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Regional Loss of the Mitochondrial Membrane Potential in the Hepatocyte Is Rapidly Followed by Externalization of Phosphatidylserines at That Specific Site during Apoptosis*

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The spatio-temporal relationship between a decrease in the mitochondrial membrane potential (MMP) and externalization of phosphatidylserines (PS) during induction of apoptosis was investigated in single freshly isolated hepatocytes. Apoptosis was induced in the hepatocytes in three different ways: attack by activated Natural Killer cells, exposure to ATP, or exposure to the inhibitor of protein synthesis cycloheximide. Fluorescence microscopy showed staining of externalized PS at those areas where the staining for MMP was lost whereas in other areas the mitochondria remained intact for longer periods of time, indicating coupling between local loss of MMP and local PS exposure. To discriminate whether the decrease in MMP itself or a decrease in ATP induced PS externalization, hepatocytes were treated with rotenone, which resulted in a rapid collapse of cellular ATP but left the MMP intact for a much longer period. Addition of fructose prevented the decrease of ATP to ~30% and also delayed the collapse of the MMP. This indicates that ATP was needed for the maintenance of the MMP probably via reverse action of the ATP synthase. In a subsequent study hepatocytes were incubated with Natural Killer cells for induction of apoptosis followed by addition of rotenone to deplete ATP. Under these conditions the PS staining co-localized with mitochondrial MMP indicating that PS externalization does not require a collapse in MMP. Moreover, exposure of PS was evenly distributed over the whole plasma membrane. In conclusion, we propose that after an apoptotic stimulus some mitochondria start to lose their MMP, which results in cessation of ATP production and perhaps even consumption of ATP. This results in an overall decrease in cellular ATP. ATP-consuming enzyme reactions most distal from still intact mitochondria will be most sensitive to such a decrease. Apparently the translocase that keeps phosphatidylserines inward-oriented is such a sensitive enzyme.

It has been demonstrated in many cell types, using a large variety of inducers, that mitochondria often play a crucial role in development of apoptosis and necrosis (reviewed in Refs. 1 and 2). Already in the 1980s a relation was observed between

elevated calcium concentrations and the mitochondrial membrane potential (MMP)¹ on the one hand and cell death on the other hand upon exposure of hepatocytes to toxic compounds or after ischemia reperfusion (3). It was shown that opening of a high conductance permeability transition pore after oxidative stress in the mitochondrial inner membrane abruptly increases the permeability of the mitochondrial inner membrane to solutes of molecular mass up to 1500 Da (4, 5). The opening of the pore is associated with a collapse of the MMP, perturbation of intracellular and mitochondrial Ca²⁺ homeostasis, and subsequent cell death. The mitochondrial outer membrane is also the site of competition between the pro- and anti-apoptotic proteins of the Bcl-2 family and is associated with the opening of the pore (6–9). Moreover, the mitochondria are a source of a number of the Bcl-2 family members and, in lymphoid cells, of the apoptosis inducing factor, a protein associated with opening of the pore and induction of apoptosis (10). Other mitochondrial factors that are associated with liver apoptosis *in vivo* are proteases that are released during cholestasis (11). Finally several studies showed that after induction of apoptosis mitochondria release cytochrome *c* (12), which is associated with opening of the mitochondrial pore (13). Cytochrome *c* forms a complex with apoptotic protease activating factor-1, procaspase-9, and ATP. Formation of this complex, the apoptosome, leads to formation of active caspase-9, which that can subsequently activate other caspase proteins. In this way the apoptotic signal is amplified (reviewed in Ref. 14). Associated with the activation of the caspases is exposure of phosphatidylserines (PS) in the outer leaflet of cells (15, 16). These molecules function as a signal for macrophages or other cells from the reticuloendothelial system to engulf and digest apoptotic bodies (17). In this way release of intracellular components and a subsequent immunological reaction is prevented. The exposure of PS is either the result of inhibition of an ATP-dependent aminophospholipid transporter (18, 19) or activation of a calcium-dependent scramblase (20–22). In addition, synthesis of PS through a calcium-dependent exchange of the polar head group of pre-existing phospholipids has been described (23).

