6. Summary

The caspase family cysteine proteases have emerged as central regulators of apoptosis. To date three plausible pathways for the cleavage and thus the activation of executioner caspases during apoptosis have been identified: (a) A receptor mediated recruitment of caspase-8 into the **d**eath **i**nducing **s**ignalling **c**omplex (DISC). (b) A mitochondrial pathway in which cytochrome c, Apaf-1, and caspase-9 interact as a caspase activating complex (APOPTOSOME). (c) An Endoplasmic Reticulum (ER) specific pathway, in which caspase-12 is activated in response to ER stress.

Density arrested AKR-2B cells die rapidly in response to serum starvation or treatment by Anisomycin. Cell death is associated with typical hallmarks of apoptosis including membrane blebbing and chromatin condensation but lacks energy dissipation in mitochondria and intranucleosomal fragmentation. During apoptosis a considerable DEVDase activity has been detected which seemed to be represented by a single enzyme. This enzyme had typical effector caspase characteristics, like caspase-3, but exhibited an unusual high K_M values of ~100 µM and its large subunit exhibited a molecular weight of 19 kDa, instead of expected 17 kDa.

In the present study, this enzyme was identified to be caspase-3 with the help of the generation of recombinant mcaspase-3 protein. N-terminal sequencing of the recombinant mcaspase-3 protein revealed that its prodomain cleavage site differs from that in the human homologue (Asp-9 instead of Asp-28). Thus the large subunit of active caspase-3 was found to be 19 kDa. Furthermore the K_M value of recombinant mcaspase-3 was ~100 µM in perfect agreement with that found in cell extracts. Affinity labeling in combination with 2D-GE confirmed that indeed caspase-3 is activated as the main executioner in AKR-2B cells during apoptosis. Since the receptor mediated pathway has already been excluded previously [129], a possible involvement of mitochondria mediated pathway in the activation of caspase-3 was examined. Gel filtration experiments revealed that caspase-3 is mainly eluted as free enzyme and in lower levels within the differently sized high molecular weight complexes of ~600 kDa and 250 kDa in response to serum starvation or Anisomycin treatment. Though the apparent molecular weight of the complexes containing caspase-3 are in accordance with recently published data, they were devoid of Apaf-1 and caspase-9. Apparently, mitochondria mediated pathway is also not involved since neither formation of high molecular weight complexes of Apaf-1 nor cleavage of caspase-9 was observed. Thus, the activation of caspase-3 is caused by a noncanonical pathway during apoptosis. In addition a new 450 kDa complex

containing activated caspase-6 was found in response to serum starvation which is clearly separated from caspase-3 containing complexes.

Generally caspase-3 has been found to be responsible for most of the morphological changes during apoptosis. One of those is intranucleosomal fragmentation. Although caspase-3 was found to be the main executioner caspase in AKR-2B cells the lack of the intranucleosomal fragmentation led to examine its localization. As detected by overexpression of the Caspase-3-GFP fusion construct in AKR-2B, procaspase-3 was localized in the cytoplasm, wheras the active caspase-3 was mainly found in the membrane blebs and partially in the cytoplasm. Clearly no nuclear localization of active caspase-3 was detected. These data gave first hints on the mechanism of degradation of AKR-2B cells demonstrating that cytoplasmic membrane is the primary site of activation of caspase-3.

The possible role of caspase-12 and ER stress mediated pathway of apoptosis was also examined in AKR-2B cells. Kinetic studies showed that caspase-12 is activated at the same time together with caspase-3 in response to serum starvation or Anisomycin treatment resulting in two cleavage products of 47 kDa and 35 kDa, respectively. It was therefore examined whether these two caspases were eluted in the same complexes. Gel filtration experiments revealed that caspase-12 is released as free enzyme during apoptosis. To date all the studies have identified that caspase-12 is specifically activated in response to ER stress. After serum starvation or Anisomycin addition there was no increase of the protein expression level of the chaperone protein Grp 78 which is known to be higly elevated in response to ER stress indicating that both treatments did not lead to ER stress. In contrast treatment with ER stressor substances i.e. Thapsigargin, A23187 (ionophore) induced an ER stress in AKR-2B which lead to unspecifically degradation of caspase-12. Thus it is unlikely that caspase-12 is activated in response to ER stress in AKR-2B cells. However, after the in *vitro* addition of recombinant caspase-3 to cytosolic extracts caspase-12 is cleaved into 47 kDa and 35 kDa fragments similiar to those observed *in vivo*. In conclusion the present data demostrated that caspase-12 is activated in AKR-2B cells during apoptosis triggered through pathways that do not involve (the) ER stress and provided evidence that caspase-3 might be involved in activation of caspase-12.

Thus the present study in AKR-2B cells gives hints for the existence of additional pathways for apoptosis other than the classical ones.