INTRODUCTION

Most, if not all, animal cells have the ability to self-destruct by activation of an intrinsic suicide program when they are no longer needed or have become seriously damaged. Apoptosis is a mode of cell death characterized by a series of specific morphological changes. The majority of programmed cell deaths from insects to mammals exhibit striking consistent morphological features, typical for apoptosis, suggesting that a molecular machinery for death has been conserved during evolution. This machinery, which is still largely uncharacterized, executes a series of changes in the cells resulting in condensation of the nucleus, cleavage of chromatin, disruption of the nuclear lamina network and plasma membrane changes like membrane blebs, resulting in recognition and engulfment of apoptotic cells by their neighbours (for review see Steller, 1995; Martin et al., 1994; McConkey and Orrenius, 1994; Whyte, 1996; Ameisen et al., 1995; Bortner et al., 1995; White and Steller, 1995; Schulze-Osthoff, 1994; Cohen, 1993; Kaufmann, 1996). Recent studies have revealed important physiological triggers for apoptosis. These include the Fas (CD5/Apo1) cell surface molecule, which is a member of the tumor necrosis factor/nerve growth factor receptor family (Enari et al., 1995; Ju et al., 1995; Los et al., 1995; Tewari and Dixit, 1995). Some of the intracellular signals generated in the cell after activation of these receptors have now been partly characterized. Recent results demonstrate the involvement of ICE-like proteases in this process (Kumar, 1995; Lazebnik et al., 1994; Miura et al., 1993; Nicholson et al., 1995; Tewari et al., 1995; Wang et al., 1994).

SUMMARY

AKR-2B cells disintegrate after serum removal. After a delay of ~90 minutes, cell death began and reached after six hours a plateau of 40-50% remaining living cells. We used time-lapse video microscopy to monitor dynamic structural changes and to measure the time span of individual cells to die. The first change was the rapid appearance of membrane blebs. Membrane vesicles were rapidly extruded and reintegrated by the cell. This highly dynamic process of an affected cell stopped after 80±20 minutes with its death. Conductivity measurements showed that at that time the membrane was electrically permeable. By using fluorescence double staining with propidium iodide and Hoechst 33258, we show that membrane leakage leading to disintegration is accompanied, and for some cells preceded, by nuclear condensation. The energy state of the intact cells was monitored by measuring the intracellular ATP content which remained high (6 mM) throughout the entire time of investigation. Mitochondrial potential was determined by rhodamine 123 fluorescence in parallel to the measurement of membrane permeability via uptake of propidium iodide and lead to the detection of a cell population that exhibits a high mitochondrial potential and an uptake of propidium iodide indicating a membrane disruption of cells which still have a high energy charge. It is shown by electron microscopy that mitochondria were swollen and damaged in parallel to nuclear condensation. There was no DNA fragmentation as shown by two independent methods. Addition of the ICE-like protease inhibitor tyr-val-ala-aspartylchloromethylketone immediately after serum starvation lead to an almost complete survival of the cells up to 6 hours. A pronounced protection was still observed after 24 hours, suggesting an involvement of this type of protease in the onset of cell death after serum removal. Apparently, serum withdrawal activates a succession of initial events that are similar to those defined as ‘apoptosis’, i.e. nuclear condensation and membrane blebbing. These steps are, however, accompanied or rapidly followed by cell lysis and disruption of mitochondria, both of which are characteristic of necrosis.

Key words: Apoptosis, Necrosis, Fibroblast, Protease, Serum removal

Cell death of AKR-2B fibroblasts after serum removal: a process between apoptosis and necrosis

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The survival of density-inhibited fibroblasts like Balb/c-3T3 or AKR-2B cells is critically dependent on the presence of growth factors or serum (Simm et al., 1994; Tamm and Kikuchi, 1991, 1993; Tamm et al., 1991, 1992). Upon serum removal a significant portion of these cells die within 6 hours. The decrease in viable cells follows approximately a first order kinetic until a residual fraction remains in steady state. For AKR-2B this fraction amounts to 40-50% of the initial cell number (Simm et al., 1994). The death process can be prevented by the addition of growth factors, e.g. platelet growth factor or second messenger agonists (Simm et al., 1994; Tamm and Kikuchi, 1993). Though Balb/c-3T3 and AKR-2B fibroblasts in many regards behave similarly, differences are reported concerning the precise involvement of second messenger signalling pathways (J. Hoppe et al., unpublished).