In thymocytes exposure of PS occurs only in those cells that have lost their MMP (15, 24). In other cell types the opposite was found; in L929sAh cells transfected with the FAS receptor and treated with anti-FAS initially PS were exposed and followed later by a drop in MMP (25). We described the involvement of the mitochondria in the induction of apoptosis in hepa-

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¹ The abbreviations used are: MMP, mitochondrial membrane potential; A-NK, interleukin-2-activated natural killer cells; ANV, annexin V; CLSM, confocal laser scan microscopy; PI, propidium iodide; PS, phosphatidylserine(s); TMR, tetramethylrosamine; TOTO-3, 1,1'-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-(3-methyl-2,3-dihydrobenzo-1,3-thiazolyl-2-methylidene)quinolinium] tetraiodide.

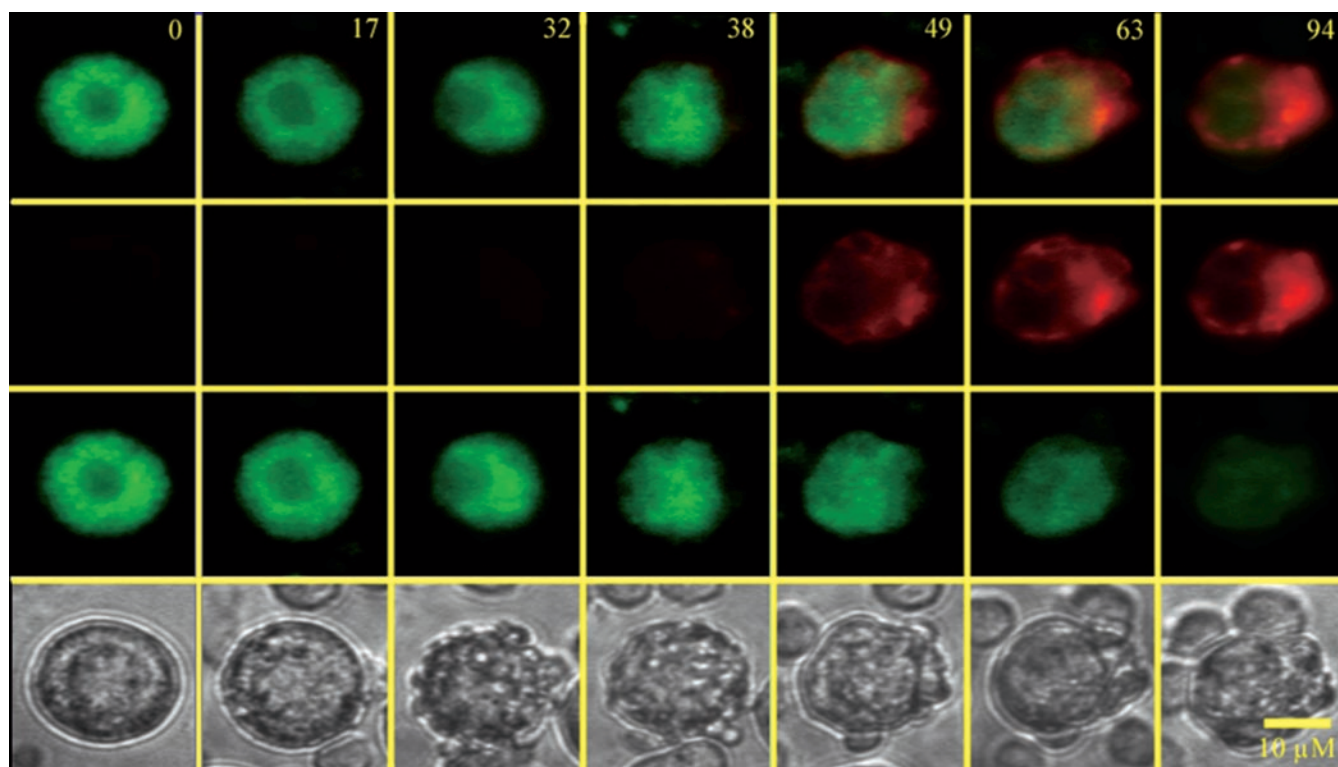


FIG. 1. Time course of disruption of the MMP and the externalization of PS in isolated hepatocytes exposed to A-NK cells. Freshly isolated rat hepatocytes were exposed to A-NK cells. ANV and PI were added to hepatocytes that were preloaded with 0.2 mM tetramethylrosamine for 15 min; subsequently images of the same cell were taken using video microscopy. At $t = 0$ A-NK cells were added, and at the indicated time points (in min) images were taken of the PS externalization (red ANV staining), MMP (green TMR staining), and morphology. The non-permeable probe PI was added to the cells to verify that the loss of MMP was not caused by a leakage of fluorescent probe because of plasma membrane permeabilization. The upper row shows the merged images of PS externalization and decrease in MMP.

