5. Discussion

5.1 Cell death model of AKR-2B cells

Density-arrested AKR-2B fibroblasts die rapidly after serum deprivation. Death after serum removal starts after a delay of 90 min. Dying of the cells ceases after 5-6 h with a survival of 50% [126-127]. In response to Anisomycin treatment, a potent inhibitor of protein biosynthesis AKR-2B cells display characterstics of cell death similar to serum starvation [141]. Treatment of AKR-2B fibroblasts with Anisomycin (10μM) led also a rapid disintegration of the cells ($t_{1/2}$ = 5h) which was 100% after 24h [141]. In serum deprivation or Anisomycin treatment, cell death is associated with typical hallmarks of apoptosis including membrane blebbing, chromatin condensation [126-141]. But remarkably there is no dissipation of the mitochondrial potential and no internucleosomal fragmentation [141].
One of the most striking finding of this study was the observation that upon serum deprivation or treatment with Anisomycin caspase-3 is activated as the main executioner caspase. Further experiments which were adressed to examine the activation of caspase-3 showed that high molecular weight complexes of caspase-3 are formed and there was no complex formation of Apaf-1 with caspase-9 in both cases. Interestingly, caspase-12 was also activated by Anisomycin treatment or serum deprivation and this activation was not coupled to ER stress.
Thus, these data strongly suggests that cell death in AKR-2B cells induced by serum starvation or by treatment with Anisomycin utilizes similar pathways if not identical. Moreover this study gives hints for the existence of additional pathways for apoptosis other than the classical ones. In this regard AKR-2B cells display an interesting death model. Further work with these cells would be useful for apoptosis research to find out novel regulation mechanisms.

5.1.1 Expression and purification of recombinant caspase-3

It is evident that caspases are activated in AKR-2B cells. Considerable increase in DEVDase activity has been detected beside the constitutive VEIDase and IETDase activities. Partially purification by ionexchange chromatography has also supported that the major part of the caspase activitiy is due to DEVDase [129]. Generally DEVDase activities are assigned to the active effector caspases i.e. caspase-3 and caspase-7 which recognize the tetrapeptide motif DEVD [20]. Therefore the activation of caspase-3 has been tested in earlier studies in AKR-2B cells. A single enzyme has been detected by affinity labeling exhibiting a molecular weight of 19 kDa which might be active caspase-3 [129]. But, the molecular weight of active

caspase-3 had been reported to be 17 kDa in the literature [28]. Additionally, K_m values of this endogenous caspase have been also determined for the substrates DEVD.pNA and VEID.pNA. [129]. Compared to that of the human homologue the K_m values of the endogenous caspase in AKR-2B cells were untypical high. Because of these discrepancies DEVDase activity could not be assigned unequivocally to caspase-3 in AKR-2B cells.

Here, in this study these discrepancies were clarified and DEVDase activity is assigned to active caspase-3 in AKR-2B cells with the help of the generation of recombinant mouse caspase-3. Generation of a mcaspase-3 was a prerequisite to be able to define the precise molecular weight of active mcaspase-3 and for the comparison of the K_m values of the both recombinant and endogenous mcaspase-3.

Generally ist is known that expression of procaspase-3 in bacteria yields processed active enzyme that is indistinguishable from the native enzyme [16,28]. Therefore full length mcaspase-3 gene was cloned into a bacterial expression vector in frame with a His_6 tag. Soluble active recombinant caspase-3 protein was produced in E.coli using a T7 expression system. The enzyme was readily purified from the lysate using metal chelate affinity chromatography. Purified mcaspase-3 was composed of two subunits with molecular masses of 19 and 14 kDa (Fig. 4.4.-4.5.) which indicates that the proenzyme has been processed to the mature enzyme as described before for the human homologue. Two possible explanations have been proposed for the caspase-3 proenzyme processing either caspase-3 processes itself or a bacterial protease processes caspase-3. But the first possibility is mostly favoured since there is evidence that invitro translated caspase-3 can also cleave its own prodomain after p17/p12 cleavage is made. As determined by N-terminal sequencing the active mcaspase-3 enzyme was generated by the cleavage of proenzyme at Asp-10 and Asp-175. Remarkably, N-terminal sequence analysis also showed that mcaspase-3 prodomain cleavage site is different from that of the human. Active mCaspase-3 was generated by the cleavage of the proenzyme from the tetrapeptide motif of **TSVD** at **Asp-9**(Fig.4.6). Whereas the human homologue was generated by cleavage of the proenzyme from the tetraeptide motif **ESMD** at **Asp-28** (Fig.4.6.). Thus the large subunit of mouse caspase-3 is found to be extended by ~2kDa compared to the human homologue (Fig. 4.10). These results revealed the precise molecular weight of active caspase-3. Because of the 2 kDa extension the large subunit of active m caspase-3 was detected at a molecular weight of 19 kDa (Fig. 4.12).

