2 cells.

2. Demonstration that timed, sequential paclitaxel treatment can favor one cell type over another having different cell cycle characteristics:
    a. Characterization of the cell cycle of Jurkat cells.
    b. Examination of the effectiveness of consecutive paclitaxel treatments (optimized to the Sp2 cell line) on co-cultures containing both Jurkat and Sp2 cells.

3. Finally, we aimed to follow the changes of O-GlcNAc state in the course of cell cycle. For this reason, we performed the following experiments:
    a. Detection of O-GlcNAc pattern of paclitaxel-synchronized HeLa cells.
    b. Analysis of O-GlcNAc pattern following synchronization with double thymidine block and mitotic shake-off.
    c. Detection of the O-GlCNAc state of nucleoporins during mitosis.
    d. Investigation of subcellular O-GlcNAc distribution pattern compared to tubulin and actin staining.

MATERIALS AND METHODS

1. Cell culturing, synchronization

Experiments were performed on HeLa (ATCC CCL-2 human cervix cacinoma epithelial cells), Sp2 (ATCC CRL-1581 mouse hybridoma), Jurkat (ATCC TIB 152 human acute T-cell leukemia; transformed to transiently express green fluorescence protein (GFP)) cell lines, cultured according to the manufacturer's instructions. (The cell lines were the kind gift of F. Boldizsar and P. Balogh from the Dept. of Immunology and Biotechnology of the University of Pecs.).

For paclitaxel synchronization, 6 mg/l stock solution of paclitaxel was freshly prepared before each experiment from commercially available, 6 mg/ml paclitaxel solution used in human medicine (Teva Magyarorszag Ltd.). Cells were synchronized with 0.05 mg/l paclitaxel, in complete media. Next, the cells were centrifuged at 500 RCF for 5 min. and washed in complete media 3 times to remove any trace amount of paclitaxel then the cells were re-suspended in complete, paclitaxel-free media and left for various time periods to recover. In some of the experiments, a second paclitaxel treatment (0.05 mg/l for 8 hours) and a second recovery time occurred. Throughout the experiments, the cells were kept at 37°C, 5% CO_2 in a humidified incubator.

Double thymidine block was performed on adherent HeLa cell line. Briefly, HeLa cells were grown in tissue culture flasks until ~40% confluency. Next, cells were incubated for 19 h at 37 °C in the presence of 2 mM thymidine, and then for 9 h in complete medium without thymidine. Finally, another 2 mM thymidine was added to the medium for 16 h. At the end of the process, the large majority of the cells were in G_1 phase. The cells were collected after synchronization as follows: G_1 phase cells were collected by scraping immediately after the end of the double thymidine block treatment. S phase cells were collected by scraping 4 h after thymidine block release. Mitotic cells were collected in 20–25 min. fractions between 9–13 h post-synchronization by vigorously shaking the culture flask to detach round-shaped, mitotic cells (mitotic shake off). For the mitotic cells, we pooled the first six fractions (M1) and the last three fractions (M2) prior to lysis. G_2 phase cells were collected by scraping the still attached cells after the last fraction of round-shaped cells were removed.

2. Cell counting

In order to assess the amount of living cells, trypan-blue exclusion dye staining was used. Cells not stained by trypan-blue were considered living cells. Cell numbers were averaged from counting at least 18 separate regions of the hemocytometer and then divided by the average cell number measured at the start of the experiments.

In experiments when Jurkat and Sp2 cells were co-cultured, we analyzed the relative number of cells in homogenous cell suspensions by flow cytometry (Cytomics FC 500 flow cytometer, Beckman Coulter). To discriminate GFP-positive Jurkat cells, Sp2 cells and cell debris, forward scatter (proportional to cell size) and fluorescence intensity at 525 nm (FL1 channel) was simultaneously detected. The same selection of regions was used and 10000 particles were counted in all experiments. The relative number of cells was expressed as the ratio of Sp2 / Jurkat cells.