This article therefore describes basic characteristics of death of AKR-2B cells occurring after serum deprivation. The rapidity of the cell death for AKR-2B as well as for Balb/c-3T3 cell lines has initially led to the proposal that this cell disintegration is different from apoptosis which usually requires a longer time to occur. By using several techniques including time-lapse video microscopy, fluorescence double staining of DNA, flow cytometric TUNEL assay, and determination of the energy state we found several features characteristic for apoptosis: (1) nuclear condensation already after 1-2 hours; (2) membrane changes leading to a berry like structure of the cells and an increased permeability; (3) possible involvement of an interleukin-1-β-converting enzyme like protease. However, we could not find further characteristics of apoptosis like DNA fragmentation. Instead, a breakdown of mitochondria parallel to nuclear condensation was observed.

MATERIALS AND METHODS

Platelet derived growth factor (PDGF) was prepared as described (Hoppe et al., 1989, 1990). The dyes Hoechst 33258, rhodamine 123, ethidium bromide and propidium iodide were purchased from Sigma (Deisenhofen, Germany). Cell culture reagents were purchased from Gibco (Eggenstein, Germany). The TUNEL kit was from Boehringer (Mannheim, Germany). The inhibitor tyr-val-alα-asp-chloromethylketone (YVAD-cmk) was from Bachem (Heidelberg, Germany).

Cell number and volume

Stock cultures of AKR-2B mouse fibroblasts were propagated in antibiotic-free McCoy-5A medium with 5% Hyclone calf serum for less than 3 months to minimize fluctuations. During that time the responses to PDGF did not change. Cells were prepared for stimulation with various reagents using the protocol originally described by Shipley et al. (1984). Briefly, experimental cultures were seeded at a density of 5x10^5 cells/cm^2 into 24-well plastic dishes (Falcon) and grown for 5 days in McCoy-5A medium containing 5% calf serum (Hyclone) without a medium change. For the measurement of cell survival, cells were washed twice with MCDB 402 medium without serum. Immediately after, the respective tested compounds were added. The number of viable cells was determined by using the CASY-1 system (Scharfe, Reutlingen, Germany) based on the Coulter Counter principle. Osmolarity of the media was measured with an Osmomat 030i from Gonotec (Berlin, Germany).

Northern blot analysis

For northern blot analysis cells were grown in 6-well plates. Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). Total RNA (10 μg) was denatured at 65°C in a solution containing 1.2 M formaldehyde, 35% formamide, and ethidium bromide. The RNA was electrophoresed in a 1.25% agarose gel containing 6.7% formaldehyde. After vacuum-transfer onto nylon sheets (Amersham Hybond N) in 6x SSC (1x SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) the RNA was crosslinked by u.v. irradiation (120 mJ/cm^2 for 30 seconds). The blot was hybridized at 60°C in 10x SSC, 10x Denhardt’s solution, 0.2% SDS, 300 μg/ml denatured herring sperm DNA. The DNA probes were labeled by random priming using an oligolabeling kit from Pharmacia (Freiburg, Germany) with [α-32P]dCTP (Hartmann Analytic, Braunschweig, Germany). The filters were washed in solutions containing decreasing contents of SSC in 0.1% SDS and 2 mM EDTA. The final wash stringency was 0.4% SSC at 65°C. Radioactivity was detected and quantified by a phosphoimager. Standardization was done with a β-actin probe. The following probes were used: v-fos, 1 kb PstI/PvuII fragment (Oncor, Hamburg, Germany); β-actin, 770 bp fragment (Oncor); c-myc, 2.8 kb Xbal/HindIII fragment from pBSVcmyc1 (Land et al., 1983) containing exon 2 and 3; odc, 1 kb HindIII fragment from odc48 (McCoulologue et al., 1984).