Does this 2kDa extension leading to an altered function *i.e.* in enzyme activity or substrate binding ? To adress this question mcaspase-3 protein was expressed and purified after restoring the cleavage site as in the human. The tetrapeptide motif (**ESMD**) containing the

Asp-28 in human caspase-3 was found to be altered to **KSVD** in mouse (Fig.4.6.). Generally caspases are more stringent in P_4 and P_1 for the tetrapeptide recognition. For example they required an absolute Asp in P_1. Therfore only the P_4 site was mutated as in the human by a single amino acid mutaion $K_{25}E$. N-terminal sequence analysis of this *mt* type showed that the prodomain was cleaved from the motif of **ESVD** at **Asp28** . Almost identical K_m values of both *wild type* and *mutant* were determined for the substrates DEVD.pNA and VEID.pNA, respectively (Table 5.1.). Thus the 2 kDa extension most likely does not cause an altered function. On the other hand with the finding that these K_m values were similar to that of the endogenous mcaspase-3 the DEVDase activity could be assigned to caspase-3.

Determination of the K_m values of both *wild type* and *mutant* for the substrates DEVD.pNA and VEID.pNA allowed us to compare them those of the human homologue, as well. K_m values for the substrate DEVD.pNA differs around 12 fold between human and mouse caspase-3. Whereas the K_m values for the substrate VEID.pNA were almost identical. These results showed that although their active centers are highly conserved the efficiencies of hydrolysis of their optimal substrate DEVD differs strikingly. This would imply that although the binding determinants are identical in two enzymes there are subtle differences in the orientation or chemical nature of the residues involved in binding and catalysis. One of the main structural feature that contributes to the differences in substrate specificity of these enzymes is the presence or absence of flap like loops that projects over the active site [28]. This flap is present in caspase-3 like enzymes. In agreement with the sequence alignments mcaspase-3 has acquired two mutations in this flap like loop (Fig. 4.6. residues 248-259), compared to hcaspase-3, which might be a possible explanation that mcaspase-3 cleaves the same substrate with less efficiency.

Table 5.1. Comparison of the K_m values of mouse and human caspase-3

K_m values (µM)	Natural hcaspase-3	Rec. hcaspase-3	Natural mcaspase-3	Rec. mcaspase-3
DEVD.pNA	9.7	11	132±4	116±20
VEID.pNA	ND	250	530±140	211±53

ND: not determined

Generally caspase substrates and their effective inhibitors have been designed according to their preferred cleavage sites in the natural substrates. For example DEVD mimics the site in PARP cleaved by caspase-3, or VEID mimics the site in lamins cleaved by caspase-6.

Although such observations lead to the generalization that DEVD based reagents are caspase-3 specific or VEID caspase-6 specific, this is clearly an oversimplification. Since here it was demonstrated that the eficiency of the hydrolysis of these substrates by caspase-3 differs only 2 fold. Thus caspases have overlapping substrate specificities which suggest at least partially overlapping functions. However, the usefullnes of peptide substrates which were recognized similiarly by caspases with similar specificities limits their use as specific probes of these enzymes.

5.1.2 Caspase-3 is the main executioner caspase- in AKR-2B cells

Although those results obtained from the analysis of recombinant mouse caspase-3 protein somehow allowed us to assign the DEVDase activity to caspase-3, it might be also possible that the other effector caspases are present which can cleave the tetrapeptide motif DEVD as effectively as caspase-3. To address this question a previously developed affinity labeling method was used which directly identifies the activated caspase in apoptotic cells [143]. It has been reported that this approach generates snap shots that simultaneously identifies multiple labeled caspases, estimates their relative abundance and indicates their post translational modifications [143].

Here, in this study by this approach it was shown that caspase-3 is activated as the main effector caspase in the cell death of AKR-2B cells induced by either serum starvation or Anisomycin treatment (Fig.4.14., 4.16). It was also shown that most likely it is present as multiple species.