In case of synchronized adherent HeLa cells, the mitotic cells had to be counted at different time points. The cells were observed with a Leica DM IL inverted microscope (Leica Microsystems) and the average number of round-shaped cells per field of view were counted using a 10× objective from at least 5 separate regions of the flask. After each counting, round-shaped cells were discarded by vigorously shaking the flasks; consequently, only newly formed mitotic cells were included in the next counting.

3. Cell cycle analysis

Cell cycle analysis was performed by flow cytometry of permeabilized cells, after propidium iodide (PI) staining. First, adherent cells had to be detached from the cell culture vessels by incubation in phosphate-buffered saline (PBS) containing 0.25% trypsin and 0.5 mM EDTA. Resuspended cells were washed in complete medium to neutralize trypsin, next in PBS. The following steps were identical in case of suspension and adherent cell cultures. Cells were fixed in 1 ml ice-cold ethanol. The fixed cells were kept at 4 °C for at least 15 min, then washed with PBS 3x and resuspended in PI solution (containing 0.2 mg/mL RNase A). After 30 min. incubation in the dark at room temperature, the fluorescence intensity of PI dye per cell was detected at 620 nm (FL3 channel) with a Cytomics FC 500 flow cytometer (Beckman Coulter). Gating and selection of regions (G_1, S and G_2/M phase) were performed on asynchronous, control cells and identical selections were utilized for all samples.

4. Western blot, Immunoprecipitation

HeLa cells were washed twice in ice-cold PBS buffer and harvested in RIPA buffer, kept on ice for 30 min, and centrifuged for 10 min at 4 °C at 3000 rpm. From the supernatant, the total protein concentration was determined using a Bio-Rad DC Assay Kit (Bio-Rad). Next, samples were completed by the addition of 4x Laemmli sample buffer and boiled for 5 min. Where specified, the centrifugation step was omitted to obtain samples containing all proteins including RIPA-insoluble ones. For immunoprecipitated samples, we lysed the cells in IP buffer (25 mM Tris pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 v/v% Triton-X 100, 5 v/v% glycerol, 1 tablet/10 mL protease inhibitor cocktail, 0,05 w/v% Na-azide and 100 μM O-(2-acetamido-2-deoxy-d-glucopyranosylidene)-amino-N-phenylcarbamate (PUGNAc)). Immunoprecipitation was done overnight at 4 °C with mouse monoclonal anti-nucleoporin antibody MAb414 (Biolegend), followed by 3 h incubation with Protein A-Sepharose (Sigma-Aldrich). Captured proteins were eluted by 0.1 M glycine, pH 2.8 and completed by the addition of 4x Laemmli sample buffer and boiled for 5 min.

Proteins were separated by 8 w/v% SDS-PAGE and transferred onto Polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were probed with the anti-O-GlcNAc antibody CTD110.6 (monoclonal mouse IgM (Sigma-Aldrich, 1:2000) in 1 w/v% casein blocking buffer followed by horseradish peroxidase (HRP) conjugated goat anti-mouse IgM (Thermo Fisher Scientific, 1:5000). Blots were also probed with mouse (monoclonal) anti-O-GlcNAc antibody RL2 (Thermo Fisher Scientific, 1:1000), rabbit polyclonal anti–actin antibody (Sigma-Aldrich, 1:1500) and mouse monoclonal antibody MAb414 (1:1000) according to the manufacturers' protocol followed by their respective HRP conjugated secondary antibodies (1:2500). Blots were developed using Femto chemiluminescent substrate (Thermo Fisher Scientific) and signal was visualized by Kodak Image Station 2000R (Eastman Kodak Company). Kodak 1D (ver3.6.1, Eastman Kodak Company) and ImageJ analysis software were used to quantify the intensity of bands.

