OTKA-PD 109539 **Crul Tim**

DOES THE PHYSICAL STATE OF THE PLASMA MEMBRANE MODULATES HEAT SHOCK FACTOR-1 TO REGULATE AN ONCOGENIC SUBSET OF HEAT SHOCK PROTEINS – A DETAILED ANALYSIS IN A B16 MOUSE MELANOMA MODEL

FINAL PROGRESS REPORT

Introduction

Being the first contact with the external environment, the plasma membrane acts as an important hub for transferring external cues through receptor proteins and ion channels. Due to their specific characteristics, plasma membrane lipids rapidly and dynamically associate to form membrane subdomains with enriched lipid species within the membrane plane. These so-called lipid rafts are defined as short-lived fluctuating nanoscale assemblies of cholesterol- and sphingolipid enriched domains present in the membranes of most cells [1,2]. Due to cholesterol-induced membrane packing, these microdomains represent a more ordered lipid environment than the surrounding membrane, which limits the lateral diffusion of the residing proteins [3]. As many of these proteins were shown to be involved in cell signalling, lipid rafts have been implicated in regulation of signal transduction [4]. Since proteins are included and excluded from lipid rafts in a quick and dynamic process, the rafts establish concentrating platforms for individual receptors upon ligand binding activation which provides protection against membrane-residing non-raft enzymes which would otherwise affect the signalling cascade [4].

One example of these membrane-originating signalling cascades is molecular stress sensing. Heat shock proteins (HSPs) are molecular chaperones which assist newly formed proteins to reach their native state or, under stressful conditions, avoid protein damage and promote survival. At the transcriptional level, Hsps are regulated by heat shock factors (HSF), including HSF1. The classic model predicts that under normal conditions HSF1 exist as cytoplasmic inactive monomers in complex with hsps. Proteotoxic stress liberates HSF1 which trimerizes and accumulates in the nucleus where it drives hsp expression. However, hsp induction can occur without proteotoxic stress. Hence, the non-exclusive membrane sensor model postulates that, even in the absence of proteotoxic stress, the fluidity state of

the plasma membrane and its associated lipid rafts are important drivers of HSP expression [5-7]. Plasma membrane-originating signalling cascades acting upon HSF1 include the PIP2-driven inositol triphosphate/diacylglycerol and phosphatidylinositol triphosphate cascades, and the ceramide-driven sphingosine- and cholesterol-dependent cascades [8].

The bottleneck for most cancer treatments is the responsiveness and/or development of resistance to anticancer drugs and radiation. Importantly, at the molecular level, the development of anticancer drug resistance and the associated poor prognosis correlates with increased levels of heat shock proteins (HSP) favouring carcinogenesis by (1) chaperoning client proteins essential for cancer cell growth [9], and (2) supporting autophagy through stabilized lysosomal membranes ultimately promoting tumour survival [10]. Several solid tumours are characterized by higher fluidity of their cell-membranes [11-15] correlating with their proliferative and invasive potential and their metastatic abilities [16-18], while melanoma tumour cells with a higher metastatic potential are characterized by higher lateral mobility of membrane receptors in metastasis while showing a reduced cholesterol/phospholipid ratio [19]. Hence, enhanced membrane fluidization and /or lipid raft modulation might be an underlying mechanism for enhanced cancer cell survival. Based on this, I hypothesised that subtle membrane modulations are able to selectively influence the HSP expression levels upon exposure to stress.

Considering the central role of cholesterol in lipid raft functionality, removal of cholesterol from cells is thought to disrupt the structure of these microdomains as signalling platform. Hence, a wide variety of chemical approaches focussing on cholesterol have been used to study the involvement of lipid rafts in the regulation of raft-residing signalling proteins. For example, the cyclic oligosaccharide cyclodextrin (CD) disrupt lipid rafts through cholesterol uptake into its hydrophobic cavity [20]. Three forms of CD exist, alpha, beta, and gamma, consisting of six, seven, or eight glycopyranose units, respectively. To enhance water solubility, a methylation modification is added [21,22]. Considering their characteristics, methyl-beta-CD (mb-CD) is a widely used tool to manipulate the plasma membrane cholesterol content of cultured cells [20]. Based on its earlier described capacity to prevent mb-CD-induced cholesterol depletion from in vitro monolayers [23], I chose to use the

membrane-intercalating hydroximic acid derivative BGP-15 [24] as a putative "cyclodextrin inhibitor".