DNA fragmentation

Cells were seeded in 10 cm dishes and grown as described in McCoy medium supplemented with 5% Hyclone calf serum. After treatment for 2 or 4 hours with MCDB 402 medium without serum, dead cells were detached by vigorous shaking of the dishes and collected by centrifugation. In some control experiments the entire population of the cells was harvested with a rubber policeman. Cells were collected and lysed in 1 ml of 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS. Lysates were incubated for 4 hours at 56°C with 0.2 mg/ml proteinase K. After extraction with an equal volume of phenol:chloroform: isoamylalcohol (25:24:1, v:v:v), followed by reextraction with chloroform:isoamylalcohol (24:1, v:v), DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 5.3, and one volume of isopropanol at −70°C for at least 1 hour. Precipitated DNA was collected by centrifugation at 14,000 g for 30 minutes at 4°C, washed with ice-cold 80% ethanol and air dried. DNA was then resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and treated with 0.5 mg/ml RNase at 37°C. DNA samples were fractionated by electrophoresis in a 1.8% agarose gel buffered with 0.1 M trishydroxymethylamethane, 0.0415 M boric acid, 1 mM EDTA, pH 8.3 (TBE) at 100 V and visualized by ethidium bromide staining (Jimenez et al., 1995).

Observations of living cells

Cells were grown on coverslips (12 mm diameter) until confluency was reached using the standard protocol. The slides were then submersed in MCDB 402 medium containing 5 μg/ml Hoechst 33342 dye for 15 minutes to stain the nuclei. The coverslips were then mounted in a home made culture chamber attached to an inverted microscope (Leica DMIRB) and were kept in a humidified atmosphere containing 5% carbon dioxide at 37°C. Cells were photographed at intervals of 15 minutes either at phase contrast or for fluorescence staining of the nuclei using the filter set A (excitation: band pass 340-380 nm; emission: long pass 425 nm). Magnification was 200-fold.

Time-lapse video microscopy

To determine more precisely the time dependency of morphological changes, time-lapse video recording was used. Cells were seeded using standard conditions into a home-made tissue culture chamber of 27 mm² which was mounted in an inverse light microscope (Leitz Labofert, Wetzlar, Germany). A magnification of 200-fold was used. Cells were cultured in humidified air supplemented with 5% carbon dioxide at 37°C. After the cells were grown to confluency in 5% Hyclone calf serum, they were washed twice with MCDB 402 medium containing no serum and were kept in this medium. Images were recorded with a video camera (Panasonic WVBF310. Mat-
sushita, Osaka, Japan) and a time-lapse video recorder (Panasonic AG-6730, Wiesbaden, Germany) using a rate of approximately 20 pictures per minute. Digitalization of these pictures was carried out with a frame grabber (Movie Machine II, Fast Multimedia AG, München Germany) using an IBM-compatible computer. Statistical analysis was made from the observation of 20 individual cells. The entire experiment was done three times.

**Staining with propidium iodide and Hoechst 33258**

Cells were grown under standard conditions on coverslides. At the indicated time after serum deprivation propidium iodide was added at a concentration of 20 μg/ml. After 10 minutes the cells were washed twice with phosphate buffered saline (PBS) supplemented with Ca²⁺ and Mg²⁺. Cells were fixed with 3% paraformaldehyde in PBS following an incubation for 5 minutes in 0.1% Triton X-100. After washing twice with PBS, cells were stained for 20 minutes with Hoechst 33258 (1.2 μg/ml in PBS). After rinsing three time with PBS and a final wash with deionized water, the slides were mounted. Cells were inspected with an Axioskop microscope from Zeiss (Jena, Germany) under phase contrast, and blue and red fluorescence. Magnification was 630-fold.

**DNA fragmentation assayed by terminal transferase labelling**

For the determination of DNA strandbreaks by the terminal deoxynucleotidyl transferase (TUNEL) assay a commercial kit from Boehringer was used. Briefly, AKR-2B fibroblasts were trypsinized and spun down at 200 g for 5 minutes. The cells were washed twice in PBS/1% BSA at 4°C and adjusted to 1x10⁷ cells/ml. The suspension was transferred into V-bottomed 96-well multi-well plates (100 μl/well) and fixed for 30 minutes at room temperature with 100 μl/well 4% freshly prepared paraformaldehyde on a shaker. Cells were centrifuged at 300 g for 10 minutes and the supernatant was removed. Cells were washed with 200 μl/well PBS, resuspended in permeabilization buffer (0.1% Triton X-100 in 0.1% sodium citrate, pH 7.4) and incubated for 2 minutes on ice. Cells were then washed twice with PBS (200 μl/well), resuspended in 50 μl TUNEL reaction mixture and incubated for 60 minutes at 37°C in the dark. The cells were washed twice in PBS, resuspended in 500 μl PBS and analysed by flow cytometry with an Coulter Epics Elite ESP.