5. Immunofluorescence microscopy

HeLa cells were grown on coverslips until ~50% confluency. Next, cells were washed twice in ice-cold PBS and fixed in 10 v/v% PBS-buffered formaldehyde for 30 min at room temperature. To avoid formaldehyde autofluorescence, the coverslips were quenched with 50 mM ammonium chloride for 10 min. The cells were permeabilized with 0.25 v/v% Triton-X

100 for 10 min. Nonspecific sites were blocked with 5% bovine serum albumin (Sigma-Aldrich) in PBS for 30 min. and then the coverslips were incubated at room temperature with the primary antibody (or antibodies) for 2 h in 5 w/v% BSA/PBS. The primary antibodies and their dilutions used were the following: CTD110.6 (1:200), RL2 (1:100), and anti-α-tubulin (Sigma-Aldrich, 1:100). After rinsing 3 times with PBS, samples were incubated with the secondary antibody for 1 h in dark. For visualizing actin, phalloidin-Alexa Fluor 488 conjugate (Thermo Fisher Scientific, 1:20) was used. Nuclei were counterstained with Hoechst dye. Finally, coverslips were mounted with Vectashield (Vector Laboratories) mounting medium. Image acquisition was performed with a Zeiss Axiovert 35 (Carl Zeiss Microscopy GmbH) inverted fluorescence microscope equipped with CellD software (Olympus Soft Imaging Solutions GmbH). Confocal images were captured using a Zeiss LSM 710 confocal scanning microscope (Carl Zeiss AG) equipped with ZEN software (Carl Zeiss AG) and a 63x objective. Alexa Fluor 488 and Texas Red fluorescence channels were used for image acquisition (ex.: 488 nm and 594 nm, em: 518 nm and 624 nm, respectively).

6. Data analysis

Statistics were performed by Student's t-test and one-way ANOVA plus Bonferroni's post-hoc test in case of multiple comparisons, using GraphPad Prism software. Statistically significant differences between groups were defined as p values < 0.05 and are indicated in the legends of figures.

RESULTS AND DISCUSSION

Cell cycle dependent changes in paclitaxel cytotoxicity

Results

1. Characterization of the cell cycle of Sp2 cells

We were able to transiently block the Sp2 cells in G_2/M phase by a 0.05 mg/l, 14-hour paclitaxel treatment. After releasing the block by vigorously washing out paclitaxel from the cell culture medium, cells entered the next cell cycle. Moreover, the cells remained relatively synchronized until the end of the monitoring; up to 24 hours after treatment. The ratio of cells in $G_{0\text{-}1}$, S and G_2/M phase was calculated every 4 hours. The elapsed time between the same

phases (e.g., S–S or G_2/M-G_2/M) was approximately 16 hours, therefore the average time of one complete cell cycle of Sp2 cells lasted 16 hours. We have also estimated the duration of each cell cycle phase based on the following equation:

$$T_d + T_{phase} = T_{total\ phase}.$$

where T_d is the delay between the first and the last cell entering a given cell cycle phase, T_{phase} is the average time a cell spends in that phase and $T_{total\ Phase}$ is the total time between the first cell entering and the last cell exiting the phase. Applying this equation for each cell cycle phase resulted in the following estimations for the duration of the cell cycle phases: $G_{0\text{-}1} \approx 1.5$ hours, $S \approx 9.5$ hours, $G_2/M \approx 5$ hours and $T_d \approx 6.5$ hours.

2. Significance of timing in paclitaxel's cytotoxicity

Since paclitaxel mainly acts during mitosis, we assumed that synchronized Sp2 cells are more susceptible for a subsequent treatment in G_2/M phase. To test this hypothesis, we synchronized Sp2 cells and after various delay periods, we exposed them to a second paclitaxel treatment. The duration of the second treatment – 8 hours – proved to be a good compromise: long enough to cover most of the cells entering G_2/M phase but short enough that experiments with various delay periods would not overlap too much.

We have found that the second treatment was most effective when it occurred between 12-14 hours after the end of the first treatment. In contrast, if the second treatment occurred 22–30 hours after the end of the first treatment, significantly more cells survived. This difference between optimal and suboptimal timing could be followed up to 2 days after the experiments.