The affinity labeling approach in combination with 2D-GE by using the irreversible tetrapeptide inhibitor YVKD-(bio)-aomk, allowed the identification of the labeled caspases by affinity blotting. The high concentration of the label, the irreversible nature of the binding and the extended time of labeling all favour the labeling of even those caspases with low affinity for the label. In fact, the YVKD tetrapeptide motif of the inhibitor is a poor motif for the effector caspases, but it labels it neverthless. Therefore if active unlabeled caspases were present, then their specificity must have been quite different from caspase-3. However considering that specificity of the effector caspases to peptide substrates overlaps with caspase-3 the number of caspases that should avoid labeling is likely to be small. A more probable explanation is that only active caspase-3 is detected because other caspases were either absent or at very low levels present. This is also supported by the absence of caspase-7 protein AKR-2B cells (Fig. 4.17.).

In earlier studies it has been reported that the relative abundance of active caspases present in apoptotic cells may reflect the hierarchy of the caspase cascade [14, 17, 21]. According to this

model, the finding that caspase-3 is the major active caspase suggests that it is the end product of the caspase cascade in AKR-2B cells. The end product of a protease cascade is often described as carrying out the main function of the whole system, suggesting that the major active caspase in apoptotic cells are directly involved in cell destruction.
The present data also indicate that active caspase-3 is present most likely as multiple species. some of the detected spots indicate that they are cleaved at distinct sites and differ in their molecular weight, suggesting an alternative or multistep processing of caspase-3. However other species differs in their isoelectric points suggesting that most likely posttranslational modifcations are present other than proteolysis.
In summary with affinity labeling and 2D-GE caspase-3 is identified as the main active executioner caspase in AKR-2B cells.

5.1.3 Involvement of complex formation in cell death of AKR-2B fibriblasts induced by serum starvation or Anisomycin treatment

Growing evidence in biology suggests that many of the key signalling events that take place within the cell are initiated and/or carried out within very large protein complexes. In general complexes efficiently bring the signalling molecules in close proximity to one each other and often constitute a convergence point for various signalling pathways. Examples to the proteins that are activated in complexes include the IκB kinase (IKK) complex (~700-900 kDa), cytosolic chaperonins (~800-1000 kDa), and the 26S proteasome complex (~2MDa) [144]. During apoptosis, complex formation is required for the activation of caspases, either in receptor mediated (DISC) or stress induced pathways (APOPTOSOME) [95-98, 106-110]. In stress induced apoptosis together with dATP and cytochrome *c*, Apaf-1 forms a multimeric complex so called apoptosome that activates procaspase-9. Caspase-9 in turn activate executioner caspases, including caspase-3 [106-110]. The formation of this complex occurs through a multistep process and serves as a key commitment step for activation of caspase-9 and downstream caspases including caspase-3 and -7 [106-110]. Cell death is thought to result from the futher proteolysis of cellular substrates by active caspase-3 and -7. Most recent studies indicate that in native cell lysates when incubated with cytochrome c and dATP, Apaf-1 oligomerizes in to two high molecular weight complexes corresponding to ~1.4 MDa and ~700 kDa, respectively [109-110]. However only the latter complex leads to the activation of the effector caspases -3 and –7 [110].
In the present study, activation of caspase-3 was shown in response to serum starvation or Anisomycin treatment as the main effector caspase. Since the receptor mediated pathway

which requires the formation of DISC and active caspase-8 has already been excluded in AKR-2B cells [129], an apoptosome like caspase activation mechanism could be involved. To validate this hypothesis gel filtration experiments have been done by using the extracts of apoptotic cells, induced by either serum starvation or Anisomycin treatment. Surprisingly these experiments suggest an alternative pathway to apoptosome complex formation which became a real classical phenomenon in the stress induced activation of caspases during the last years.

The most striking finding of the present study was the observation that effector caspase activity DEVDase was eluted in three regions during gel filtration corresponding to the molecular weight of ~600, ~250 and 60 kDa, respectively (Fig 4.19, 4.25.). Remarkably the sizes of the complexes corrobrate exactly with those reported by Cain et al. for human monocytic tumor cells (THP.1) a ~700 kDa apoptosome, ~250 kDa microapoptosome and uncomplexed caspase-3 (~80 kDa) [109]. But the distrubution of the active caspase-3 in these complexes differs strikingly. Evidently in AKR-2B cells most of the active caspase-3 was found to be uncomplexed in consistent with the DEVDase profile, whereas it was reported to be found predominantly in the microapoptosome in THP.1 cells[109]. Interestingly although after 3h starvation most of the active caspase-3 was found uncomplexed, kinetic studies of the complex formation suggested that caspase-3 is activated in the complexes and then released as free enzyme (Fig. 4.20). DEVDase activity was firstly found in the complexes of 600 and 250 kDa after 1h serum starvation wheras it was detected in the region of free enzyme with a delay of one hour. Thus these data indicate that the induction of apoptosis most likely induce the complex formation leading to the activation of caspase-3 in the complexes and exclude the possibility of the distribution of the active caspase-3 in to the complexes.