Findings of the current project based on the proposal layout

1. Limited plasma membrane cholesterol removal results in enhanced thermotolerance and cisplatin resistance

Acquired thermotolerance (ATT) is an experimental process induced by pre-exposure to elevated but non-lethal temperatures which enables cells to survive a subsequent severe heat stress that would be lethal in the absence of the first priming heat treatment. Based on the higher fluidity of cancer cell membranes which correlates with their proliferative and invasive potential, I wondered if modulations in plasma membrane fluidity could result in enhanced survival upon severe stress. B16-F10 cells were treated for 2 min with 10 mM mb-CD with or without BGP-15 followed by 1 hour heat stress at 42^{o}C and, after overnight recovery, 30 min at 45^{o}C for. A very brief mb-CD exposure prior to the priming heat resulted in a small but significant increase in ATT compared to control whereas BGP-15 could limit the mb-CD-induced increase in ATT

This finding indicated that specifically these cells with more fluid plasma membranes are more likely to acquire resistance to imposed stresses such as elevated heat or likely, and more relevant to cancer, chemotherapy. Thus, next I wondered if a very brief mb-CD exposure prior to priming heat would result in the development of chemo resistance. B16-F10 cells were treated for 2 min with 10 mM mb-CD with or without BGP-15 followed by 1 hour heat stress at 42^{o}C and, after overnight recovery, exposed to 75 μM cisplatin for 24h. Similar as observed for ATT, a brief mb-CD exposure prior to the priming heat resulted in a small but significant higher survival upon cisplatin, suggesting reduced cisplatin sensitivity. Equally as in the case of ATT, BGP-15 exposure prevented the mb-CD-induced cisplatin resistance.

2. Limited plasma membrane cholesterol removal affects heat-induced hsp profile

Considering the effect on ATT, I focussed on heat induced hsp expression levels. B16-F10 cells were exposed to 10 mM mb-CD for 5 minutes followed by heat exposure for 1 hour at

42,5^{o}C. Immediately after heat, RNA was isolated. As expected, heat exposure for 1 hour at 42.5^{o}C resulted in increased levels of hspb1 and hspa1a. Cells with more fluid plasma membranes (due to a limited plasma membrane cholesterol-depletion by mb-CD) were hampered in their inducibility of hspb1 while retaining full inducibility of hspa1a upon exposure to the same heat shock regime. Interestingly, in a recent comparative study comparing chemotherapy-sensitive versus chemotherapy-resistant breast cancer patients, a significant downregulation of HSPB1 in the chemotherapy-resistant group was reported[11].

Considering the blunted expression of the small heat shock protein hspb1 upon cholesterol depletion followed by heat compared to heat alone, the effect on other small heat shock proteins was investigated. Expression levels of both hspb5 and hspb8 were blunted upon cholesterol depletion followed by heat compared to heat alone (similar as hspb1), while the expression levels of hspb6 and hspb11 were unaffected.

Importantly, pre-treatment of B16-F10 cells with BGP-15 significantly maintained the inducibility of the small hsp expression levels upon mb-CD treatment followed by heat shock for 1 hour at 42.5°C.

3. Limited cholesterol depletion changes the post translational profile of HSF1, the hsp master regulator, suggesting altered activity

Hsp expression is regulated by members of the heat shock factor (HSF) family, of which four different members (HSF1 to HSF4) are described. Heat-induced hsp expression is mainly regulated by the interplay between HSF1 and HSF2. The activity of HSF1 itself is regulated by a complex intertwined mechanism consisting of a myriad of post translational modifications (PTM) and protein-protein interactions [25].

3a Analysis of HSF1 PTM profile

The activity of HSF1 is regulated by multiple PTM including phosphorylation (19 sites), acetylation (9 sites), and sumoylation (1 site) [25]. Whereas the functional relevance for most of these PTM is unknown, the presence or absence of certain modifications was suggested to have an activating or repressive character. For instance, phosphorylation on serine-326 was reported to result in more active HSF1 [26-28]. In the current proposal, the use of mass spectrometry to identify the HSF1 PTM profile and interactome was suggested. However,

the analysis of HSF1 by mass spectrometry was complicated by several factors. 1) The pull down of significant HSF1 quantities needed for mass spectrometry analysis of the PTM profile and interactome was complicated by very low endogenous HSF1 expression levels. Although the use of overexpressed and/or tagged HSF1 has been described, the biological relevance in relation to its PTM profile and interactome is questionable. 2) The trypsin cleavage pattern of HSF1 is not ideal for analysis of all PTM described. 3) Sub-fractions of HSF1 which are modulated by different PTM run closely together on SDS-PAGE. This complicates the excision of these specific sub-bands from the gel for subsequent mass spec PTM analysis.