toocytes after attack by interleukin-activated Natural Killer (A-NK) cells (16). Fluorescence-activated cell sorter analysis after staining for extracellular-oriented PS with fluorescent-labeled annexin V (ANV) demonstrated that A-NK cells induced apoptosis in the hepatocytes (10% apoptotic cells after 30 min and 38% after 60 min of co-incubation). In these apoptotic cells the overall MMP was $\sim 60\%$ of the value as compared with non-apoptotic hepatocytes. The decrease in MMP and exposure of PS in hepatocytes is apparently tightly coupled, because all cells with a lowered MMP exposed PS. In comparison with other studies (15, 24, 25) the decrease in MMP was relatively moderate, and the exposure of PS was very rapid. Microscopical examination showed that ANV staining was unevenly distributed over the cell membrane.

To gain more insight in the spatio-temporal relationship between a decrease in the MMP on the one hand and exposure of PS on the other hand the present study was undertaken. Time-lapse video microscopy and confocal laser scan microscopy of hepatocytes that were attacked by A-NK cells was performed. In addition, two totally unrelated apoptosis-inducing stimuli were applied: addition of extracellular ATP or cycloheximide (26, 27). We report that in all cases a local loss of MMP of a limited number of mitochondria in the cell results, within minutes, in exposure of phosphatidylserines at that particular spot.

MATERIALS AND METHODS

Collagenase, recombinant protein ANV, and HEPES were obtained from Roche Molecular Biochemicals. Tetramethylrosamine (TMR), rhodamine 123, propidium iodide (PI), TOTO-3, and the AlexaTM protein labeling kit were from Molecular Probes. Fluorescent ANV was prepared by labeling with AlexaTM488 or AlexaTM633. Bovine serum albumin (type V), the luciferin/luciferase kit, and poly-L-lysine were from Sigma. Mouse anti-rat monoclonal antibody OX18 (anti-

total rat major histocompatibility complex class I) was prepared as described before (28).

Isolation and Activation of Natural Killer Cells

Isolation of natural killer cells and activation of these cells were done as described (16). Briefly, a cell suspension was prepared from spleen isolated from a 4–5-month-old male Wag rat (RT1^u), a Wistar-derived strain, purchased from Charles Rivers Wiga (Schulzfeld, Germany). The splenocytes were separated from other cells (*i.e.* B cells, macrophages) by nylonwool adherence. The non-adherent cells contained $\sim 30\%$ A-NK cells, which were collected and cultured in RPMI 1640, Dutch modification (Invitrogen), supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 50 $\mu\text{g}/\text{ml}$ streptomycin, and 50 units/ml penicillin (all Invitrogen). The culture medium was supplemented with 1000 Cetus units/ml human recombinant interleukin-2 (Chiron) and 50 μM 2-mercaptoethanol. After 24 h non-adhering cells were removed, and the remaining adhering cells, A-NK cells, were cultured for another 6 days. The population thus obtained consisted for $\geq 95\%$ of CD161A-positive cells and for $\leq 5\%$ of T cell receptor-positive cells.

Isolation and Incubation of Rat Hepatocytes

Liver parenchymal cells were isolated by collagenase perfusion from male Wistar rats (200–230 g) (29), purchased from Charles Rivers Wiga (Schulzfeld, Germany) and housed at least 1 week at the animal facilities of the Sylvius Laboratories. The rats were fed *ad libitum* and kept at a 12-h day-night cycle. Prior to the experiment the rats were fasted for 24 h. Viability of the freshly isolated cells was $>95\%$ as determined by trypan blue exclusion. After isolation cells were kept on ice until use.