It is generally believed that the characteristics of the apoptosome complex is its size and composition, especially the distribution of oligomerized Apaf-1 in the lysates of apoptotic THP.1 cells. In AKR-2B cells although the sizes of the active caspase-3 containing complexes corroborate with the apoptosome complexes, composition differs strikingly in regard to the distribution Apaf-1 or caspase-9. In AKR-2B cells gel filtration experiments and western blotting analysis of extracts obtained from either serum starved or Anisomycin treated cells revealed that Apaf-1 did not oligomerize in to the high molecular weight complexes corresponding to the 1.4 MDa or 700 kDa (Fig.4.24., 4.26.) Apaf-1 was entirely found in its native state at a molecular weight of ~200 kDa in the extracts of apoptotic AKR-2B cells most likely existing as dimer or bound with another protein (for serum starvation Fig 4. and anisomycin induced Fig 4.24., 4.26). Importantly, no coelution of Apaf-1 or caspase-9 was

observed excluding their interaction in AKR-2B cells (Fig. 4.21., 4.24. and Fig.4.26.). The other striking difference is that caspase-9 was not processed in AKR-2B cells in response to serum starvation or Anisomycin treatment (Fig. 4.21., 4.26).

In a previous study it has been proposed that the activation of caspase-9 requires a three step reaction [106-108]. First, dATP/ATP binds to Apaf-1 through its consensus nucleotide binding domain and is hydrolized to dADP or ADP , respectively, second cytochrome c binds to Apaf-1 and promotes the multimerization of Apaf-1 cytochrome c complex third, once the multimeric complex is formed, procaspase-9 recruited to this functional complex in a 1:1 molar ratio to Apaf-1 and it becomes activated through autocatalysis. Thus cytochrome c and dATP mediated oligomerization of Apaf-1 is a prerequisite for the activation and processing of caspase-9 [145]. Importantly, cytochrome c seems to play an additional role in processing since it is still detectable after recruitment of caspase-9 in to the functional complex. Holocytocrome c which has the apoptosis promoting activity exists exclusively in the intermembrane space of mitochondria in living cells [102]. The newly translated apocytochrome c in cytosol does not have apoptosis promoting activity [102]. However when cells undergo apoptosis in response to a variety of stimuli, holocytochrome c is released from mitochondria to the cytosol where Apaf-1 is located. Remarkably in AKR-2B cells neither the membrane potential of mitochondria is changed nor is there cytochrome c efflux [141] which might be the possible explanation of the question why Apaf-1 did not oligomerized in to the complexes and caspase-9 is not processed. Since caspase-9 can also act as zymogen without proteolytic processing [38], its possible involvement can not be entirely excluded. It has been described that caspase-9 has proteolytic activity similar to its cleaved form because the zymogenicity (i.e. the ratio of the processed protease activity to the non processed protease activity) of caspase-9 is approximately 10 wheras the zymogenicity of caspase-3 is greater than 10.000 [38]. Moreover the proteolytic processing of procaspase-9 has only a minor effect on the ezymes catalytic activity, wheras the key requirement for caspase-9 activation is its association with a dedicated protein cofactor Apaf-1. Along this line, it has been also reported that in certain circumstances caspase-9 zymogen, but not its (L/V/IEHD) cleaving activity is needed for cell death [146]. Clearly caspase-9 was not cleaved and predominantly eluted in an uncomplexed form in the extracts of apoptotic AKR-2B cells. If caspase-9 is involved, its assembly in to the possible complexes must be transient and weak. Thus the peresent data in this study strongly suggests that activation and complex formation of caspase-3 might be brought about by an alternative pathway.