3. Distinct effectivitiy of timed sequential paclitaxel treatment according to cell cycle kinetics

We tested whether discrimination between two cell lines that have different cell cycle characteristics is possible by consecutive paclitaxel treatments. For this reason, we have chosen Jurkat cells having an approximately 24–36 hours population doubling time under the same cell culture conditions used for Sp2 cells. The Jurkat cell line was expressing GFP which was useful to distinguish between the two cell lines.

First, we compared the cell cycle characteristics of the two cell lines in asynchron cultures and also after 14 hours of 0.05 mg/l paclitaxel treatment (optimal only for Sp2 cells) which resulted in a significantly lower effectivity on Jurkat cells, producing only a low synchronity rate. Then, a 1:1 ratio of Sp2 and Jurkat cells' mixture was treated for 14 hours with 0.05 mg/l paclitaxel, then the ratio of the two cell types was measured by flow cytometry one, two or three days after the treatment. If no subsequent treatment followed Sp2 cells quickly

overcame the number of Jurkat cells, despite that the first paclitaxel treatment was intended to be more effective on them than on Jurkat. Similarly, when a second treatment occurred at a suboptimal time period, the ratio of Sp2 cells compared to Jurkat cells was almost as high as in the absence of the second treatment. On the other hand, if the first treatment was followed-up by a second, optimal timed paclitaxel treatment, the ratio of Sp2/Jurkat cells remained significantly lower.

Discussion

We demonstrated that paclitaxel's cytotoxicity could be significantly improved by timed, sequential treatments based on the cell cycle characteristics of the aimed cell line. We could also differentiate by the subsequent treatmens between cell types with various cell cycle characteristics. These results suggest that analyzing the cell cycle properties of malignant cells and scheduling chemotherapeutic regimes based on this information could improve the efficacy of antitumor therapy. There are a number of methods available to measure cell cycle properties. In vivo administration of BrdU followed by delayed biopsy allows the estimation of potential doubling time (Tpot) of malignant tumors. (Bertuzzi et al. 1997; Riccardi et al. 1988) Analyzing the amount of DNA and cell cycle distribution in cells from biopsies by flow cytometry or microscopy using DNA stains such as propidium iodide is also an option. Non-invasive techniques such as MRI has been used to estimate the proportion of dividing cells as well.(Xu et al. 2011) However none of these techniques are without limitations. E.g. flow cytometry techniques require suspended individual cells from the tumor mass which might be challenging in some types of malignant tissues. On the other hand, *in vitro* cultured isolated cells could show cell cycle characteristics not necessarily reflecting the *in vivo* conditions. Probably the biggest difficulty can be cancer inhomogeneity, when part of the tumor mass is necrotic, part of it is growing slower than Tpot would allow.(Yano, Zhang, Miwa, et al. 2014) Thus, collecting biopsies representative of the whole tumor or improving non-invasive imaging techniques are of utmost importance to enhance the intra-tumoral cell cycle analysis capabilities.

Some degree of cell synchronization is a long known consequence of cancer therapy. Paclitaxel was successfully applied in synchronizing and sensitizing malignant cell lines against radiotherapy.(Wenz et al. 1999) Interestingly, Wang et al. demonstrated on cell cultures that synchronization prior to paclitaxel treatment also improved paclitaxel's cytotoxic potency.(Wang et al. 2013) However, to our knowledge there is no current clinical or pre-

clinical application of the combined use of cell cycle analysis and subsequent therapy which incorporates these specific cell cycle data.

