

## **Intracellular Adenosine Triphosphate (ATP) Concentration: A Switch in the Decision Between Apoptosis and Necrosis**

By Marcel Leist, Barbara Single, Anna F. Castoldi, Simone Kühnle, and Pierluigi Nicotera

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*From the Department of Molecular Toxicology, Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany*

### **Summary**

Apoptosis and necrosis are considered conceptually and morphologically distinct forms of cell death. Here, we report that demise of human T cells caused by two classic apoptotic triggers (staurosporin and CD95 stimulation) changed from apoptosis to necrosis, when cells were pre-empted of adenosine triphosphate (ATP). Nuclear condensation and DNA fragmentation did not occur in cells predepleted of ATP and treated with either of the two inducers, although the kinetics of cell death were unchanged. Selective and graded repletion of the extramitochondrial ATP/pool with glucose prevented necrosis and restored the ability of the cells to undergo apoptosis. Pulsed ATP/depletion/repletion experiments also showed that ATP generation either by glycolysis or by mitochondria was required for the active execution of the final phase of apoptosis, which involves nuclear condensation and DNA degradation.

Apoptosis and necrosis are two forms of cell death, with clearly distinguishing morphological and biochemical features (1). However, these two types of demise can occur simultaneously in tissues or cell cultures exposed to the same stimulus (2–5). Often, the intensity of the same initial insult decides the prevalence of either apoptosis or necrosis (6, 7). This suggests that while some early events may be common to both types of cell death, a downstream controller may be required to direct cells towards the organized execution of apoptosis. Previously, we have shown that intracellular energy levels are dissipated in necrosis, but not in apoptosis of neuronal cells (3). To investigate the role of ATP in apoptosis and necrosis here, we used Jurkat cells, a lymphoid cell line, and two well-established inducers of apoptosis: (a) anti-CD95 antibodies ( $\alpha$ CD95) that elicit apoptosis by activating cell surface CD95 receptors (8, 9); (b) the protein kinase inhibitor staurosporin (STS) that, at high concentrations, triggers apoptosis in a wide variety of mammalian cells (10). Neither of these stimuli requires a functional respiratory chain for the induction of apoptosis (11, 12).

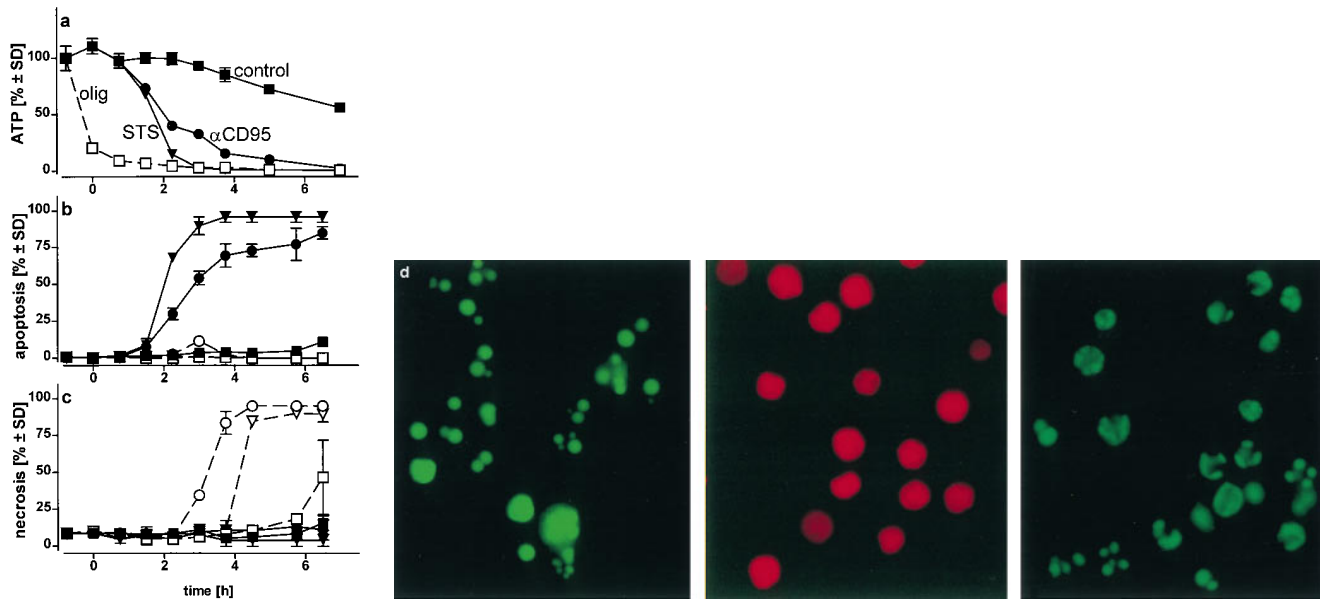
We examined the mode of cell death elicited by these typical apoptosis triggers under conditions of intracellular ATP depletion, which was obtained by blocking mitochondrial and/or glycolytic ATP generation. After the initial findings that ATP is required for the progression of apoptosis, we defined the threshold ATP concentration needed to execute fully the apoptotic program, and the time window during which the intracellular ATP level decides the mode of cell death in this system.