Recently additional CED-4/Apaf-1 family members have been identified. CARD4, CARD7, CARD12 and Nod1 and Nod2. Whereas both CARD4 and Nod2 seem to be involved in the activation of NFκB, CARD7 and 12 may play a role analogous to Apaf-1 and mediate caspase activation [147-148]. Nod 1 have been demostrated that it has an Apaf-1 like activity. It binds caspase-9 via CARD domain and promotes its activity. An interaction of CARD7 and CARD12 with caspases -2 and –1 has been also demostrated [147]. But to date these experiments have been done under *in vitro* conditions. For a better understanding of physiological roles in vivo experiments are required.

In a recent study [149] an alternative mechanism was proposed for the activation of caspases in which dependancy receptor DCC (deleted in colorectal cancer) induces apoptosis and activation of caspase-3 in a caspase-9 dependent but Apaf-1 independent fashion. Dependancy receptors *i.e.* $p75^{NTR}$, the common neutrophin receptor, the androgen receptor, and as recently described DCC create cellular states of dependence on their respective ligands by inducing apoptosis when the ligands are absent, but inhibiting apoptosis in the presence of ligand [149]. In the recent study the authors showed that DCC-induced cell death requires caspase-9 but does not involve cytochrome c release and subsequent apoptosome associated caspase-9 processing. DCC directly interacted both with caspase-9 and caspase-3 [148]. Moreover they showed that this mechanism of activation of caspase-3 is not only independent of mitochondria, cytochrome c release and apoptosome formation but also independent of caspase-8 activation. But the combination of DCC, caspase-3 and caspase-9 was not sufficient to activate caspase-3 *in vitro*. Activation required S-100 lysates to allow the formation of a functional complex, which strongly indicates to the involvement of other proteins present in the lysate [149].

The present data in the current study resembles in some regards those for DCC. Apparently there is no involvement of the Apaf-1 and of the caspase-8 pathway in the activation of caspase-3 in AKR-2B cells.

In the present study beside to the 600 kDa and 250 kDa caspase-3 activating complexes a new complex of 450 kDa was also described in response to serum starvation for the first time in which activated caspase-6 is present (Fig. 4.21.). This 450 kDa complex is clearly separated from the caspase-3 containing complexes (Fig.4.21.). Active caspase-6 was detectable as free enzyme, as well (Fig.4.21.). Surprisingly activated caspase-6 as detected by western blotting analysis did not coelute with the major VEIDase activity in the high molecular weight range (>450 kDa), though this activity was inhibited by the compound VEID.cho (Fig. 4.19.). Furthermore there was only marginal VEIDase activity in those fractions which revealed the

presence of active caspase-3 (Fig.4.21). The assessment of a VEIDase activity in the low molecular weight range is compromised by the presence of active caspase-3 which exhibits considerable activity towards the substrate VEID.pNA (Fig.4.19, 4.21). But an indirect evidence for the activation of caspase-6 in AKR-2B cells came from a recent study that has demonstrated the cleavage of the lamins, cellular substrates of caspase-6 [129]. Thus, it is evident that caspase-6 is activated in AKR-2B cells but it is not clear if it is activated in this 450 kDa complex since the zymogen is only detected as free enzyme and at very small amounts in the region of 600 kDa (Fig. 4.21.). Interestingly active caspase-6 was totally released as uncomplexed free enzyme in response to Anisomycin treatment (see Appendix S-300 data of Anisomycin treatment) being the only differences between the cell deaths induced by the serum deprivation or Anisomycin treatment. It might be possible that caspase-6 is cleaved as free enzyme and then complexed together with other proteins to be translocated to the cellular compartments where it can cleave its substrates.

The 26-S proteasome complex is important in the nonlysosomal degradation of proteins and suggested to be involved in apoptosis signalling [150]. Regulatory molecules that are involved in apoptosis have been identified as substrates of the proteasome. Recently the coelution of the proteasome complex with VEIDase/IETDase activities was demonstrated [109]. In this study it was also shown that in response to serum starvation the proteasome assembles from precursor proteasome complexes in to the ~2 MDa complex, which seems to represent the catalytically active 26-S proteasome complex (Fig.4.24.). Importantly the proteasome complexes can be clearly separated from the caspase activating complexes (by Superose 6 HR column). Cain et al.[109], speculated that high molecular weight VEIDase and IETDase activities may be uncharacterized proteolytic activities of the proteasome, this assumption is supported here 1) the active proteasome complex of 2MDa coelutes with VEIDase/IETDase activities which are not inhibitable by their corresponding inhibitors (Fig. 4.19, 4.23) and the precursor proteasome complexes which are demostrated not to be catalytically active, eluted in control gel filtration experiments in fractions lacking the VEIDase/IETDase activities (Fig.4.24).