Incubation of Hepatocytes and A-NK Cells

To allow non-self recognition and induce the cytotoxic response by A-NK cells, the major histocompatibility complex class I protein of hepatocytes was blocked with the OX18 antibody (27). This masking leads to recognition of the target cell as foreign and activation of the killing machinery (29, 30). The liver cells were preincubated with the OX18 antibody at 4 °C for 45 min in Hanks/HEPES buffer (pH 7.4, 4 °C) composed of 120 mM NaCl, 5 mM KCl, 4.2 mM NaHCO₃, 1.2 mM

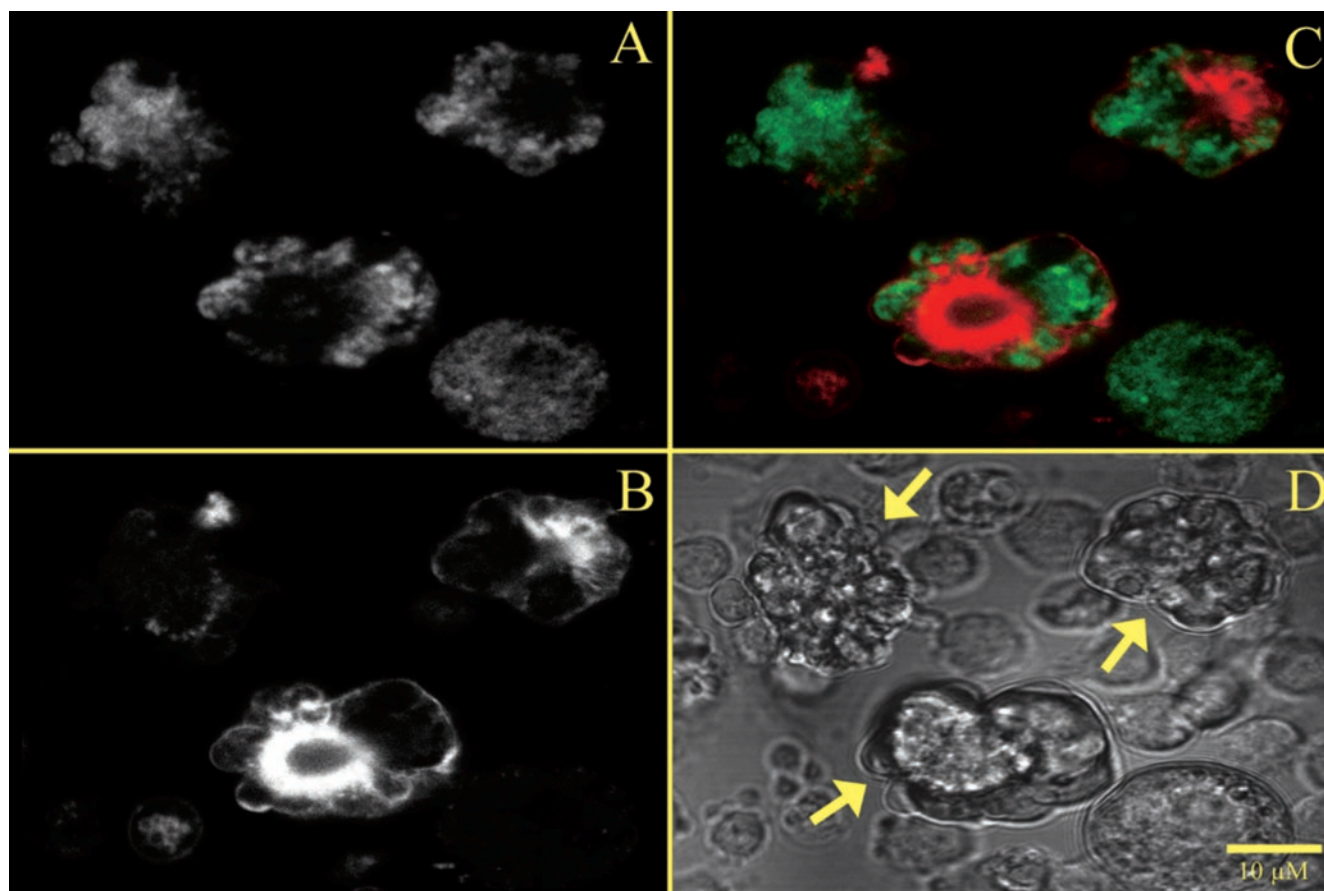


FIG. 2. Confocal laser scan photomicrograph of disruption of the MMP and the externalization of PS in isolated hepatocytes exposed to A-NK cells. Conditions were the same as for Fig. 1. TMR was excited with the green laser (A), and ANV was excited with the blue laser (B). These images were pseudo-colored and merged (C). The morphology is shown in D; arrows indicate apoptotic cells. Hepatocytes were exposed to A-NK cells for 95 min.

