Glycoprotein synthesis in the endoplasmic reticulum as a novel promising anticancer target

PhD thesis

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Introduction

The endoplasmic reticulum (ER) is the largest membrane-enclosed organelle in the eukaryotic cells. Its size and structure can vary to a great extent depending on the cell's function. The rough ER plays a central role in the synthesis, co- and posttranslational modifications, folding, quality control mechanism and degradation of secretory, membrane-bound, and some organelle-targeted proteins. N-glycosilation is one of the major co- and post-translational modifications in the ER. An oligosaccharide of a 14 subunits ($Glc_3Man_9GlcNAc_2$) is transferred to the asparigne residue of the polypeptid chain. This nascent oligosaccharide moiety gets sequentially trimmed by glucosidase and mannosidase enzymes. The process is initiated by the action of glucosidase I (membrane-bound protein), which removes the outer α1,2-linked glucose residue, and continues with the removal of the remaining two α1,3-linked glucose residues by glucosidase II. The removal of the first two glucose units is an uncontrolled process; contrarily the removal of the proximal glucose is a much slower and rate-limiting reaction. Glucosidase II plays a central role in the quality control of newly synthesized glycoproteins by removing this certain proximal glucose unit, which serves as a marker of immaturity. The quality-control system is responsible for the proper folding of newly synthesized proteins, and it prevents that unfolded or misfolded proteins reach their final destinations.

The calnexin-calreticulin cycle is the best characterized quality control mechanism for N-glycoproteins. This mechanism is responsible for the proper folding of glycoproteins, for the retention of unfolded or misfolded glycoproteins in the ER (until they reach their native conformation), furthermore it plays a role in the degradation of folding-defective proteins. The initiation and termination of calnexin/calreticulin

cycle are regulated by two separate enzymes. Glucosidase II removes the special proximal glucose label and thereby the glycoprotein dissociates from the chaperon. Deglucosylated proteins, having achieved their native conformation, can be forwarded to the secretory pathway. However, they can also get reglucosylated by UDP-glucose:glycoprotein-glucosiltranferase once judged immature and the chaperons can retry to correct the protein folding. Alternatively, one of the distal mannose units can be removed by α1,2-mannosidase I enzyme, which directs the protein to proteasomal degradation through a process called ER associated degradation (ERAD). Therefore, glucodase II enzyme plays a central role both in the glycoprotein maturation and quality control mechanism.

Imbalance between protein folding demand and capacity leads to accumulation of immature (unfolded or misfolded) proteins in the ER lumen, which triggers the unfolded protein response (UPR). This complex cellular response is initiated through activation of three ER transmembrane receptors: PERK („pancreatic ER kinase"), ATF6 („activating transcription factor 6") and IRE1 („inositol requiring enzyme 1"). In resting cells, all three ER stress receptors are maintained in an inactive state through their association with ER chaperon, GRP78. The accumulating immature proteins engage GRP78 and hence lead to its dissociation from these receptors. The receptors activated this way can trigger the three main branches of the UPR. The UPR fundamentally aims to restore the balance between protein folding demand and capacity (attenuation of translation, induction of ER chaperones and foldases, enlargement of the ER network, stimulation of ERAD). The active PERK phosphorylates eukaryotic initiation factor 2 α (eIF2α), which leads to the attenuation of „cap-dependent" translation. If the ER functions cannot be restored by the UPR and the ER-stress persists, apoptosis is triggered through several molecular pathways. Induction of

CCAAT-box enhancer binding protein-homologous protein (CHOP) and c-Jun N-terminal kinase (JNK) leads to the commitment to apoptosis. Ultimately, the different transcription factors, kinases and Bcl2 family members lead to caspase activation, resulting in cell death. Caspase-12 (in rodents) and caspase-4 (in human) are also proposed as key mediators of ER-induced apoptosis. Since the above mechanisms can contribute to tumor cell death, ER-stress and ER-related apoptosis are getting into the focus of cancer research lately.