The above described difficulties resulted in extensive trial and error. However, no satisfying and reproducible results could be obtained. Hence, I ultimately opted to analyse individual PTM sites by site-specific antibodies. Considering its described effect on HSF1 activation, phosphorylation on serine-326 was analysed. B16-F10 cells were exposed to 10 mM mb-CD for 2, 5, and 10 minutes followed by heat exposure for 1 hour at 42,5^{o}C. Immediately after heat, proteins were isolated and probed for phosphorylation on HSF1 serine-326. As expected, heat exposure resulted in enhanced phosphorylation on serine-326 in control samples indicating HSF1 activation. However, exposure to mb-CD prior to heat resulted in reduced phosphorylation of HSF1 serine-326 in a mb-CD exposure time-dependent manner suggesting reduced/altered HSF1 activity. These data are conform with an altered heat-induced hsp expression profile upon mb-CD exposure. Also, these data clearly indicate a functional link (represented by signalling cascades) between cholesterol depletion from the plasma membrane and HSF1 activity.

3b Analysis of HSF1 binding to DNA

Upon activation, HSF1 trimerizes and adopts a DNA-binding competent state. Based on the observed reduced phosphorylation on HSF1 serine-326, I aimed to analyse HSF1 binding to DNA with chromatin immunoprecipitation (ChIP). Similar as described for mass spectrometry, these experiments proved however complicated due to low expression levels of endogenous HSF1 and the consequent need of high cell numbers (= multiple plates) per sample. Consequently, a tight fine-tuning of the time each plate is exposed to MBCD is required. As short exposure times of 2 and 5 minutes were planned, every second of

exposure could alter the observation. As a result, this high background noise resulted in poor reproducibility.

3c Analysis of HSF1 expression

Being a transcription factor, the expression level of HSF1 is generally considered to be more or less constant. To analyse the effect of limited mb-CD-induced plasma membrane cholesterol depletion on HSF1 expression levels, B16-F10 cells were exposed to 10 mM mb-CD for 5 minutes followed by heat exposure for 1 hour at 42,5^{o}C. Immediately after heat, RNA was isolated. A limited removal of plasma membrane cholesterol followed by heat resulted in increased mRNA expression levels of hsf1 compared to heat alone. Furthermore, when comparing the increase in mRNA expression of both hspa1a and hsf1 after limited removal of plasma membrane cholesterol followed by heat, a strong positive correlation between both (R^2=0.9387) was found. With western blotting however, no significant differences in HSF1 protein expression levels were observed.

Recently, loss of NF1 was shown to result in the activation of HSF1 and promote carcinogenesis [29]. Considering enhanced HSF1 mRNA levels upon mb-CD-treatment followed by heat, NF1 expression levels were analysed in our system. Interestingly, treatment of cells with mb-CD followed by heat resulted in decreased NF1 mRNA levels compared to cells exposed to heat only. Moreover, a negative correlation between NF1 and HSF1 levels was found. Based on this, it is tempting to speculate on a potential role of NF1 in our system.

4 Modulation PIP3/PI3K signalling

Phosphoinositol-3-kinases (PI3K) are activated by growth factor receptor kinases resulting in the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-biphosphate (PIP2) and subsequent parallel activation of AKT1 and Rac1 [30]. During heat shock, activation of PI3K [31] and HSF1 [32-34] have been shown resulting in enhanced HSP levels [35]. Earlier strategies to manipulate inositol lipids in the cell expressed either a kinase-active or kinase-dead version of an enzyme that enhances or inhibits, respectively, the making of a particular lipid at its natural membrane location. However, while all of these manipulations target specific pools of the lipids made by specific enzymes working at particular compartments, they expose the membranes to an altered inositide level for a period of