### **Materials and Methods**

**Cell Cultures.** Jurkat cells were grown in RPMI-1640 medium supplemented with 10% FCS. Before the experiments, cells were washed and resuspended in serum-free medium without glucose containing 2 mM pyruvate. After adaptation to this medium, cells were exposed to 0 or 2.5  $\mu$ M oligomycin for 45 min, and then challenged with STS or an agonist mAb (clone CH-11) against CD95.

**Viability Assays and Membrane Alteration.** Cultures were stained with a mixture of the membrane permeant dye H-33342 (500 ng/ml) and the membrane impermeant dye SYTOX (500 nM) (Molecular Probes, Eugene, OR). Necrotic (damaged plasma membrane; noncondensed nuclei) and apoptotic cells (intact plasma membrane; characteristically condensed or fragmented nuclei) were scored. Alternatively, cells were stained with a combination of 0.5  $\mu$ M calcein-AM, 1  $\mu$ M ethidium homodimer (EH-1), and 500 ng/ml H-33342. Stained cells (immobilized on poly-L-lysine-coated cover slips at the bottom of a 35-mm chamber) were examined with a Leica TCS-4D confocal microscope simultaneously for membrane integrity (calcein retention and EH-1 exclusion) and morphological changes of the nucleus and the cell (polarization contrast and H-33342 stain). Results obtained with both methods were similar. Digital images of SYTO-13/EH-1-stained cells were acquired on a Leica DM-IRB microscope (40 $\times$  lens) coupled to an imaging system (Imaging Corporation, St. Catherins, Canada). Apoptotic nuclei appeared hyperfluorescent and condensed. In necrotic nuclei, the fainter green SYTO-13 stain was displaced by the red EH-1 stain (3). Surface phosphatidylserine expression was analyzed by annexin V-staining and FACS<sup>®</sup> analysis as described (13).

**ATP Determination.** ATP was determined luminometrically (14). Addition of glucose ( $\leq$ 10 mM) to cells in the absence of



**Figure 1.** ATP-depletion and the shape of cell death: apoptosis or necrosis. Cells, in a glucose-free medium supplemented with 2 mM pyruvate, were incubated with solvent (control) (squares), 1.2  $\mu$ M STS (triangles), or 100 ng/ml  $\alpha$ CD95 (circles). Pretreatment with 2.5  $\mu$ M oligomycin (olig) is shown by the open symbols; (a) intracellular ATP concentration was measured at the time indicated and expressed as percentage of untreated controls (1.6 nmol/mg protein; 4.5 nmol/ $10^6$  cells). ATP concentrations in cells treated with oligomycin plus STS or  $\alpha$ CD95 did not differ from those of cells treated with oligomycin alone (open squares); (b and c) the percentage of apoptotic and necrotic cells was determined after staining of cultures with H-33342 plus SYTOX; in d, Jurkat cells were incubated with 1.2  $\mu$ M STS (left), STS plus oligomycin (middle), or STS plus oligomycin plus 5 mM glucose (right) for 4 h, before staining with SYTO-13 (green) and EH-1 (red). Data are means  $\pm$  SD of triplicate determinations and representative of at least four experiments.

oligomycin resulted in increases of the ATP concentration  $\leq 20\%$  of the initial value (data not shown) and did not modify apoptosis. With ATP concentrations between 30% and 50% of the resting control level intermediate forms of cell death (e.g., partially condensed nuclei in ruptured cells), and a slight delay in the kinetics of necrosis were occasionally observed after stimulation with either  $\alpha$ CD95 or STS. Addition of oligomycin decreased the intracellular ATP concentration (as compared with initial control levels: 60% within 10 min, 75% within 20 min, and  $\geq 90\%$  within 30 min) irrespective of the presence of other medium supplements or stimuli. Conversely, after treatment with glucose, ATP rose to  $\geq 70\%$  within 5 min and reached a new steady state after 15 min.

