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An attempt to determine the mechanism of early apoptotic spectrin aggregation and physiological role of this event.

Summary

In previous studies aggregation of spectrin associated with early apoptosis induced by mixture of chemotherapeutics in tumor cells was observed. The presence of PKCθ was detected in these apoptotic aggregates, and PKCθ inhibition with pseudo-substrate, accelerated the formation of spectrin aggregates. Since aggregation of spectrin has been observed in early stages of apoptosis, signal to form the aggregates should appear shortly after apoptosis induction. Molecular mechanism underlying this phenomenon remains unexplored, so the project which is an object of this thesis attempts to solve this mechanism of spectrin aggregation.

The experiments were conducted on lymphoid leukemia cell line – Jurkat T and spectrin aggregation was observed by quantitative analysis of the protein in Triton X-100 insoluble fraction or by immunofluorescence. The first stage of the study was to establish the aggregation of spectrin from the induction of apoptosis pathway. Apoptosis was activated using two inducers: TRAIL protein (receptor apoptosis pathway) and hydrogen peroxide (mitochondrial apoptosis pathway) and after induction the dynamics of spectrin aggregates formation was observed. It was found that spectrin forms aggregates after induction of both pathway of apoptosis, however, slightly faster after use of TRAIL. PKCθ was also observed in apoptotic aggregates after induction of apoptosis with TRAIL.

As spectrin aggregates are formed after the activation of the receptor TRAIL-R, but prior to activation of caspase-8, it was decided to check the interval between the two stages, which may be the initiation of aggregation of spectrin. For this purpose stable cell line with decreased expression of a gene encoding FADD was generated. In cells *FADD*-KnD, FADD protein level was reduced by more than 50%. These cells were less susceptible to apoptosis induced by TRAIL, compared to control cells. Most importantly, reduced expression of FADD caused a delay in spectrin aggregation after induced apoptosis. In addition, in this cell line PKCθ is localized predominantly in the cell membrane as compared to the cytosolic localization in the control cells. This observation suggests that the TRAIL – FADD signaling pathway may be associated with the process of spectrin aggregate formation.

Based on previously published studies and the results mentioned above, it was decided to further investigate the relationship between kinase PKCθ and aggregation of spectrin. It has been shown that the presence of kinase PKCθ in the membrane fraction is not a factor inducing spectrin aggregation. It has been demonstrated that the stable cell line with reduced level of expression of a gene encoding a PKCθ, Jurkat T *Prkcq*-KnD cells, was found that spectrin forms aggregates much faster after apoptosis induction by TRAIL than in control cells Jurkat T. It was also found that spectrin in the aggregates is phosphorylated, but this is not a signal to aggregation. To explore the effect of PKCθ on the formation of spectrin aggregates, we transfected cells with a DNA construct encoding the C-terminal fragment of spectrin (Spc-GFP). After initiation of apoptosis it was observed that endogenous spectrin aggregates were formed faster in cells that overexpressing the C-terminal fragment of spectrin. Furthermore, in cells overexpressing the C-terminal fragment of spectrin, it was found that PKCθ translocation from cytosol to the membrane after activation of TRAIL-R, was delayed.

On the other hand, the project included analysis of impact of spectrin on the process of apoptosis. For this purpose a stable Jurkat T cell line with lowered level of spectrin (*Sptan1*-KnD) was obtained. Experiments have shown that caspase-8 is activated earlier in the cells *Sptan1*-KnD compared to control cells. This may be due to the fact that amount of the active form of caspase-8 is higher in *Sptan1*-KnD cells. Inactive form of caspase-8 in Jurkat T cells is localized in the cell membrane and cytosol, but in *Sptan1*-KnD mainly in the membrane zone. It was observed that the caspase-8 precursor did not co-localize with spectrin aggregates. Destabilization of the actin skeleton does not affect the distribution of the precursor caspase-8, however, it was observed that the precursor caspase-8 is localized in the membrane with vimentin and protein is 4.1 only in *Sptan1*-KnD.

The aim of presented here project was to define the mechanism of spectrin aggregation during apoptosis. Through series of experiments we were able to show that spectrin aggregates form faster in cells with lower concentration of PKCθ, but slower in cells with lower level of FADD. It seems that PKCθ localized in the area of membrane plays as a negative regulator on spectrin aggregation, but on the other hand the lower concentration of spectrin in cells can lead to faster apoptosis by an example, gives an open space in the membrane for proteins like caspase-8 or 4.1. In our study we show, that PKCθ can play protective role in spectrin aggregation, and FADD is its inductor. Understanding the molecular regulation of spectrin aggregation will be helpful in defining its role in early apoptosis.