**Determination of nucleotide content**

For determination of the nucleotide contents, cells were grown in 6-well plates. For the termination of the reaction, cells were washed briefly with MCDB medium and subsequently lysed in 60% methanol. After filtration through reversed phase chromatography material, the filtrate was dried and the residue was taken up in 40 μl water. Capillary electrophoresis was performed on an ABI 270 HT instrument using a standard 50 μm capillary with a length of 50 cm and a buffer consisting of 50 mM sodium phosphate, 2 mM tetrabutylammonium-hydrogensulfate and 50 mM octylglucoside, pH 7.8.

**Determination of the mitochondrial potential by flow cytometry**

AKR cells were trypsinized, resuspended and centrifuged at 200 g in 0.5% calf serum in PBS; 1x10⁶ cells were resuspended in 250 μl MCDB in the case of the serum deprived cells or in 250 μl McCoy-5A/5% serum in the case of the control. Rhodamine 123 (10 μg/ml, 26 μM) was added and the cell suspension incubated for 30 minutes at 37°C. The suspension was diluted with 750 μl HBS (0.15 M NaCl, 5 mM Hepes, pH 7.35); 40 μg/ml propidium iodide (PI, 60 μM) was added and the cells incubated for 5 minutes at room temperature. The fluorescence of the labeled cells was measured by flow cytometry with an Epics Elite ESP (Coulter) using an argon laser with an excitation wavelength of 488 nm and an emission wavelength of 525 nm for rhodamine 123 fluorescence and of 630 nm for PI fluorescence, respectively.

**RESULTS**

**Serum deprivation leads to rapid disintegration of AKR-2B cells**

To exclude the possibility that osmotic differences between the medium McCoy-5A containing 5% calf serum and the medium MCDB 402 are the cause of the membrane breakdown, the osmolarities were measured. A value of 280 mOsmol was found for McCoy-5A supplemented with 5% Hyclone calf serum and a value of 270 mOsmol was found for MCDB 402. Osmolitic effects were therefore excluded.

Fig. 1. shows the effect of serum removal in density arrested AKR-2B fibroblasts. As reported previously, the cells rapidly stopped and the number of cells remained constant for at least 48 hours. In order to exclude the possibility that a genetic selection had occurred, in one series of experiments starved cells were stimulated to divide by refeeding them with McCoy medium containing 5% calf serum. After two days a final density is reached that is approximately 30 to 40% higher than that prior to the first starvation. Remarkably, the second serum deprivation lead to the same results as the first one, i.e. cells decayed with an identical kinetic and again, about 50% of the

![Fig. 1. Time dependence of cell disintegration after serum removal.](image-url)

(■) AKR-2B cells were grown in 24-well plates for 5 days in McCoy-5A medium supplemented with 5% Hyclone calf serum. This medium was then replaced by MCDB 402 medium without serum or protein. The number of viable cells was determined as described in Materials and Methods after the indicated time intervals. (□) Cells were grown for five days in McCoy-5A medium containing 5% calf serum and starved for one days in MCDB 402 medium without serum. Cells were then refeed with McCoy-5A medium supplemented with 5% calf serum for two days and were starved for a second time in MCDB 402 medium without additions.
cells died. Furthermore, there was no cell death in sparse growing cultures induced by serum removal (data not shown). These data argued strongly against the existence of two different cell populations, which differ in their sensitivity toward serum removal.