**DNA Fragmentation.** Field-inverted gel electrophoresis (FIGE) was performed as described previously (3) after embedding  $5 \times 10^5$  cells into 40- $\mu$ l agarose blocks. Yeast chromosomes and  $\lambda$ DNA concatemers were used as molecular weight markers. Conventional agarose gel electrophoresis (CAGE) was performed on 1.0% agarose gels (15). Gels were stained with SYBR green (Molecular Probes, Eugene, OR). Alternatively, histone-containing DNA fragments were determined quantitatively by ELISA (Boehringer Mannheim, Germany) (16). In this assay we used a lysate obtained from  $\sim 500$  cells.

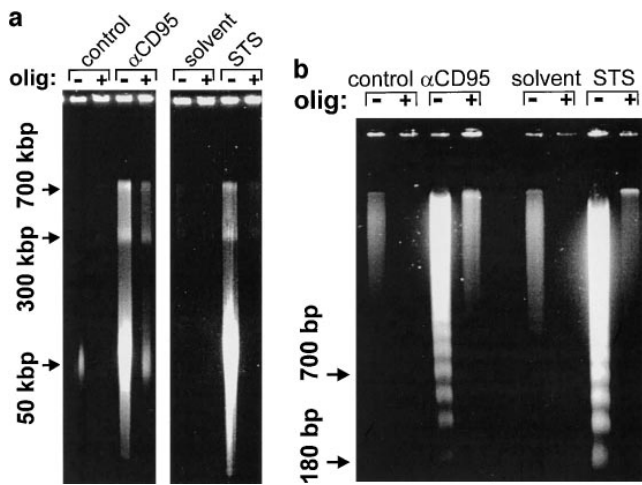
**Lamin Proteolysis.** Western blots were performed using an anti-human lamin B antibody (clone 101 B7, Oncogene Research Products, Cambridge, MA). All lanes of 10% polyacrylamide gels were loaded with 10  $\mu$ g protein, and homogeneous transfer to nitrocellulose membranes was controlled by Ponceau red staining. For immunocytochemistry, cells were fixed with 80% MeOH. Incubation with anti-lamin B antibody (101 B7) was followed by staining with a secondary BODIPY-coupled anti-mouse antibody and 1  $\mu$ g/ml H-33342. Cells were visualized by three-

channel confocal microscopy with a 100 $\times$  UV lens (polarization, lamin, chromatin).

## Results and Discussion

Initially, we examined the characteristics of cell death caused by the two inducers under conditions of intracellular ATP depletion. Jurkat cells were maintained in a glucose-free RPMI-1640 medium supplemented with 2 mM pyruvate to allow only mitochondrial ATP production. Exposure to either STS or  $\alpha$ CD95-induced apoptosis. Intracellular ATP levels were maintained during the first 90 min, but eventually declined after the appearance of the first apoptotic cells (Fig. 1, a and b). When cells were pre-empted of ATP using oligomycin, a blocker of mitochondrial ATP synthesis, death occurred by necrosis (Fig. 1, a and c).

We asked ourselves whether maintenance of a certain ATP level was needed to prevent an early breakdown of the plasma membrane. In this case, the development of apoptosis, which would not necessarily require ATP, could have been precluded by a premature demise with apparent necrotic features. However, it became clear that necrosis elicited by either of the two inducers in ATP-depleted cells occurred with a similar or rather longer time course than apoptosis. In this case, cell demise had entirely different morphological and biochemical features (Fig. 1 d). Nuclei of cells pretreated with oligomycin and then exposed to STS/ $\alpha$ CD95 had normal size and showed no apoptotic chroma-