In conclusion these data revealed that caspase-3 is activated in noncanonical complexes which are involved in the cell death of AKR-2B fibroblasts induced by either serum starvation or Anisomycin treatment and importantly these data suggest an alternative pathway independent of the known major pathways such as formation of the apoptosome complexes containing Apaf-1, caspase-9 or cytochrome c. Since only the active forms of the effector caspases were associated within the complexes, and given the sizes of the complexes (600 kDa, 450 kDa or

250 kDa) it is likely that other proteins were part of these complexes. Future work in AKR-2B cells which is adressed to identify the proteins composing these complexes would be useful to get more information about this alternative pathway.

5.1.4 In vitro reconstitution of caspase activating complexes

In vitro reconstitution of the apoptosome complexes in AKR-2B cells was also demonstrated in the present study by using the human monocytic tumor cells (THP.1) as a control. In contrast to the in vivo situation, the addition of 200 µg cytochrome c, 2mM dATP and 5 mM $MgCl_2$ to the extracts of untreated AKR-2B cells resulted in the distribution of Apaf-1 in to the apoptosome complexes (1.4 MDa and 700 kDa, respectively), (Fig. 4.27.), though the major part still eluted in the mono-/dimer region. In THP.1 cells most of the Apaf- 1was found to be associated with the 700 kDa apoptosome (Fig. 4.27.). Thus the present study provide the evidence to compare the *in vitro* and *in vivo* complex formation by addition of cytochrome c + dATP or serum starvation, respectively and suggest the differences between these i*n vitro* and *in vivo* situations.

It is generally believed that complex formation can be reconstitute also by the addition of recombinant caspases *i.e*. caspase-9. In this regard, to examine the role of active caspase -3 in the complex formation, recombinant active caspase-3 was added to the extracts of untreated AKR-2B cells (Fig. 4.28.). Interstingly as confirmed by gel filtration experiments and by western blotting analysis, active caspase-3 was found in the similar complexes as detected in the extracts from serum starved or Anisomycin treated AKR-2B cells (Fig. 4.28). Moreover no active caspase-3 was detected in the complexes by the addition of the inhibited recombinant caspase-3 to the extracts (Fig. 4.28). This suggested that most likely an active caspase-3 is required for the complex formation.

5.1.5 Localization of caspase-3 during apoptosis

In mammals caspases (principally caspase-3) are activated in a caspase cascade leading to a rapid disablement of key structural proteins and important signalling, homeostatic and repair enzymes [150]. Caspase-3 is the most frequently activated protease in mammalian cell apoptosis. Earlier works have demonstrated that caspase-3 is important for cell death in a variety of tissue, cell type or death stimulus and is essential for some of the characteristic changes in cell morphology and certain biochemical events associated with the execution and completion of apoptosis i.e. nuclear fragmentation [151].

In AKR-2B cells although caspase-3 was found to be the main executioner active caspase during apoptosis, internucleosomal fragmentation was nor detected [141]. To adress this unusual behaviour, localization of caspase-3 was demonstrated here. By using a GFP+Caspase-3 construct localization of procaspase-3 is found to be mainly cytoplasmic (Fig. 4.31.) consistent with the other studies [152]. By immunofluorescence staining upon apoptotic stimuli, active caspase-3 was localized particularly to the membrane blebbings and partially to the cytoplasm (Fig. 4.29.). Remarkably, no nuclear staining of active caspase-3 was detected indicative to nuclear localization of active caspase-3 (Fig. 4.29.). Thus these data suggested that caspase-3 might be involved in the morphological changes i.e. in membrane blebbing during apoptosis but not nuclear fragmentation in AKR-2B cells. One possible effect of this particular localization of caspase-3 might be to be involved in a destabilization and leakage of the membrane. Different subcellular localization during apoptosis might allow caspase-3 to access to a distinct set of apoptotic substrates. In addition sequestration of procaspases in various cellular compartments might restrain their activation and separate them from their substrates in living cells.