In order to obtain more information about the disintegration of individual cells, time-lapse video microscopy was used (Fig. 2A). Pictures of cells were recorded continuously for 24 hours after serum removal. Cells were monitored at bright field. With this technique, confluent viable cells which have low contrast are barely visible. On the contrary, cells undergoing death are easily recognized. (For a better resolution see Fig. 2B.) In Fig. 2A, cells were monitored over a time span of 270 minutes. First disintegrating cells are noticed after 45 minutes. In agreement with the results obtained by cell counting, massive cell death is initiated after 90-120 minutes and ceased after ~6 hours for the majority of the cells. A single cell marked with the arrow was selected to demonstrate the key morphological change. The initial event (45 minutes) is a rounding up of the cell. Multiple membrane vesicles are shed from the cell or appear at its surface. During a short period (45 minutes to 105 minutes) this structure rapidly changes: shed vesicles are re-integrated and new particles are extruded. After that time the morphological changes of the cell stop suddenly, and the cell...
is considered as dead. A final structural change occurred after 140 minutes with a loss of the membrane vesicles and a prominent appearance of the nucleus. This cell structure remains unchanged for a long time of at least 12 hours. By analyzing 20 individual cells the following parameters were obtained: the time lapse between a normal structure and the full established bleb-like structure, i.e. initiation of death, is rather short (5.6±2.1 minutes). Rapid membrane structural changes occurred during 80±23 minutes. This time is considered as the dying period of a cell.

A higher resolution is presented in Fig. 2B. Cells were photographed using phase contrast which allows us to distinguish the outlines of healthy cells (upper part). In parallel, nuclei were stained with the membrane permeable dye Hoechst 33342 (lower part). Healthy cells are characterized by a flat morphology in the phase contrast together with a dark blue staining of the nuclei. Condensed nuclei in dying cells are distinguished by a light blue staining. The arrow indicates a cell showing the development of bleb structures without apparent alteration in the morphology of the nucleus. Arrowheads indicate some typical cells showing blebs together with nuclear condensation.

These results show that alterations in the plasma membrane occur prior to morphological changes of the nucleus.

**Energy state of dying cells**

Bleb structures are characteristic of apoptosis but may also appear in necrotic cells that are energy deprived and have lost an intact cytoskeleton (Kabakov and Gabai, 1994). We have
therefore analysed the ATP content of AKR-2B cells during serum deprivation. The ATP concentration is high in AKR-2B cells grown in McCoy medium (cellular content ~6 mM). Furthermore, the energy charge \( E = \frac{[\text{ATP}]+1/2[\text{ADP}]}{[\text{ATP}]+[\text{ADP}]+[\text{AMP}]} \) of 0.90 is extremely high (Table 1). These values did not change during starvation. Thus these results demonstrate a high energy state of the intact cells and exclude the possibility that an energy drop in the living cells is the initiating event for cell death. They do not exclude a mechanism which implies membrane damage leading to a drop in the ATP concentration that eventually caused the appearance of membrane changes.

Apoptotic and necrotic cells may be distinguished by their mitochondrial potential. Necrotic cells lose their potential rapidly after disintegration of their membranes. In the canonical pathway of apoptosis, the cytoplasmic membrane remains intact, granting a high mitochondrial potential (Cohen, 1993; Kaufmann, 1996; Steller, 1995). Membrane permeability is monitored by an uptake of propidium iodide and the mitochondrial potential by staining with rhodamine 123. Thus, necrotic cells are characterized by a high propidium iodide fluorescence and a low rhodamine 123 fluorescence. In contrast, apoptotic cells do not take up propidium iodide (low PI fluorescence) and maintain a high mitochondrial potential (high rhodamine 123 fluorescence).

After staining of the AKR-2B cells with both dyes the fluorescence intensities as well as the side and forward scattering signals of single cells were measured by flow cytometry. Cells with a high rhodamine 123 fluorescence and low PI fluorescence are indicated by a pink colour; cells with high PI fluorescence and low rhodamine fluorescence are indicated by a yellow colour. Fig. 3 shows that, as expected, control cells exhibit a high mitochondrial potential and no PI uptake. Surprisingly, after one hour of serum deprivation a cell population (green) appeared showing PI-uptake together with a high rhodamine 123 fluorescence. This population represents cells that became leaky and still possess their mitochondrial potential. After a longer incubation time (4 hours) these cells apparently lose their potential leading to the appearance of necrotic cells (yellow). Side scattering and forward scattering were recorded after 4 hours of serum deprivation in parallel with the fluorescence intensities of single cells. This measurement indicates morphological changes associated with functional (fluorescence) alterations of the cells. There is clearly a single population of intact cells (pink) with high mitochondrial potential and no PI-uptake. Necrotic cells (yellow) exhibit the lowest scatter signals, whereas the green population (leaky cells with high potential) shows mainly a reduced forward scatter signal. This population probably reflects those cells showing membrane blebs. As described above, these blebs are not static but undergo rapid fluctuations. In order to maintain this dynamic process, a high energy state of the cell is required.