The polyphenolic tea cathechins (flavanols) are natural agents of remarkable anti-cancer potential. Polyphenols represent 3-10% of the dry matter in black tea leaf, and 30-42% of the dry matter in green tea leaf. The difference is due to the different processing of black and green tea leaves. The catechines are converted by polyphenol oxidase into tearubigins and teaflavins in black tea (so-called fermentation). Contrarily, polyphenol oxidase is heat-inactivated in green tea leaves; thereby the cathecines can preserve their original structure. Tea constains a wide array of cathecines: epicathecin (EC), epicathecin-gallate (ECG), epigallocatechin (EGC), epigallocatechin-gallate (EGCG), catechin and gallocathecin (GC). The most abundant and best studied cathecin is EGCG, which represents 50-65% of total cathecin content and its beneficial health effects are the most widely investigated.

Both animal models and human studies have demonstrated the anti-cancer and chemopreventive properties of tea flavanols. It has been revealed, that tea cathechins can inhibit proliferation and angiogenesis. Moreover, they can induce apoptosis in tumor cells.

Objectives

The Er is strongly related to the control of cell proliferation and apoptosis. Several regulator proteins of mitogenic or apoptotic pathways are synthesized and processed in the ER. Moreover, proapoptotic signals are also generated in the ER, which is getting into the focus of scientific interests. We proposed the hypothesis that modulation of ER functions might contribute to the anti-tumor effects of green tea catechins. In the present study we investigated whether tea catechins – particularly EGCG – interfere with glycoprotein maturation and quality control, and whether this mechanism leads to ER-derived apoptosis. The novel mechanism of action revealed in our study further highlights the ER and protein maturation as promising anticancer targets.

It was the core of our hypothesis that glycoprotein maturation in the ER is disturbed by EGCG. This assumption was tested by using tyrosinase enzyme, which is a suitable and widely used model of glycoprotein maturation. However, the phenomenon was also investigated using another glycoprotein, vascular endothelial growth factor (VEGF), which plays a direct role in angiogenesis, tumor growth and metastatic dissemination.

Answers to the following questions were sought:

- Do the tea flavanols affect the activity of glucosidase II, the key enzyme in glycoprotein quality control?
- Does the inhibition of glucosidase II lead to ER stress in hepatoma cells, and can ER-derived pro-apoptotic signals be detected as part of the UPR?
- Does EGCG treatment reduce the level of (endogenous) tyrosinase enzyme in melanoma cells?

- Is the reduction of tyrosinase level due to disturbance of post-translational modifications (folding process) and can it be counteracted by the inhibition of proteasomal degradation?
- Do the hindrance of glycoprotein maturation – we propose it being a general effect – affect the synthesis of other glycoproteins (e.g. VEGF), wich play direct roles in tumor growth?

Methods:

Rat liver microsomes, mouse hepatoma Hepa1c1c7 cells and human melanoma Sk-Mel28 cells were used in our study. The rat liver microsomes contain mainly ER membrane vesicles, which preserve their original orientation; hence they are suitable for the examination of ER enzymes. We investigated the effect of EGCG, other catechins and N-butil-deoxynojirimycin (a known inhibitor of glucosidases I and II) on glucosidase II in rat liver microsomes using two specific artificial substrates, methylumbelliferyl glucoside and nitrophenyl glucoside. The production of fluorescent methylumbelliferon was measured by fluorimeter and the nitrophenol was quantified using HPLC.

The liver-derived hepatoma cells are characterized with an excessive ER network and an intensive protein processing. They are, therefore appropriate cellular models to study the ER-stress and UPR mechanism as well as the ER-derived apoptosis. ER stress markers (CHOP/GADD153, caspase-12, phosphorylated/dephosphorylated eIF2α, and major ER chaperons) were monitored in cell lysates using Western blot analysis. Induction of apoptosis by EGCG was studied with fluorescence microscopy after staining the samples with annexin and propidium-iodide. Apoptosis

index (the number of apoptotic cells/bodies in 100 cells) was determined after counting the annexin-stained stells.