NaH_2PO_4 , 1.3 mM CaCl_2 , 0.4 mM MgSO_4 , 25 mM HEPES supplemented with 10 mM glucose and 1% (w/v) bovine serum albumin and gassed for 30 min with 95% O_2 /5% CO_2 . Then, cells were washed and resuspended in either William's E supplemented with 10% fetal calf serum or the Hanks'/HEPES buffer supplemented with 1% (w/v) bovine serum albumin.

For microscopical experiments isolated liver cells were allowed to attach to circular glass coverslips coated with poly-D-lysine (1 mg/ml in water) (16). The coverslips were placed in a microscope chamber, and 500 μl of cell suspension (3.0×10^5 cells/ml) was carefully added to the glass coverslip; the hepatocytes were allowed to attach for 45 min. The cells were kept at 37 °C throughout the experiment. The experiments with the A-NK cells were started by removal of the medium and addition of A-NK cells in William's E supplemented with 10% fetal calf serum in the effector:target ratio of 20:1. This ratio was chosen, because previous experiments showed that it resulted in apoptosis in a large number of hepatocytes (16). In other experiments ATP or cycloheximide were added directly to the cells.

Batch incubations were performed in the same Hanks' buffer on a rotary shaker that was kept at 37 °C. Cells were incubated at a density of 3.0×10^5 cells/ml. At the selected intervals 0.5-ml samples were taken for flow cytometry and ATP determinations.

Imaging Techniques

The video microscopy system consisted of an IM35 inverted microscope with a 100-watt mercury arc lamp (Zeiss) and a Nikon $\times 40/1.4$ NA numerical aperture Fluor objective. ANV staining was detected using a 475-nm band pass filter for excitation, a 510-nm dichroic mirror, and a 540-nm band pass emission filter. TMR was visualized using a 535-nm band pass filter for excitation, a 580-nm dichroic mirror, and a 590-nm long pass filter for emission. Images were recorded using a CCD instrumentation camera, controlled by a CC200 camera controller (Photometrics, Tucson, AZ).

For confocal laser fluorescence microscope (CLSM) an upgraded Bio-Rad MRC-600 system was used (31). The first filter block of the CLSM

contained a triple dichroic mirror (488/543/633) and an emission filter (488/543/633). ANV was excited with the 488-nm argon laser, TMR with the 543-nm HeNe laser, and TOTO-3 with the 633-nm HeNe laser.

Staining Techniques

PS Externalization and Plasma Membrane Permeabilization—Exposition of PS on the extracellular side of the plasma membrane of hepatocytes was visualized by staining PS with ANV labeled with fluorescent AlexaTM488 (1 $\mu\text{g}/\text{ml}$ ANV and AlexaTM488 in a stoichiometric complex of 1:1) (4). 0.2 μl ANV and 2 μl of a 5 mM solution of the cell-impermeable dye TOTO-3 were added to 500 μl of cell suspension in the incubation chamber of the microscope.

Determination of the Relation among Externalization of the Phosphatidylserines, Mitochondrial Membrane Potential, and Cell Death—Hepatocytes were pre-incubated for 15 min with 0.2 μM TMR. Subsequently ANV and TOTO-3 were added. After recording of the baseline level of TMR the apoptotic stimuli were given.

Determination of the Degree of Co-localization of Fluorescent Signals of Different Dyes—Co-localization of the signals was measured by comparing the equivalent pixel positions in each image and generation of a co-localization scatter plot using Image Pro software (Media Cybernetics, Silver Spring, MD). These scatter plots were analyzed using Pearson's correlation. The result of this analysis is a number between +1 and -1. The former indicates perfect correlation, and the latter indicates no correlation (32, 33).

Determination of the Relation among Externalization of the Phosphatidylserines, Intracellular Free Calcium ($[\text{Ca}^{2+}]_i$), and Cell Permeabilization

For determination of intracellular free calcium the hepatocytes were loaded with 40 μM Fura-2/AM for 30 min. Then, the cells were washed carefully with Hanks'/HEPES buffer at 37 °C. Next, ANV and A-NK cells were added. From a group of cells the 470-nm emission images after 340- and 380-nm excitation were recorded using a dichroic mirror of 395 nm and a 470-nm long pass emission filter. Images were cor-