**Nuclear condensation and mitochondrial disruption**

Nuclear condensation is considered to be part of the cellular events involved in apoptosis. Usually, the condensation is followed by a cleavage of DNA at the internucleosomal region leading to fragments with sizes of multiples of 180 base pairs, approximately (Steller, 1995). These fragments, the ‘DNA ladder’ can be visualized by standard agarose gel electrophoresis in the presence of ethidium bromide. The first series of experiments was performed in order to demonstrate nuclear condensation. The further aim was to investigate the temporal relation between membrane cleavage and nuclear condensation. Cells were subjected to serum deprivation and incubated with propidium iodide, which does not penetrate cells with an intact membrane. After the indicated time, propidium iodide was carefully washed out and after fixation of the cells, the entire cellular DNA was stained with Hoechst 33258. Cells were photographed at phase contrast, and fluorescence with the appropriate filters for the two dyes. Pictures were taken after 2 hours starvation (Fig. 4). At this time several stages were detected: (1) normal non fragmented nuclei stained with Hoechst and unstained by propidium iodide as expected for viable cells; (2) nuclei that show a high staining by Hoechst 33258 at the periphery; (3) condensed nuclei stained by Hoechst and not by propidium iodide; (4) nuclei stained by

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both dyes. Here the cytoplasmic membrane was permeable before or simultaneously with nuclear condensation. Apparently, in dying AKR-2B cells the processes of nuclear condensation and loss of membrane integrity overlap.

Cells were further analysed by electron microscopy. Fig. 5 shows a picture of a healthy cell and cells after three or six hours serum deprivation. In the healthy cell the structure of the nucleus and the size and shape of the mitochondria are apparently normal. In the cell shown after three hours of serum deprivation the chromatin condensation is quite obvious. Surprisingly the mitochondria had significantly enlarged forming globular structures accompanied by a disruption of the cristae. Quite similar mitochondrial structures were observed after six hours. Here the nuclear condensation is more advanced leading to a small particle which is shed from the nucleus. These pictures confirm the above results suggesting an unusual type of cell death in which apoptotic events take place in parallel with membrane damage, thus leading to energy deprivation.

DNA was isolated from AKR-2B cells at the time where maximum cell death occurred (2 and 4 hours). There was no detectable DNA fragmentation after DNA agarose gel electrophoresis (data not shown). To increase the sensitivity, dead cells were selectively isolated by shedding off. As shown in Fig. 6A, there was no indication for DNA fragmentation. As a second independent method we tried to label 3'-DNA ends with biotinylated dUTP. The biotinlation can then be quantified. As shown in Fig. 6B, there was no indication of additionally incorporated biotinylated dUTP in dead cells compared to control cells. The results thus demonstrate that though nuclear condensation occurred, there was no DNA fragmentation. Similar results have been reported recently for thymocytes and MOLT-4 cells (Cohen et al., 1992; Falconeri et al., 1993).

Cell death is not associated with a high expression of the protooncogene myc

Upregulation of the myc oncogene has been reported to predispose cells for apoptotic death after serum removal (Evan et al., 1992; Gibson et al., 1995; Harrington et al., 1994; Milner et al., 1993). The expression of this protooncogene together with fos and ODC (ornithine decarboxylase) was investigated by northern blotting after serum removal. As a control, cells received 50 ng/ml PDGF-BB at the time point of serum deprivation. As shown in Fig. 7, the expression of all three RNAs was substantially stimulated only in the presence of PDGF-BB. After 30 minutes a maximum was observed for fos. There was
a faint stimulation of fos of 2-to 3-fold by serum deprivation which is far less than that by PDGF-BB of 50-fold. myc expression was maximum at 60 minutes. Induction of myc expression after PDGF addition was less pronounced than that for fos, with a 3-fold increase over control. Remarkably there was no induction after serum removal but instead a slight loss of myc-RNA. The reduction was about 30% of control and remained constant for at least 48 hours (data not shown). ODC was induced by PDGF-BB and serum removal had no effect. These results rule out the necessity of an induction of myc expression in order to initiate the death of AKR-2B cells.