In our experiments, we used paclitaxel to both synchronize and to kill malignant cells at 0.05 mg/l or ~60 nmol/l. In human therapy, the plasma concentration of paclitaxel ranges from around 80 nmol/l to 500 nmol/l, but the intracellular paclitaxel concentration was found to be much higher, up to 1–9 mmol/l.(Weaver 2014; Rowinsky et al. 1999) Thus the conditions in our experiments are comparable to the lower end of the therapeutic range. Sp2 and Jurkat cells represent a proper model for rapidly growing tumor cells and normal cells dividing in a lower rate. However, cell production rate of cancers have a very wide range. In theory, paclitaxel could be administered to patients over a long period of time to increase the amount of malignant cells entering and subsequently arresting at G_2/M phase. Although longer exposure also increases the severity of cytotoxic damage done to non-malignant cells, the success or failure of the first treatment to synchronize the cells will greatly impact the effectivity of the subsequent treatments. Another limitation is that *in vivo*, a large percentage of cancer cells are quiescent (G_0/G_1 phase) and are resistant to chemotherapy and to synchronization. To circumvent this, a number of techniques have been proposed to force quiescent cells to pass the restriction point in G_1.(Yano et al. 2013; Yano, Li, Han, et al. 2014)

Despite these limitations, we think that even a slight improvement of the traditional treatment by sequential therapy would be welcomed in oncotherapy. In the future, more extensive studies of sequential treatment could discover new strategies to overcome some of the limitations. E.g. the combination of paclitaxel with other drugs could improve or expand the applicability of this method significantly.

In summary, our experiments demonstrated that analyzing the cell cycle properties of an individual cell type and timing the therapy based on this information could improve the effectivity of the cytotoxic treatment. We successfully used a single drug, paclitaxel in sequential treatments at therapeutically relevant concentration to synchronize and subsequently kill cells in a cell culture model. Moreover, with careful choice of treatment times we could improve the selectivity of the treatment for a particular cell type and reduce the „collateral” damage done to a different cell type.

Cell cycle dependent changes of protein O-GlcNAcylation in mitotic HeLa cells

Results

1. O-GlcNAc changes after paclitaxel synchronization

Investigating the O-GlcNAc pattern with western blot technique, we found slightly elevated protein O-GlcNAc levels in HeLa cells synchronized with paclitaxel for 4 or 8 hours.

2. Synchronization of HeLa cells with double thymidine block

To further investigate the changes of O-GlcNAc pattern during the cell cycle, we aimed to use a method with less potential side effects. Thus, double thymidine block was used to gain synchronized cell populations in the subsequent experiments which results in a transient block in the G_1/S transition.(Pederson & Robbins 1971; Bostock et al. 1971) Accordingly, by applying the method on HeLa cells, we mainly gained G_1 phase populations.

According to the DNA content of the cells determined by propidium iodide, four hours after the release of the block the cells progressed into S phase and after eight hours most of the cells reached the G_2 phase. After 12 h, approximately half of the synchronized cells underwent mitosis. In line, we use the term 'early G_2/M phase' to describe the time period when most cells are in G_2 phase but did not undergo mitosis yet, and 'late G_2/M phase' to describe the time period a significant number of cells already underwent division, while the rest of the cells are about to enter mitosis.

3. Changes in O-GlcNAc levels during the G_2/M - G_1 transition remain hidden by synchronization

For the investigation of the transition from G_2/M to G_1 phase, HeLa cells were synchronized by double thymidine block and were allowed to continue their cell cycle for 9, 10, 11 or 12 h. Our data showed that mitosis started between 10–11 h post-synchronization and about half of the cells already passed mitosis 12 h post-synchronization.

Therefore, we collected cells from synchronized cultures in a time period when most cells progressed through mitosis (8–13 h) but no extra band or any obvious change could be detected in O-GlcNAc levels detected from the samples by Western blotting with CTD110.6 labeling.

4. Selective collection of mitotic cells resulted in detection of distinct changes in O-GlcNAc pattern

Although in our synchronized cultures up to 70% of the cells were in the same phase, the individual mitotic events are spread over several hours. Thus, in the next step we collected mitotic cells in ~25 min. fractions from 9 to 13 h after synchronization by vigorously shaking the cell culture flasks to detach these cells from the surface. The first six fractions (M1) and the last three fractions (M2) were pooled together. Moreover, in this set of experiments, all samples were lysed directly in Laemmli sample buffer; consequently, the lysate represented the protein content of the whole cell. O-GlcNAc levels were analysed by RL2 and CTD110.6 antibodies. Both antibodies detected altered O-GlcNAc patterns in the mitotic and G_2 fractions, compared to other cell cycle phases. Most notably, a strong protein band at ~100 kD appeared in the mitotic cells.