Tyrosinase enzyme, a widely used model of glycoprotein maturation, was used to study the interference with glycoprotein maturation. Tyrosinase is a glycoprotein with six N-glycosilation sites, and it catalyzes the rate-limiting steps of melanin synthesis in melanocytes. The level of tyrosinase was monitored in Sk-Mel28 human melanoma cells using a semi-quantitative in situ tyrosinase assay based on the melanin deposition due to tyrosinase activity and Western blot analysis in the cell lysates. VEGF is also expressed in melanoma cells, and plays a crucial role in tumor vascularization. We examined the levels of VEGF and HIF1α in cell lysates with Western blot analysis, and we used β-actin protein as control.

RNA was also isolated from the melanoma cells after treatment with glucosidase inhibitors. The alterations of tyrosinase, VEGF and HIF1α mRNA levels were assessed using quantitative real-time PCR.

Three independent measurements were performed in triplicates. The quantified results are shown as mean values±S.D. and were compared using ANOVA with Tukey's multiple comparison post hoc test. Differences of $P < 0.05$ were considered significant.

Results

The effect of tea flavanols on glucosidase II activity was studied first in isolated rat liver microsomes. N-butil-deoxynojirimycin, a widely used specific inhibitor of glucosidase I-II was applied as a positive control. The transport of arteficial substrates across the membrane turned out to limit the rate of luminal hydrolysis, thus the measurements were undertaken after permeabilization of the vesicular membranes with the pore-forming

antibiotic alamethicin to allow free access of substrates and inhibitors to the enzyme.

The investigated tea catechins exerted concentration dependent inhibitory effect on glucosidase II with significantly different efficiency. EGC and GC, which lack the gallate group, were much less effective than the other investigated tea flavanols. It is remarkable that the configuration of the gallo moiety also largely influenced the inhibitory effect: the IC_{50} and K_i values of GCG were comparable with those of NBDJ, while EGCG was less efficient. Although it became evident that EGCG is not the most efficient inhibitor of glucosidase II, this flavanol was used in the concomitant experiments becuase it is the most abundant catechin in green tea, and consequently it is the most widely studied and characterized catechin.

The kinetics of the inhibitory effect of EGCG on glucosidase II has been further studied in rat liver microsomes. Glucosidase II activity showed Michaelis-Menten kinetics with both substrates although the kinetic parameters were different. EGCG-treatment significantly decreased the v_{max} values while K_M remained essentially unaffected in case of either substrate. Therefore, the inhibitory effect turned out to be non-competitive from kinetic point of view.

Once the inhibitory effect of EGCG on glucosidase II had been estabilished and characterized in liver microsomes, the same phenomenon was also investigated in Hepa1c1c7 mouse hepatoma cells. A remarkable inhibition was obsereved both in alamethicin-permeabilized cells and intact cells at similar extent, which indicates that EGCG affects the enzyme directly rather than interfeing with the transport of the substrate, methylumbelliferyl-glucoside. Inhibition of glucosidase II by EGCG was

permanent for at least 12 h in intact cells, which indicates that a persistent inhibition can be maintained in long incubations.

Glucosidase II enzyme plays a key role in glycoprotein maturation and quality control mechanism in the ER. It is, therefore, likely that glucosidase II inhibitors, such as EGCG can lead to ER stress and trigger the UPR, which in turn can generate proapoptotic signals. This assumption was tested in the hepatoma cells by detecting ER stress markers and pro-apoptotic UPR elements after treating the cells with EGCG or deoxynojirimycin. In line with our original hypothesis, EGCG was shown to induce the UPR (phosphorylation of eIF2α, induction of CHOP, activation of caspase-12), which was in accordance with the progressive increase in the apoptosis index also observed in the EGCG treated cells. Interestingly, some pro-survival elements of the classic UPR – induction of ER chaperones – could not be detected in the EGCG treated cells. Our studies revealed that EGCG induces ER stress and a partial UPR in hepatoma cells characterized by an intensive protein processing activity, and this effect likely contributes to the induction of apoptotic cell death.