**Possible involvement of an ICE-like protease**

Having demonstrated that death of AKR-2B cells after serum depletion is not due to osmotic imbalance and is an active process involving nuclear condensation and membrane structure alterations, we tried to identify pathways involved in the cell death process. A recent report suggested the involvement of reactive oxygen species in the onset of apoptosis. In some cases the addition of 10 mM antioxidant, e.g. acetylcysteine, was sufficient to block apoptosis (Mayer and Noble, 1994). For AKR-2B cells the addition of 10 mM acetylcysteine did not prevent cell death (data not shown). Several reports showed that an ICE-like protease is involved in fas/Apo mediated cell death in the hemopoetic system. Inhibition of this protease rescued cells from death (Enari et al., 1995; Faucheau et al., 1995; Lazebnik et al., 1994; Los et al., 1995; Nicholson et al., 1995; Tewari et al., 1995). We therefore added the specific peptide based protease inhibitor tyr-val-alasp-chloromethylketone to cells after serum removal. Two other protease inhibitors that are specific for trypsin (tosyl-lysylchloromethylketone – TLCK) or chymotrypsin (tosylphenylchloromethylketone – TPCK) and contain the same reactive chloromethylketone moiety were chosen for control. Cell number was determined after 6 hours and 24 hours, respectively. The results are shown in Fig. 8. The compound tyr-val-alasp-chloromethylketone afforded almost complete protection at 100 μM after 6 hours. After 24 hours the effect of protection was still 80%. There was a slight protective effect of TPCK after 6 hours at 100 μM. Higher concentrations were toxic resulting in less than 20% surviving cells. After 24 hours none of the two compounds (TLCK and TPCK) had any protective effect and concentrations of >100 μM were toxic. Thus, the protective effect was not due to the reactive chloromethylketone moiety. The high specificity of the peptide based protease inhibitor lead to the suggestion that an ICE-like protease was inhibited. We therefore assumed the involvement of a protease of this type in the process of death initiated by serum removal.

**DISCUSSION**

Apoptosis, or programmed cell death, is a naturally occurring process of cell suicide that plays a crucial role in the development and maintenance of metazoans by eliminating superfluous or unwanted cells. In cell culture experiments cell death can be initiated by activation of specific receptors like TNF-receptor or fas/Apo receptor or alternatively simply by withdrawal of serum or appropriate growth factors (Enari et al., 1995; Evan et al., 1992; Jimenez et al., 1995; Ju et al., 1995; Tamm and Kikuchi, 1991, 1993; Tamm et al., 1991, 1992; Tewari and Dixit, 1995). There is a series of criteria used to distinguish programmed death (apoptosis) from necrosis. At the morphological level, apoptosis is characterized by nuclear changes, i.e. aggregation of chromatin at the nuclear membrane, membrane blebbing without loss of integrity, chromatin fragmentation, and formation of membrane bound vesicles (apoptotic bodies; Steller, 1995). These processes are not associated with a disintegration of organelles like mitochondria. Apoptotic bodies are finally phagocytosed by adjacent cells or macrophages. Necrosis might be initiated by chemical or physical insults including osmotic imbalance, or energy deprivation. It has been proposed that due to loss of ATP a breakdown of the cytoskeleton occurs that leads to bleb like structures which are prone to shear stress (Kabakov and Gabai, 1994). Further morphological changes include swelling of cells and disintegration of organelles.

It is evident from the results presented above that not all criteria of either apoptosis or necrosis are found during the death of AKR-2B fibroblasts after serum removal. The osmolarities of the serum-containing and serum-free media do not significantly differ, and moreover no cell swelling was observed. We therefore exclude the possibility that cell death was initiated by osmotic stress. Secondly, the energy state of E = 0.9 was extremely high with an ATP concentration of 6
mM in the cell. Though we can only determine the nucleotide contents in intact cells and not of those cells that became membrane permeable, these results show that there was no bulk energy deprivation before cell disintegration started.