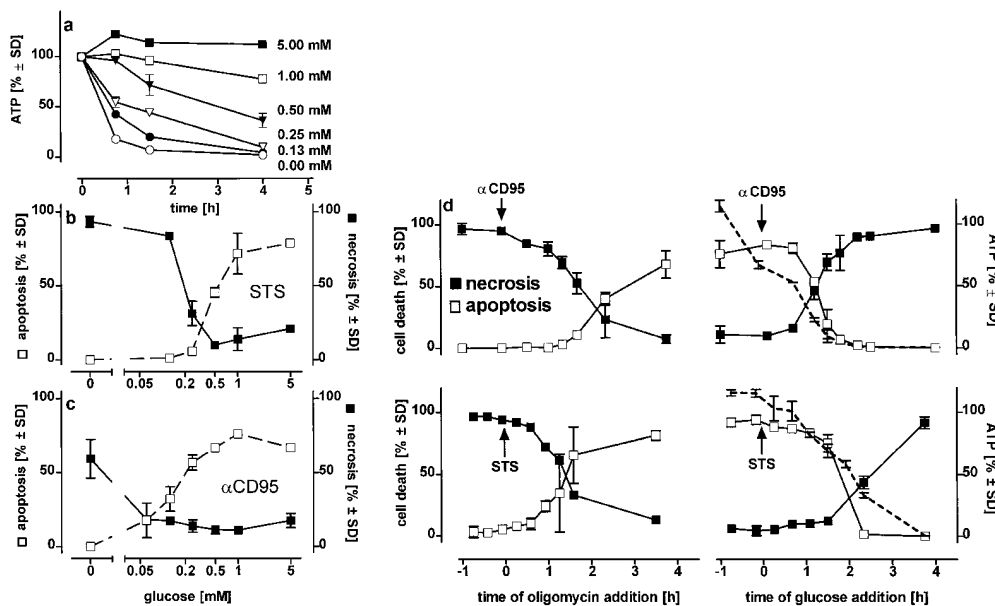


**Figure 2.** Lack of apoptotic DNA cleavage in ATP-depleted cells. Jurkat cells were incubated with  $\alpha$ CD95 or STS in the presence or absence of oligomycin (*olig*). (a) After a 90 min incubation, high molecular weight DNA fragmentation was determined by FAGE; (b) after 3 h, low molecular weight oligonucleosomal fragments were determined by CAGE. At the selected time points, the two types of DNA cleavage had reached the maximum as determined by pilot experiments.

tin condensation, while the cell membrane was clearly permeable to fluorescent dyes. The typical apoptotic DNA cleavage into 700/300 kbp, 50 kbp, and oligonucleosomal fragments did not occur in Jurkat cells preempted of ATP (Fig. 2). In addition, exposure of phosphatidylserines on the outer surface of the plasma membrane, a process leading to recognition of apoptotic cells (13), did not occur in

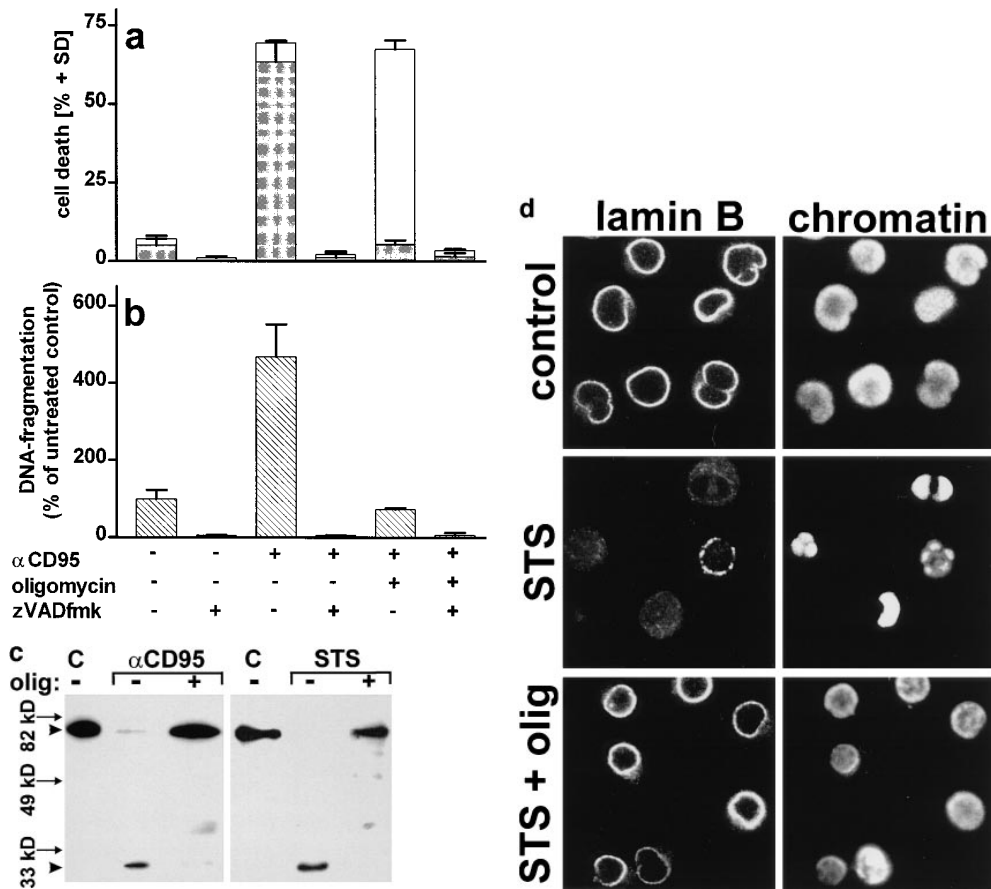
ATP-depleted cells, which later underwent necrosis (annexin binding cells after 210 min: STS,  $85 \pm 9\%$ ; oligomycin,  $19 \pm 4\%$ ; STS + oligomycin,  $13 \pm 7\%$ ; control,  $11 \pm 5\%$ ). This suggests that ATP was required for the execution of several distinct processes in the apoptotic program.