Our results strongly supported our hypothesis that EGCG interferes with protein processing and quality control mechanism in the ER lumen presumably due to inhibition of glucosidase II. Henceforth we decided to prove this suggested mechanism with further examinations. Interference with N-glycoprotein maturation and quality control was studied in Sk-Mel28 human melanoma cells producing endogenous tyrosinase enzyme, a widely used model of glycoprotein maturation. The effect of 72 hour long EGCG and deoxynojirimycin treatment on the level of active tyrosinase protein was first examined using an in situ semi-quantitative assay. The treated melanoma cells produced remarkably less melanin pigment compared to control. Since EGCG and deoxynojirimycin do not directly

affect tyrosinase activity, reduction of melanine production represents a reduced expression of active tyrosinase. This conclusion was further supported by Western-blot analysis and native gel electrophoresis. Both revealed a remarkable decrease in tyrosinase protein levels in the melanoma cells treated with EGCG in a concentration- and time-dependent manner. These result corresponded with the morphological alterations detected in the cells using fluorescent microscopy. EGCG treatment decreased the number of melanosomes, and also induced evident changes in the melanosome morphology according to the obstructed production of their protein components.

In order to elucidate whether the observed inhibition of glycoprotein synthesis by EGCG occurs indeed at post-translational phase (i.e. at protein processing) as hypothesized; first we performed quantitative real-time PCR analysis to rule out any alteration at mRNA level. The mRNA expression did not change significantly in glucosidase inhibitor treated cells, therefore the reduction of tyrosinase glycoprotein expression was caused by translational or post-translational modifications.

If our original hypothesis is valid and these agents disturb protein glycosylation in the ER, a great amount of immature glycoproteins should be directed towards ERAD by the quality control mechanisms, which can be responsible for the reduced tyrosinase protein expression. These immature proteins can be spared from ERAD by means of inhibition of proteasomal degradation, which is therefore expected to efficiently reduce the inhibitiory effect of EGCG on tyrosinase production.

The semi-quantitative tyrosinase activity assay as well as the native electrophoresis and Western blot did not reveal any obvious difference between the tyrosinase enzyme levels and activities in lactacystine treated or control melanoma cells. This indicates that proteasomal degradation

plays a minor role in the normal control of tyrosinase protein expression in the untreated cells. However, the proteasome inhibitor lactacystine counteracted the inhibition of tyrosinase production by either EGCG or deoxynojirimycin, which indicates a remarkable intensity of proteasomal degradation in the glucosidase inhibitor treated cells. According to our results, the immature tyrosinase enzyme, which contains a glucose residue as a marker of immaturity is forwarded to the ERAD for degradation, which is responsible for the lower protein levels in glucosidase inhibitor treated cells. Once these proteins escape proteasomal degradation, they can achieve active conformation, which could explain our observation, that inhibition of ERAD increases not only the level but also the activity of tyrosinase protein.

However, tyrosinase enzyme was primarily used in our study as a well assessable model system for glycoprotein processing and quality control. We proposed that the disturbance in tyrosinase maturation is just part of a general derangement in glyoprotein-processing.

The inhibitory effect of EGCG and deoxynojirimycin was also demonstrated on the maturation of another glycoprotein, VEGF in melanoma cells. VEGF protein levels were remarkably reduced despite unaltered HIF1α level and mRNA expression in glucosidase inhibitor treated cells. The proteasome inhibitor lactacystin could efficiently counteract this effect as well. Our results confirm that inhibition of glucosidase II by EGCG interferes with N-glycoprotein quality control, which in turn diverts immature glycoproteins, such as tyrosinase or VEGF to ERAD.