several hours at a minimum. By the time the cells are examined, their membrane distribution and signalling pathways will be greatly distorted by the processes initiated and maintained by the prolonged lipid changes. A recent approach combines the advantage of both a targeting strategy and the enzymatic manipulation of inositides, and complements them with chemically inducible heterodimerization based on the rapamycin-induced association of the FRB fragment of mTOR with the FKBP protein [36,37]. In this system, our enzyme of interest, 5-ptase, is stripped of its own localization determinants and fused to one of the partners of heterodimerization, namely, the FKBP12 protein yielding a cytoplasmic enzyme that has some but limited impact on membrane phosphoinositides. The other heterodimerization partner, the FRB fragment of mTOR, is targeted to the cytoplasmic surface of the plasma membrane. Addition of rapamycin [36], which does not bind endogenous mTOR, will induce the heterodimerization of FKBP12 and FRB thereby recruiting the cytoplasmic 5-ptase to the plasma membrane where the FRB was targeted earlier. Increased concentration of 5-ptase at the plasma membrane will then rapidly deplete PIP2 at the plasma membrane [38-40]. To test our hypothesis that changes in membrane physical state might provide cancer cells with enhanced PI3K activity, a B16 cell line stably expressing both the 5-ptase-FKBP12 fusion construct and the FRB fragment of mTOR [39,40] would be generated. In this newly generated cell line, PI3K signalling would be impaired upon addition of rapamycin.

Unfortunately, during the pilot experiments it turned out that rapamycin affects heat shock protein induction by itself. An alternative rapamycin analogue (rapalogue AP21967, currently sold as A/C heterodimerizer by Clontech) has a list price of 1.115.700 Ft for 5 mg. To our knowledge, only one alternative system able to specifically target PIP2 levels was published and is based on optogenetics – the methodology that allows noninvasive manipulation of cell function by genetically encoded light-sensitive probes. In this method, two plant proteins controlling the expression of genes regulating floral initiation, cryptochrome 2 (CRY2) and the transcription factor CIB1, are used. Upon blue-light illumination, a FAD molecule bound to the photolyase homology region of CRY2 absorbs a photon, causing a conformational change in this domain promoting binding to the N-terminal portion of CIB1. As such, the light-inducible interaction of these two domains can be used for the acute manipulation of PI(4,5)P2 and PI(3,4,5)P3 in the plasma membrane via

the recruitment of 5-ptases or PI3-kinase [41]. Unfortunately, this method is described as a microscopy approach and as such not feasible for our aims.

Growth factor induced activation of PI3K results in subsequent activation of AKT1 [30]. This serine/threonine kinase AKT1 links growth factor signalling with Hsp response by both inhibiting the glycogen synthase kinase-3 (GSK3) what results in subsequent HSF1 activation [27,32]. HSF1 activity is negatively regulated through phosphorylation. Mitogen-activated protein kinases (MAPKs) of the ERK family phosphorylate HSF1 and represses transcriptional function through initial phosphorylation on serine-307, which primes HSF1 for secondary phosphorylation by glycogen synthase kinase 3 on serine-303 [42]. The activity of GSK-3ß activity is regulated by site-specific phosphorylation. Full activity of GSK-3ß requires phosphorylation on tyrosine-216, whereas phosphorylation at serine-9 inhibits GSK-3ß activity [43,44]. Hence, as an alternative approach, the phosphorylation status of GSK3β was analysed to assess the activation status of the PI3K pathway. B16-F10 cells were exposed to 10 mM mb-CD for 2, 5, and 10 minutes followed by heat exposure for 1 hour at 42,5^{o}C. Immediately after heat, proteins were isolated. My preliminary data indicated that, upon mb-CD exposure followed by heat exposure, an elevated phosphorylation of GSK3β at serine-9 was noted, an observation which was most prominent after 10 min mb-CD exposure. This observation suggests that mb-CD might affect the PI3K pathway what ultimately results in GSK3β inhibition. Inhibition of GSK3β would imply activation of HSF1 due to the lack of phosphorylation on hsf1 serine-303. However, the observed mb-CD-induced increased phosphorylation of HSF1 serine-326 upon heat exposure mentioned earlier suggested an enhanced HSF1 activity rather than decreased. Recently, it was suggested that the HSF1 phosphorylation signature of HSF1 alone is not an appropriate marker for HSF1 activity, but rather a fine-tuning mechanisms for regulating the transcriptionally active HSF1 [45]. Hence, rather than focusing on the potential direct outcome of single PTM events on HSF1 (serine-326 versus serine-303), the combinatorial interplay of HSF1 PTM events should be taken into account.

