ABSTRACT

Chaperone proteins consist of a group of evolutionary conserved proteins with a broad distribution among bacteria and all eukaryotic organisms. Their presence is esssential for cell survival in eukaryotic organisms. The main functions of chaperone proteins include the correct folding of newly synthesized proteins, the formation of protein complexes and the prevention of aggregates formation by missfolded proteins. This regulatory action is mainly achieved by the interaction of chaperone proteins with their clients or target proteins, which mainly consist of protein kinases and transcription factors.

Among the plethora of chaperone proteins, the most significant and well studied are HSP90 and its co-chaperone Cdc37. HSP90 is shown to have a pleiotropic action, interacting with a large group of kinases, transcription factors and steroid hormone receptors. For this pleiotropic action, HSP90 needs to cooperate with other chaperones or co-chaperone proteins.According to current experimental data, Cdc37 seems to be the favorable HSP90 co-chaperone. The dimeric HSP90-Cdc37 through the formation of multi-protein complexes regulates the activity of a large number of protein kinases, signalling molecules and steroid hormone receptors intracellularly. Both HSP90 and Cdc37 are involved in the main mechanisms for cell survival and metastasis of cancer cells, via their chaperoning activity to the deregulated kinases, transcription factors and receptors in cancer cells. For this reason Cdc37 and HSP90 are considered as attractive candidates for chemotherapy drug targeting.

Recently it was shown by our lab that HSP90 is localized not only in the cytoplasm- as known until then- but also in the cell surface of mouse melanoma and human mummary breast cancer cells. Furthermore, it was shown that cell surface HSP90 interacts with the extracellular domain (ECD) of HER-2 receptor. Using a monoclonal antibody against HSP90, namely mAb 4C5, which was produced in our laboratory previously and has the ability to remain bound on the cell surface and not to internalized, it has been shown that functional blocking of cell surface HSP90 reduces the invasion ability of human breast cacncer cells MDA-MB 453. This reduction is correlated with the blockage of the interaction of cell surface HSP90 and the ECD of HER-2, which in turn leads to lower levels of HER-2 activation. *In vivo* experiments in mice have also shown that mAb 4C5 reduces the formation of metastatic tumors

from autologous melanoma cancer cells and expands the life expectancy in these mice.

Taking these data into account, at first we examined using immunofluorescence techniques, the cell surface localization of Cdc37 on MDA-MB 453 cells, where HSP90 was previously shown to be detected. To evaluate whether Cdc37 localization is restricted to MDA-MB 453 cells, which overexpress the HSP90 interacting HER-2 receptor, we further examined the cell surface localization of Cdc37 on MDA-MB 231 cancer cells, which do not express HER-2 but EGFR receptor instead. Cdc37 was indeed detected on the cell surface of both these cells lines by immunofluorescence. In order to verify the presence of Cdc37 on the cell surface of these cell lines, membrane protein extracts were analysed by Western blot analysis. The presence of Cdc37 was confirmed in both cell lines. Making a step forward we employed siRNA technology in order knock down Cdc37 in both cell lines. siRNA-transfected cells exhibited extremely low levels of surface immunofluorescence staining compared to controls. These experimental findings further support our original observation, while they also verified the specificity of the antibody used, i.e. to specifically bind to cell surface Cdc37.Next, we showed using antibodies against Cdc37 and HSP90, that these proteins co-localize on the cell surface of live MDA-MB 453 and MDA-MB 231 cells.

Previously we showed that cell surface HSP90 participates in the invasion of MDA-MB 453 cancer cells. Based on these data, we decided to investigate whether cell surface Cdc37 also takes part in this process. In order to confirm that the observed results would not be caused by the inhibition of intracellular Cdc37, the internalization assay was performed in both MDA-MB 453 and MDA-MB 231 cell lines using a commercial anti-Cdc37 antibody. By this assay, it was verified that the particular antibody is cell impermeable for both cell lines and remains bound to the cell surface even after 16 hours of incubation. By recruiting the “wound healing” assay it was shown that the specific anti-Cdc37 antibody significantly reduced the cell migration of both cancer cell lines, proving that cell surface Cdc37 is involved in the cancer cell invasion. By employing the same set of experiments we further demonstrate that mAb 4C5 reduces the migration of MDA-MB 231cells.

In order to clarify the molecular mechanisms underlying the involvement of cell surface Cdc37 and HSP90 in cancer cell invasion, membrane protein fractions from both cell lines were immunoprecipitated using anti-Cdc37 antibodies. The