Conclusions

The possible role of the ER as a target organelle of tea flavanols has been investigated. The effect of various tea catechins was studied on glucosidase II enzyme activity and consequently on protein processing in the ER. According to our hypothesis, the inhibition of this key enzyme of protein quality control could cause ER stress and trigger the UPR, which in turn can contribute to the proapoptotic activity of EGCG. We aimed to reveal whether EGCG, a major tea flavanol causes the accumulation of glycoproteins in the ER and consequently enhance proteasomal degradation.

The following conclusions were drawn:

1. The studied tea catechins effectively inhibit glucosidase II enzyme in rat liver microsomes. Their inhibitory effect was comaperable with that of deoxynojirimycin, a widely used glucosidase inhibitor. The non-competitive kineticsof inhibition was revealed in case of EGCG.
2. EGCG was also shown to inhibit glucosidase II and cause ER streess in Hepa1c1c7 mouse hepatoma cells. Enhanced phosphorylation of eIF2α and the main pro-apoptotic components (CHOP/GADD153 induction and procaspase-12 activation) of the UPR were detected, which might contribute to the enhanced apoptosis in EGCG treated cells.
3. The amount of two N-glycoproteins: tyrosinase and VEGF was reduced by EGCG and deoxynojirimycin in a time and concentration dependent manner. The tyrosinase and VEGF mRNA levels and HIF1α expression remained unaltered, and the effect could be counteracted by the proteasomal inhibitor lactacystin. Our results

confirm that EGCG interferes with glycoprotein maturation and quality control in the ER through inhibiting glucosidase II enzyme.

Our findings reveal a novel mechanism for the antitumor properties (pro-apoptotic and antiproliferative) of glucosidase inhibitors. Disturbence of glycoprotein processing and quality control in the ER can lead to ER stress, and induce the UPR, which triggers apoptosis through several signaling pathways. Moreover, certain glycoproteins play a direct role in the regulation of cell proliferation and tumor growth, metastasis formation at pathological conditions. Disturbed protein processing and enhanced proteasomal degradation of these proteins could contribute to the anti-tumor effect of glucosidase inhibitors as well.

This mechanism might play an important role in the antitumor effect of EGCG and it highlights glycoprotein maturation in the ER as novel promising anticancer target in tumor therapy.

The thesis is based on the following publications

1. Gamberucci A*, **Konta L***, Colucci A, Giunti R, Magyar JÉ, Mandl J, Bánhegyi G, Benedetti A, Csala M. (2006) Green tea flavonols inhibit glucosidase II. Biochem Pharmacol, 72: 640-646. IF: 3.581

   shared first authorship

2. Magyar JÉ, Gamberucci A, **Konta L**, Margittai É, Mandl J, Bánhegyi G, Benedetti A, Csala M. (2009) Endoplasmic reticulum stress underlying the pro-apoptotic effect of epigallocatechin gallate in mouse hepatoma cells. Int J Biochem Cell Biol, 41: 694-700. IF: 4.009
3. **Konta L**, Száraz P, Magyar JÉ, Révész K, Bánhegyi G, Mandl J, Csala M.: Inhibition of glycoprotein synthesis in the endoplasmic reticulum as a novel anticancer mechanism of (-)-epigallocatechin-3-gallate. BioFactors, 37: 468-476. IF: 4.933

Other publications

1. Révész K, Tüttő A, **Konta L**. (2007) [Effect of green tea flavonols on the function of the endoplasmic reticulum.] Orvosi Hetilap. 40: 1903-1907.
2. Révész K, Tüttő A, Szelényi P, **Konta L.** (2011) Tea flavan-3-ols as modulating factors in endoplasmic reticulum function. Nutr Res, 31: 731-740. IF: 1.974