To estimate the ATP concentration sufficient to allow the progression of apoptosis, we clamped the ATP concentration of oligomycin-treated cells at different levels by supplementing the medium with glucose (Fig. 3 a). Cells were then treated with the inducers. In STS-treated cells, an ATP depletion  $>50\%$  (i.e., a residual ATP concentration  $<0.8 \text{ nmol} \times \text{mg prot}^{-1}$  45 min after the addition of the stimulus, when the apoptotic features were not yet apparent) was sufficient to change the mode of demise from apoptotic to necrotic. Higher ATP concentrations instead favored the ordered continuation of the apoptotic program. With  $\alpha$ CD95, an ATP loss  $\geq 70\%$  (i.e., a residual ATP concentration of  $\leq 0.5 \text{ nmol} \times \text{mg prot}^{-1}$ ) was invariably followed by necrosis (Fig. 3, b and c). Similar results were obtained in experiments with fructose replenishment. In cells pretreated with oligomycin and then challenged with  $\alpha$ CD95, a fructose concentration (40 mM) that limited the ATP loss (i.e., residual ATP above 50%) changed the mode of cell death from necrosis to apoptosis. Addition of similar metabolic substrates, e.g., glycerol (50 mM), dihydroxyacetone (50 mM), or lower fructose concentrations (10 mM), which were not able to restore intracellular ATP levels, did not modify the shape of cell death. Thus, it appears that the availability of the ATP generated by glycolysis is sufficient to allow the execution of cell death by apoptosis. In the absence of glucose and oligomycin, mitochondrial ATP gen-



**Figure 3.** Changes in the mode of cell death after clamping the intracellular ATP concentration at defined levels or at different times. (a) Jurkat cells in glucose-free medium containing pyruvate (2 mM) were exposed to  $2.5 \mu\text{M}$  oligomycin plus the indicated concentrations of glucose. Intracellular ATP concentrations were determined at the times indicated; (b and c) Jurkat cells were preincubated for 45 min in medium containing  $2.5 \mu\text{M}$  oligomycin plus the concentration of glucose indicated. STS (b) or  $\alpha$ CD95 (c) were then added and the mode of cell death was determined after further 3.5 h; (d) intracellular ATP levels were manipulated during incubation of Jurkat cells with either STS or  $\alpha$ CD95. Left, cells were incubated in pyruvate-supplemented medium without glucose and

challenged (at  $t = 0$ ) with  $100 \text{ ng/ml}$   $\alpha$ CD95 or  $1.2 \mu\text{M}$  STS. At the times indicated oligomycin was added to deplete ATP, and the mode of cell death was determined 4 h after the challenge. Right, cells were first depleted of ATP by preincubation with oligomycin (at  $t = -1 \text{ h}$ ). At  $t = 0$ , STS or  $\alpha$ CD95 were added. At the times indicated, intracellular ATP was replenished by adding 10 mM glucose to the incubation medium. The dashed bold line indicates the cellular ATP concentration (percentage of untreated control cultures in standard pyruvate medium), which was reached 15 min after each glucose supplementation. Data are means  $\pm$  SD of triplicate determinations.