immunoprecipitates were then analyzed by Western blot, where we detected that cell surface Cdc37 interacts with cell surface HSP90 on both MDA-MB 453 and MDA-MB 231 cells. In the same set of experiments it was also revealed that cell surface Cdc37 interacts with HER-2 and EGFR in MDAMB 453 and MDA-MB 231 cells, respectively. It was also shown that cell surface HSP90 interacts with EGFR in MDA-MB 231 cells. Incubation of cells with the anti-Cdc37 antibody revealed that the surface interaction of Cdc37 with HSP90 was reduced in both cell lines and simultaneously that the interaction of Cdc37 with HER-2 and EGFR was also reduced. When cells were incubated with mAb 4C5, the interaction of cell surface Cdc37 with HSP90 was also reduced in both cell lines. In the same set of experiments incubation of cells with mAb 4C5, interaction of Cdc37 with HER-2 and EGFR was also reduced in MDA-MB 453 and MDA-MB 231 cell lines, respectively. These data suggest that a chaperoning system of receptor tyrosine kinase (RTK) exists on the cell surface of breast cancer cells, similar to the intracellular chaperoning machinery, with the formation of multiprotein complexes of Cdc37-HSP90-HER-2/EGFR. This model is further supported by our finding that functional inhibition of surface Cdc37 by the cell impermeable anti-Cdc37 antibody reduces the phosphorylation/activation of Akt in MDA-MB 453 cells as well as the phosphorylation/activation of EGFR, Akt and MEK in MDA-MB 231 cells. In MDA-MB 231 cells the functional inhibition of cell surface HSP90, using the cell-impermeable anti-HSP90 antibody mAb 4C5, also reduced the phosphorylation/activation of EGFR,Akt and MEK.

In a parallel study, our team showed that HSP90 is secreted in the culture media of both MDA-MB 453 and MDA-MB 231 cells. The secreted HSP90 seems to participate in the activation of matrix metalloproteinases MMP-2 and MMP-9.

As a next step in this study, we sought to investigate the role of cell surface HSP90 in the metastasis of breast cancer cells. For this purpose we used the two cell lines (MDA-MB 453 and MDA-MB 231) in two xenograft models using the immunodeficient mice SCID. In the first model the metastatic deposits of MDA-MB 453 cells in mice lungs were examined either with pre-incubation with the chimeric anti-HSP90 antibody ch4C5 or without pre-incubation. It was shown that cancer cell deposition in the lungs was significantly lower in SCID mice that had received cells pre-incubated with ch4C5. In the second experiment, SCID mice received MDA-MB 231 cells by intravenous injections and were treated for 2 weeks with mAb 4C5 or

PBS (controls). Seven weeks later mice that had received mAb 4C5 treatment had reduced cancer metastatic nodules in their lungs.

Finally, in this thesis the effects of *Vitis vinifera* fruit (grape) extract on on chaperone proteins were examined in MDA-MB 453 cells. It was shown that, although there was no effect on the dimeric HSP90-Cdc37 interaction or the total levels of HSP90 protein expression, it reduced the expression of total Cdc37 protein. Furthermore, the extract seems to affect the expression of HSP70 and proteolysis through the ubiquitination of cell proteins, in a differential manner. At this end, it has been shown that the particular plant extract induces cell death of MDA-MB 453 cancer cells and also prevents the completion of cell mitosis.

The data presented in the current thesis strengthen the notion of an extracellular chaperoning system, which is similar to the classical intracellular chaperoning machinery, where chaperone proteins act synergistically through multiple interactions in order to regulate a group of biomolecules. This system consists an attractive target for metastasis treatment.

ΠΕΡΙΛΗΨΗ ΧΑΜΙΤΙΕ

The main functions of chaperone proteins include the correct folding of newly synthesized proteins, the formation of protein complexes and the prevention of aggregates formation by misfolded proteins. Among the chaperone proteins, the most significant and well studied are HSP90 and its co-chaperone Cdc37. Recently it was shown by our lab that HSP90 is localized not only in the cytoplasm, but also in the cell surface of mouse melanoma and human mammary breast cancer cells and it is involved in cancer cell invasion and metastasis. The aims of this study was to investigate the possible presence of Cdc37, the main co-chaperone of HSP90, on the cell surface of cancer cells, the investigation of possible interactions with receptor tyrosine kinases as well as the participation of Cdc37 and other chaperones in metastasis. In this study, Cdc37 was detected on the cell surface of breast cancer cells MDA-MB 453 and MDA-MB 231, by immunofluorescence and Western blot. Then, by using an experimental method namely wound healing assay, it was illustrated that Cdc37 participates in cancer cell invasion, in vitro. Co-immunoprecipitation experiments revealed that cell surface Cdc37 interacts with surface HSP90 as well as with receptor tyrosine kinases HER-2 and EGFR. The formation of the multi protein complex HSP90-Cdc37-HER-2/EGFR seems to be important for the downstream signal transduction through the MAP kinases and Akt pathways. Furthermore, by using the zymography technique, it was shown that extracellular HSP90 is necessary for the activation of MMP-2 and MMP-9 metalloproteinases. In vivo experiments with immunodeficient SCID mice revealed that inhibition of cell surface HSP90 by the monoclonal antibody mAb 4C5 reduces the metastatic deposits and metastatic formations in the lungs. Finally, the effect of grape extract in breast cancer cells was examined. In these experiments it was shown that the expression of Cdc37 and HSP70 is altered and cells are driven to apoptosis. The data from the present thesis suggest an extracellular chaperoning system of receptor tyrosine kinases by Cdc37 and HSP90, which contributes to the regulation of these receptors and participates in the mechanisms of invasion and metastasis of cancer cells.