The surface blebbing during apoptosis is typical for disintegrating cell. Proteolysis of α-fodrin (a major component of the cortical cytoskeleton of most eukaryotic cells) had earlier been suggested to contribute to the structural rearrangements including blebbing during apoptosis [48]. This is supported by the fact that fodrin has binding sites for microtubules, calmodulin and actin [48]. In a previous study it has been shown that introduction of caspase-3 cDNA into MCF-7 (caspase-3 (-) cells) restores α-fodrin cleavage as well as membrane blebbing [48] is clearly showed that caspase-3 is essential for the cleavage of α-fodrin which may contribute to the membrane blebbing. It has been also demonstrated that caspase-3 mediated cleavage of PAK-2 and gelsolin lead to the morphological changes associated with apoptosis [19]. In case of gelsolin, caspase-3 cleaves a fragment that severs actin filaments [49]. The cleavage of gelsolin is important, because the enforced expression of the gelsolin fragment in several cells causes them round up and detach [49]. In addition gelsolin deficient cells have an extremely delayed onset of membrane blebbing [48]. In summary these studies demonstrated that membrane blebbing is mediated at least in part by cleavage of gelsolin and fodrin by caspase-3 during apoptosis. It is likely that active caspase-3 is also involved in the cleavage of gelsolin and fodrin in AKR-2B cells.

5.1.6 Examination of the activation of Caspase-12 in cell death of AKR-2B cells

The Endoplasmic Reticulum (ER) is a principal site for site for protein synthesis and folding and also serves as a cellular storage site for Ca^{+2} [116]. Perturbations of Ca^{+2} homeostasis, increased production of free radicals, inhibition of protein glycolisation and accumulation of misfolded proteins in the ER can all elicit cellular stress responses. In particular ER stress signals which protect cells against changes in [Ca^{+2}] leads to a toxic build up of misfolded proteins [116-117]. Prolonged ER stress leads to cell death and is linked to pathogenesis of some neurodegenerative disorders that feature misfolded proteins including Alzheimer´s disease, Parkinson´s disease [116]. Like the mitochondria, the ER is a deposite for both proapoptotic and antiapoptotic molecules. The known proapoptotic molecules include caspase-12, P28Bap31, GADD153, whereas the antiapoptotic molecules identified to date include the ER chaperone proteins Grp78, calreticulin, protein disulfide isomerase and CRP-150 as well as DAP1 [117]. Despite the identification of these apoptotic regulators, the pathways that connect ER stress to apoptotic cell death remain unclear.
Earlier reports have indicated that ER stress induces the formation of a Grp78-procaspase-12-procaspase-7 complex [122]. Prolonged stress can result in the disruption of this multimeric complex and release of active caspase-12 that may activate caspase-9 and lead to apoptosis [151]. Other activators of caspase-12 include the IRE1-TRAF2 complex and calpain, both in response to ER stress [120-121]. However earlier studies have also suggested that calpains act as negative regulators of caspase processing by inactivating caspases -9 and -3 [154]. Thus while calpains may be required for caspase-12 activation they may not have a role in the caspase cascade leading to cell death. Activation of caspase-12 by any of the above mechanisms may therefore initiate downstream caspase processing, activation and cell death.

In AKR-2B cells involvement of the extrinsic receptor mediated pathway has been already excluded [129]. In the present study it is disclosed that the mitochondrial intrinsic pathway is also not involved in the activation of caspase-3. Therefore the possible role of caspase-12 and ER stress mediated pathway was examined. Kinetic studies clearly showed that caspase-12 is activated in response to serum starvation resulting in two cleavage products of 47 kDa and 35 kDa, respectively (Fig. 4.32.). Additionally, activation of caspase-12 was examined in response to Anisomycin treatment. These data also revealed that caspase-12 and caspase-3 were activated at the same time points, with a delay of 1h compared to serum starvation (Fig. 4.33.). Moreover the putative cleavage products of caspase-12 were also observed (Fig.4.33.). Remarkably the time points of the appearance of the cleavage products of caspase-12 were

found identical with that of the p17 subunit of active caspase-3 in response to serum starvation or Anisomycin treatment which indicated to a strong correlation between these two caspases (Fig. 4.32-33.). It is therefore examined whether these two caspases are coelute or activated in the same complexes. Interestingly gel filtration experiments indicated to the opposite. Caspase-12 is totally released as free enzyme in response to serum starvation or Anisomycin treatment (Fig. 4.34.).