5 Understanding of the molecular basis of these membrane-associated processes in cancer

Cisplatin causes DNA damage through the formation of crosslinks which prevents mitosis what ultimately results in apoptosis. To measure DNA damage, I used the well-established single cell electrophoresis or comet assay [46]. The principle of the comet assay is that unfragmented DNA maintains a well-organized structure in the nucleus, but becomes disrupted when the cell is damaged. It detects both single-strand and double-strand breaks, and has a simple and inexpensive setup. B16-F10 cells were treated for 2 min with 10 mM mb-CD followed by 1 hour priming heat stress at 42^{o}C and, after overnight recovery, exposed to 75 µM cisplatin for 24h. As expected, the comet assay indicated significant DNA damage in cisplatin-treated cells compared to non-treated control. However, mb-CD exposure prior to heat and cisplatin exposure resulted in a more or less equal DNA damage as compared to mb-CD treated cells. As the observed increase in mb-CD-induced cisplatin resistance was small, the comet assay might lack the required sensitivity to detect potential differences in DNA crosslinking [47].

Cells encountering chemotherapeutic agents rely, amongst others, on ABC transporters to actively pump out the toxic agent. Thus, I wondered if ABC transporter activity would be affected by a very brief mb-CD exposure. B16-F10 cells were 2 min exposed to 10 mM mb-CD with or without BGP-15, heat-stressed for 1 hour followed by overnight recovery after which ABC transporter activity was measured. A slight but significantly higher ABC transporter activity was observed in mb-CD-treated cells. Pre-treatment with BGP-15 did not prevent mb-CD-induced increased transporter activity. This indicates that subtle plasma membrane modulations regulate transporter activity what ultimately might result in chemo resistance. As BGP-15 was ineffective to prevent mb-CD-induced enhanced ABC transporter activity, additional mechanisms might underlie the observed cisplatin resistance. Interestingly, increased GSK3β serine-9 phosphorylation has been correlated with enhanced cisplatin resistance in ovarian cancer cells [48].

6. In depth lipidomics reveals mb-CD-induced lipid profile alterations

The activity of multiple membrane-residing receptors and channels is determined by the direct lipid environment of the surrounding plasma membrane. Although the capacity of mb-CD to modulate membrane cholesterol levels is well described, the effect of mb-CD on other membrane-residing lipid species levels is not characterized. Hence, a time course

overview of mb-CD-induced changes to the lipidome was established. B16-F10 cells were exposed for 2, 5, 10, and 20 minutes to 10 mM MBCD and immediately processed for mass spectrometry lipidomic analysis. Depending on the lipid species, mb-CD exposure had different effects. First, certain lipid species (for instance the lysophophatidylcholine species LPC-16:0, LPC-18:0, and LPC-18:1) decreased whereas others (for instance the phosphoinositides species PI-38:2, PI-36-1, PI-36:2, PI-36:3, PI-36:4, PI-34-1, PI:34-2, PI:32-1) increased in a time-dependent fashion. On the other hand, phosphatidic acid species (PA-38:4, PA-36:1, PA-36:2, PA-34:0, PA-34:1, PA-32:1) initially increased after 2 minutes of mb-CD exposure but decreased below baseline levels upon extended exposure of 5, 10, and 20 minutes. These data indicate that the widely used cholesterol depletion agent cyclodextrin has hitherto unknown effects on the cellular lipidomic profile. Although the biological effects of these changes are not known, modulations of receptor clustering and/or function is anticipated.

7. Conclusion

This proposal aimed to have more insight in the signalling events linking cellular membranes and stress responses. In the current report, I presented evidence that mb-CD-induced plasma membrane cholesterol removal alters the heat-induced HSF1 PTM profile what might correlate with an altered HSF1 activity. Consequently, I demonstrated that limited cholesterol depletion from the plasma membrane alters the stress-induced hsp profile. The biological relevance of these findings lies in the fact that limited plasma membrane cholesterol depletion resulted in enhanced acquired stress tolerance. In addition and more relevant to cancer, I provided evidence that limited plasma membrane cholesterol depletion resulted in increased cisplatin resistance. Next, the current proposal aimed to get a better understanding of the molecular basis of these membrane-associated processes in cancer. Here, I presented evidence that limited plasma membrane cholesterol depletion resulted in enhanced ABC transporter activity while a functional link between mb-CD-induced enhanced phosphorylation on GSK3β serine-9 and cisplatin resistance is suggested.

8. Dissemination of the research results

A manuscript describing the above mentioned data of the current proposal is currently under construction.

Working title Limited deprivation with huge consequences: brief cholesterol removal induces significant gain in acquired thermo- and chemo resistance in B16 melanoma cells

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