**Figure 4.** Activation of caspases in apoptotic or necrotic cell death. (a and b) Cells were preincubated for 45 min with oligomycin and/or 75  $\mu$ M zVAD-fmk as indicated and then challenged with  $\alpha$ CD95. After 4 h, (a) the mode of cell death (closed bars, apoptotic; open bars, necrotic) or (b) DNA fragmentation were determined by dual fluorescent staining or ELISA, respectively; (c) cells were incubated with  $\alpha$ CD95 or STS for 200 min in the presence or absence of 2.5  $\mu$ M oligomycin (olig). Lamin B cleavage was determined by Western blot analysis. The original lamin band and the proteolytic fragment, typically found in apoptotic cells are indicated by arrowheads; (d) Lamin disintegration was also studied by immunostaining. Cells untreated or treated with oligomycin were then challenged by STS. Lamins were stained with an anti-lamin antibody, whereas nuclei were counterstained with H-33342. Confocal images show that breaks/dissolution of the lamin structure (middle-left) as well as chromatin condensation (middle-right) did not occur in cells undergoing necrosis (i.e., treated with oligomycin) (bottom).

eration stimulated by the addition of pyruvate would naturally replenish also the extramitochondrial pool.

To determine at which phase of the death program ATP was required, we added oligomycin at different times after triggering cell death with STS or  $\alpha$ CD95. When ATP depletion was inflicted up to 90 min after the challenge with either of the two stimuli, cell death still shifted from apoptosis to necrosis (Fig. 3 d). In a complementary experimental setup, we found that glucose addition to replenish ATP in cells treated with either STS or  $\alpha$ CD95 plus oligomycin directed the death process towards apoptosis up to 2 h after the challenge (Fig. 3 d). Thus, it seemed that ATP was required immediately before the characteristic nuclear changes, perhaps to ensure that nuclear collapse, the ordered packaging of chromatin, and DNA degradation occur before cell lysis. Accordingly, DNA fragmentation was strongly reduced in necrotic cells (i.e., challenged with STS or  $\alpha$ CD95 in the presence of oligomycin) as compared with those undergoing apoptosis, although the total percentage of cell death was approximately the same (Fig. 4, a and b). Differences in the fate of the nucleus became evident when we examined lamin degradation, a process pivotal for the nuclear collapse that is effected by the activation of certain caspases during apoptosis (17, 18). Cleavage of lamin B was significantly reduced under conditions of ATP depletion leading to necrosis (Fig. 4, c and d), which suggests that activation of the

proteolytic system responsible for lamin cleavage is specific for apoptosis.

Because activation of caspases is also an integral component of the CD95 signaling system that triggers apoptosis (19, 20) we used caspase inhibitors to test whether the same signaling system would be active in necrosis and apoptosis. In this case, caspase inhibitors prevented  $\alpha$ CD95-induced cell death regardless whether apoptotic or necrotic (Fig. 4, a and b), in agreement with other findings that both types of cell death share similar signals and initial mechanisms (21–23).

A cytoplasmic controller (24) seems to direct downstream events resulting in the typical apoptotic features including proteolysis of nuclear elements and DNA degradation. Furthermore, according to a series of elegant experiments with reconstituted cell-free systems, an apoptogenic protease (25) released from mitochondria upon permeability transition (26) can promote the nuclear changes. In cells, mitochondrial permeability transition can be triggered by a variety of stimuli acting on the mitochondria directly, or by a yet unknown upstream controller (25, 26). Thus, either the upstream controller or the execution system(s) may be modulated by the availability of ATP. The latter may be provided by glycolysis or, in tissues with high metabolic demand, primarily by the mitochondria (3, 27). In the model described here, restoration of glycolytic ATP generation was sufficient to allow the ordered execution of apoptosis.

Thus, apoptosis and necrosis would be two extremes of a continuum of possible types of cell demise, whose shape and implications for the neighboring tissue would be decided by the availability of ATP, and likely by additional factors in the dying cell and in scavenger cells. This would

explain the frequent coexistence of both types of cell death in pathological situations, e.g., cerebral ischemia (28) or inflammatory liver failure (5, 6), where individual cell death within the tissue would be decided by the energy supply.

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Address correspondence to Professor P. Nicotera, Chair of Molecular Toxicology, University of Konstanz, POB 5560-X911, D-78457 Konstanz, Germany.

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