5. O-glycosylation of nucleoporins during cell cycle

However, nuclear pore proteins (Nucleoporins, Nups) are among the most heavily O-GlcNAc modified proteins, very few data are available on the regulation of Nups by O-GlcNAc. Therefore, we have enriched Nups using a mouse monoclonal antibody MAb414 to assess O-GlcNAc levels in mitotic cells. Corresponding to previous data, we have found that Nups are abundantly O-GlcNAc modified. Moreover, at ~100 kD molecular weight in mitotic cells, we have found an O-GlcNAc staining pattern similar to that observed in crude cell extracts.

6. Immunofluorescence detection of O-GlcNAc in mitotic HeLa cells

To assess the amount of O-GlcNAc and its subcellular distribution at the level of individual cells, asynchronous HeLa cells were labelled with anti-O-GlcNAc antibody RL2 and fluorescence intensity levels were measured from interphase and mitotic cells for quantification. Mitotic cells were further divided into early and late mitotic cells. Mitotic cells demonstrated a significantly higher fluorescence intensity than interphase cells. The total fluorescence of the cells late in the mitosis was significantly lower. This was expected since the size of the daughter cells is approx. 50% of the metaphase cells before the division. Average fluorescence intensity in the two mitotic groups became similar after normalizing fluorescence intensity by the size of the cells.

Since tubulin and actin cytoskeletal proteins are not only the part of the O-GlcNAc proteom but also play roles during mitosis, we investigated next their relationship with O-GlcNAc. Thus, we performed co-stainings on asynchronous HeLa cells with CTD110.6 and

either anti-tubulin antibody or phalloidin-Alexa Fluor 488 for actin. CTD110.6 staining showed similar results to RL2, and the strong fluorescence signal of the mitotic cells was clearly distinct from the signal of the interphase cells. Mitotic cells containing the mitotic spindles visualized by tubulin staining also demonstrated increased level of O-GlcNAc. Epi-fluorescence microscopy did not show any obvious morphologic similarity between O-GlcNAc and either tubulin or actin staining pattern, thus we also examined possible co-localization by confocal microscopy. Based on our results, a large-scale co-localization between O-GlcNAc and either tubulin or actin could be ruled out, but potential O-GlcNAc modification of a smaller subset of actin or tubulin unfortunately remains hidden in this experimental setup.

Discussion

We analysed the mitosis-related changes of a post-translational modification that have a well-known influence on cell cycle regulation. Global, dynamic changes of O-GlcNAc pattern were detected during different cell cycle phases in HeLa cells, after double thymidine block.

We have shown that O-glycosylation pattern did not alter during the G_1 and S phases, but a sudden elevation in M phase occurred together with specific changes in the O-GlcNAc pattern. These alterations remained hidden without the use of mitotic shake-off, which ensures a precise, almost pure mitotic cell collection.(Sakabe & Hart 2010) According to our immunofluorescence results, this modification lasts only for a short period in the telophase of the mitosis, and, at the time of cytokinesis, the amount of O-glycosylated proteins already decreases.