To date all of the studies have demostrated that caspase-12 activation is specifically mediated by ER stress [118-123]. In the present study, it was demonstrated for the first time that activation of caspase-12 was induced by other than ER stress.Treatment of Thapsigargin an inhibitor of the intracellular Calcium-ATP transporter and Ca^{+2} ionophore A23187 induces the elevation of Ca^{+2} concentrations in the cytosol [118-120] disruption of intracellular calcium homeostasis and accumulation of unfolded proteins in ER leading to the inducution of chaperone proteins, a phenomenon termed as unfolded protein response or ER stress[118-123]. In order to examine the role of ER stress in the activation of caspase-12 in AKR-2B, firstly the influences of ER stressors A23187 and Thapsigargin were examined. As it has been shown previously, the both substances causing a rapid elevation of [Ca^{+2}] in AKR-2B cells [142]. Maximal effective concentrations of A23187 and Thapsigargin treatment for the induction of apoptosis has been determined as 2.5 μM and 0.5 μM, respectively [142]. The further evidence came from the present study showing that these substances leading to ER stress in AKR-2B cells, by means of the analysis of the expression level of ER chaperone protein Grp78. In response to treatment with A23187 and Thapsigargin Grp78 protein expression level was found to be highly elevated in the total lysates of AKR-2B cells (Fig.4.36.). Furthermore analysis of the cytosolic extracts of AKR-2B cells treated with 2.5 μM A23187 and 0.5 μM Thapsigargin clearly showed that after 16 h Grp78 protein is released to the cytosol which indicated to high level of stress and entire damage of ER (Fig. 4.35.). In contrast the analysis of the expression levels of Grp78 protein in response to Anisomycin treatment or serum starvation clearly showed that Grp78 protein expression is not elevated excluding that an ER stress is not induced (Fig. 4.37.). Furthermore the previous [Ca^{+2}] measurements supported that since neither Anisomycin nor serum starvation lead to elevation of [Ca^{+2}] in cytosol in AKR-2B cells [142]. Thus Anisomycin or serum starvation do not cause any stress to ER suggesting that caspase-12 is activated by an alternative mode.

Activation of caspase-12 was also tested in response to treatment of the respective substances A23187 and Thapsigargin. Interestingly, those results revealed that, after 16h in accordance to Grp78 elevation caspase-12 is started to be degraded. But unlike to the Anisomycin treatment

or serum starvation, this was an unspecifically degradation, since no putative cleavage products were detected (Fig. 4.36.). A very low level activation of caspase-3 was also observed by means of the appearance of p17 subunit (Fig. 4.36.). Thus, it is evident that these substances induce ER stress in AKR-2B cells. However, concerning its unspecific degradation it is unlikely that caspase-12 is specifically activated in response to ER stress. A possible explanation for the presence of active caspase-3 at very low levels might be that the presence of the prolonged ER stress leading to interaction of caspase-12 with caspase-9 and activate caspase-3 as proposed reviously.

In a recent study, Rao et al. has described an alternative mode of caspase-12 activation in which caspase-7 is involved [122]. In this study they showed that caspase-7 translocates to the ER surface and cleaves and activates caspase-12 during ER stress induced apoptosis [122]. As shown in the present study, AKR-2B cells do not express caspase-7. One possibility might be that in AKR-2B cells caspase-3 cleaves and activates caspase-12 similiar to caspase-7. Kinetic studies also suggested this possiblity since these two caspases are activated at the same time. In order to examine this, recombinant caspase-3 was added to the extracts of non treated AKR-2B cells and subsequent western blot analysis clearly showed that caspase-12 is cleaved by addition of recombinant caspase-3 (Fig. 4.38.) Moreover the identical cleavage products, 47kDa and 35 kDa species were detected which were observed in response to serum starvation or Anisomycin treatment (Fig.4.38.). Thus these results suggest that caspase-3 can cleave and activate caspase-3 similarily to caspase-7. It might be also plausible, that caspase-3 translocated to the ER surface during apoptosis, if so, caspase-3 must undergo modifications causing its translocation to the ER surface, since this type of a translocation to the ER surface is not observed with other caspases. Another possible mechanism may involve binding of caspase-3 and -12 to Grp78 at the ER surface resulting in their activation. This would be similar to the activation of caspase-8 and caspase-9 both of which are thought to be activated by induced proximity brought about by adaptor molecules.

In conclusion the present data in this study suggested that caspase-12 is activated in AKR-2B cells during apoptosis triggered through pathways that do not involve the ER stress and provide evidence that caspase-3 might be involved in activation of caspase-12. Furthermore these results highlight the presence of the alternate pathways which may be important for caspase activation. Furtherwork which will be adressed to subcellular localization of caspase-12, the significance of caspase-12 activation and its downstream targets in AKR-2B cells would be helpful in understanding the mechanism of activation.