Many features of apoptosis like blebbing and chromatin condensation are preserved in AKR-2B fibroblasts. But these cells, like Balb/c-3T3 fibroblasts, die extremely rapidly after serum removal. For both cells the kinetics are quite similar and notably for AKR-2B cells like for Balb/c-3T3 cells, there was no DNA fragmentation after nuclear condensation (Tamm et al., 1991). For both cells the cytoplasmic membrane became leaky almost simultaneously with chromatin condensation. A further processing of chromatin is energy dependent and it is thus possible that these cells have already lost their ATP and are therefore unable to further proceed in the apoptotic pathway. A time scale for morphological and biochemical alteration is discussed by Cohen (1993) and Kaufmann (1996): after the activation of initial biochemical events which may vary within different cell types, a plasma membrane hyperactivity is the first morphological event, followed shortly after by high molecular mass DNA fragmentation. Somewhat later chromatin condensation occurs. Subsequent changes include internucleosomal DNA fragmentation and laminin cleavage. Later on nuclear segmentation and the formation of apoptotic bodies is observed. In the present work we show that membrane hyperactivity was one of the first signals in AKR-2B cell after serum removal. This process involves the rapid reorganization of the plasma membrane. It might be possible that in AKR-2B fibroblasts these membrane are not stable enough to warrant perfect integrity during these massive alterations. Thus the destiny of the majority of the cells is directed to necrosis rather than to apoptosis.

A clue to this type of cell death might be the detection of ICE-like proteases involved also in the cell death process of AKR-2B cells. ICE-like proteases have been shown to be involved in the death program of C. elegans as well as of mammalian cells (Steller, 1995; Kumar, 1995; Lazebnik et al., 1994; Nosseri et al., 1994). The more defined target is PARP (poly (ADP-ribose)/polymerase) which is selectively cleaved after an aspartic acid residue and is suspected to play a role in the initiation of apoptosis. Most interestingly, a member of the cytoskeleton proteins, fodrin, has been shown to be cleaved during apoptosis. The cleavage could be initiated by activation of fas, or treatment of cells with staurosporine, dexamethasone or synthetic ceramide, substances considered to activate apoptotic death (Martin et al., 1995). A relationship between cytoskeletal breakdown and the occurrence of membrane blebbing was proposed. On the other hand, as shown previously for Balb/c-3T3 cells (Tamm et al., 1992), the cytoskeleton breaks down after serum removal but this initial process could be dissociated from cell death by several criteria. Clearly, further investigations are needed to elucidate the role of the cytoskeleton during programmed cell death.

Numerous similarities between apoptosis and cell cycle regulation have been noted and it has been suggested that the mechanisms of apoptosis and mitosis could be related or even coupled. An extreme view is the idea that apoptosis may be an aberrant mitosis. Some support for such a connection has been raised from the implications for genes that play a role in the regulation of proliferation, e.g. c-myc, p53, Rb-1, c-fos. c-myc is capable of inducing apoptosis when it is aberrantly expressed (for review see Steller, 1995). In AKR-2B cells the expression of c-myc is rather low and was not stimulated after serum removal but was stimulated effectively after the addition of PDGF-BB, arguing against an involvement of this protooncogene in cell death induced by serum deprivation.

It should be noted that about 50% of the cells survived serum withdrawal. These cells form a confluent layer and are stable for several days. They are quiescent, but a very high percentage (~95%) can be stimulated to divide by PDGF-BB. Upon serum deprivation the AKR-2B undergo a differentiation process which renders the cell resistant to serum-free medium and prone to mitogenic stimulation. This differentiation process is now being studied with the aim of identifying pathways/regulators of cell survival.

In conclusion death of AKR-2B cells initiated by serum removal does not follow entirely the canonical route of ‘apoptosis’. Clearly initial events occur, i.e. membrane blebbing and nuclear condensation that are believed to reflect apoptotic events. But no further processing of DNA or engulfment by neighbouring cells was displayed. Possibly a further progression of apoptosis is ATP dependent. The cell lysis which is accompanied with an energy breakdown of the cell most likely stops this process. Interestingly enough, in a recent publication (Shimizu et al., 1996), a possible involvement of common mediators in apoptotic and necrotic signalling pathways was suggested, based on the observation that chemical hypoxia-induced necrotic cell death was retarded by Bcl-2 and ICE-inhibitors. Moreover, after experimental infarction of rats, a significant number of cardiac myocytes exhibited features characteristic for both apoptosis and necrosis (Kajstura et al., 1996; Cheng et al., 1996). These data might indicate that common pathways also exist in vivo. Programmed cell death is as yet a rather ill defined process and the underlying molecular events are largely unknown. It might be necessary to define more categories of cell death in order to classify the different observations reported so far.

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