There is increasing evidence representing O-GlcNAc modification as an important coordinator of the cell cycle. e.g., the level of O-GlcNAc-transferase (OGT) and O-GlcNAcase (OGA) the enzymes responsible for O-GlcNAc addition and removal were shown to be significantly fluctuate during various cell cycle transitions, correlating with changes of O-GlcNAc levels. Thus, nutritional substrate availability for O-GlcNAc modification could be a pre-requisite for cell cycle entry.(Slawson et al. 2010; Butkinaree et al. 2010) G_2/M transition highly depends on cyclin B1 expression, which is decreased by either OGT or OGA inhibition showing that both enzymes are needed for proper cell cycle progression.(Fardini et al. 2013)

The changes of global O-GlcNAc level during the cell cycle are controversial. Several reports showed that an increase in O-glycosylation and OGT expression occurs in G_2 phase

and these events are necessary to G_2/M transition.(Dehennaut, Hanoulle, et al. 2008; Dehennaut et al. 2007; Yang et al. 2012; Dehennaut, Slomianny, et al. 2008; Fardini et al. 2013; Tian et al. 2016) According to Yang et al., O-GlcNAc starts to elevate already in the late S phase and reaches a peak in the M phase of MEF and HEK293 cells. (Yang et al. 2012) However, other authors did not find any change or even detected a decrease in global O-glycosylation and OGT expression during M phase. (Slawson et al. 2005; Sakabe & Hart 2010; Haltiwanger & Philipsberg 1997) Differences between these findings regarding O-GlcNAc changes during mitosis may be explained by the rapid and reversible nature of O-GlcNAc modification but can also arise from the different synchronization techniques and timing of sample collection.

Previous reports suggest that O-GlcNAc influences cytoskeletal proteins during mitosis. In our experiments, we did not find any detectable co-localization between O-GlcNAc and actin or tubulin. Based on the intracellular localization and morphology of the abundant O-GlcNAc during mitosis, the elevation of O-GlcNAc is probably not directly connected to cytoskeletal re-arrangement. However, the present study was limited by the abundance of O-GlcNAc, which hinders the study of co-localization with other proteins by confocal microscopy. Slight morphological changes and interactions with cytoskeletal proteins may have remained hidden. Thus, we do not exclude the possibility that key elements of the cytoskeletal system might be reversibly O-GlcNAcylated during mitosis. However, it seems improbable that the large increase of O-GlcNAc during mitosis would be caused solely by the re-arrangement of the cytoskeletal system.

One of the limitations of our study could be that nuclear pore proteins (Nups) are strongly O-GlcNAc modified,(J A Hanover et al. 1987) which could skew the analysis of the immunofluorescently labelled interphase cells. However, it seems to be that Nups also persist during mitosis.(Theisen et al. 2008; Chatel & Fahrenkrog 2011) Our western blot data, which agreed with the immunofluorescence analysis, were based on strong lysis conditions, thus the lysate represented the total protein content, including nuclear pore proteins in both interphase and mitotic samples as well. Moreover, we have also analysed the level of O-GlcNAc modification on Nups by immunoprecipitation. While we have found both in mitotic and interphase cells abundantly O-GlcNAc modified Nups, distinct differences could be observed that corresponded to O-GlcNAc staining patterns found in whole cell lysates. This finding suggests that at least part of the changes we see during mitosis may be attributed to proteins that are strongly associated with Nups.

Although we are only starting to understand the impact of O-GlcNAc on the regulation of cell division, there is evidence that cell cycle control and metabolism are tightly connected. In modern lifestyle, an important risk factor for cancer is excessive food intake.(Dossus & Kaaks 2008) Since O-GlcNAc is an important signalling element in nutrient sensing, metabolic changes could impact cell cycle regulation through O-GlcNAc's regulatory effect. Indeed, malignant cells show both an increased nutrient uptake and elevated O-GlcNAcylation.(Fardini et al. 2013) In fact, in the future, O-GlcNAcylation detection may become a valuable diagnostic and prognostic marker. Some authors have already proposed the measurement of OGT and OGA mRNA in urine as a diagnosis tool in bladder cancer.(Rozanski et al. 2012) Moreover, O-GlcNAc related proteins, e.g., OGT, OGA or O-GlcNAcylated cell cycle regulatory proteins, could represent promising targets in the therapy of malignant diseases.(Lefebvre 2016)

PUBLICATIONS

Articles related to the thesis

Fisi V, Katai E, Bogner P, Miseta A, Nagy T. *Timed, sequential administration of paclitaxel improves its cytotoxic effectiveness in a cell culture model*. Cell Cycle. 2016 May 2;15(9):1227-33. IF: 3.53

Fisi V, Miseta A, Nagy T. *The Role of Stress-Induced O-GlcNAc Protein Modification in the Regulation of Membrane Transport.* Oxid Med Cell Longev. 2017:1308692. IF:4.593

Fisi V, Katai E, Orban J, Dossena S, Miseta A, Nagy T. *O-linked N-acetylglucosamine transiently elevates in HeLa cells during mitosis.* Molecules. [Közlésre elfogadva] IF: 2.861

Articles not related to the thesis

Nagy T, Kátai E, **Fisi V**, Takács TT, Stréda A, Wittmann I, Miseta A. *Protein O-GlcNAc Modification Increases in White Blood Cells After a Single Bout of Physical Exercise.* Front Immunol. 2018 May 3;9:970. IF: 6.429

Szijártó IA, Molnár GA, Mikolás E, **Fisi V**, Cseh J, Laczy B, Kovács T, Böddi K, Takátsy A, Gollasch M, Koller A, Wittmann I. *Elevated Vascular Level of ortho-Tyrosine Contributes to the Impairment of Insulin-Induced Arterial Relaxation*. Horm Metab Res. 2014 Sep 10. IF: 2.038

Kellermayer Z, **Fisi V**, Mihalj M, Berta G, Kóbor J, Balogh P. *Marginal Zone Macrophage Receptor MARCO Is Trapped in Conduits Formed by Follicular Dendritic Cells in the Spleen*. J Histochem Cytochem. 2014 Mar 26;62(6):436-449. IF: 2.403

Szijártó IA, Molnár GA, Mikolás E, **Fisi V**, Laczy B, Gollasch M, Koller A, Wittmann I. *Increase in insulin-induced relaxation of consecutive arterial segments toward the periphery: Role of vascular oxidative state*. Free Radic Res. 2014 Jul;48(7):749-57. IF: 2.989

Mikolás E, Cseh J, Pap M, Szijárto IA, Balogh A, Laczy B, Bekő V, **Fisi V**, Molnár GA, Mérei A, Szeberényi J, Wittmann I. *Effects of erythropoietin on glucose metabolism*. Horm Metab Res. 2012 Apr;44(4):279-85. IF: 2.145

Fisi V, Mazák I, Degrell P, Halmai R, Molnár GA, Fehér E, Németh K, Pintér I, Kovács T, Wittmann I. *Histological diagnosis determines complications of percutaneous renal biopsy: a single-center experience in 353 patients*. Kidney Blood Press Res. 2012;35(1):26-34. IF: 1.596

Balogh P, **Fisi V**, Szakal AK. *Fibroblastic reticular cells of the peripheral lymphoid organs: unique features of a ubiquitous cell type*. Mol Immunol. 2008 Nov;46(1):1-7. IF: 3.555

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Abstracts published in cited journals

Mikolas EZ, **Fisi V**, Cseh J, Pap M, Szijarto IA, Molnar GA, Beko V, Wittman I. *Effects of Erythropoietin on Glucose Metabolism.* Kidney Blood Press Res. 2010;33(6):425-426. Meeting Abstract: 24. IF: 1.5

Szijarto IA, Merei A, **Fisi V**, Fesus G, Cseh J, Mikolas EZ, Molnar GA, Wittmann I. *Does Oxidative Stress Affect the Vasoactive Effect of Insulin?* Kidney Blood Press Res. 2010;33(6):434-435. Meeting Abstract: 42. IF: 1.5

Kellermayer Z, **Fisi V**, Mihalj M, Kobor J, Balogh P. *Role of complement receptor in the acquisition and transport of marginal zone macrophage-associated MARCO scavenger receptor by follicular dendritic cells.* Eur J Clin Invest 2012 Apr; 42:7-7. IF: 3.365

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