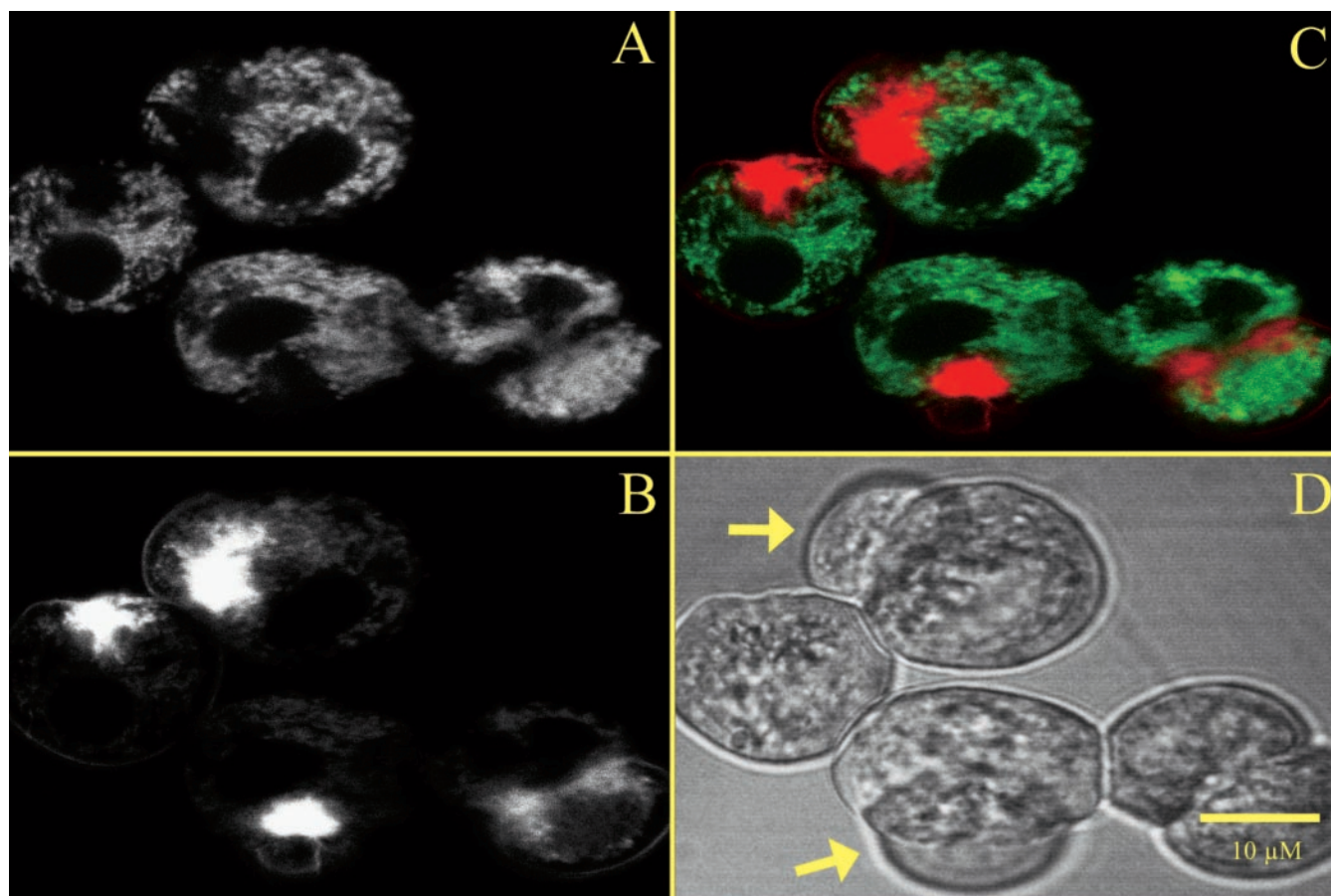


FIG. 3. Confocal laser scan photomicrograph of disruption of the MMP and the externalization of PS in isolated hepatocytes exposed to ATP. Conditions were the same as for Fig. 1. Hepatocytes were incubated with 0.4 mM ATP. *A*, TMR; *B*, ANV; *C*, merge; *D*, morphology is shown. *Arrows* indicate apoptotic cells. Hepatocytes were exposed to ATP for 27 min.

rected by background subtraction before calculation of the ratio images. Ratio images of the 340/380-nm excitation were determined by division of the 340-nm image by the 380-nm image on a pixel-to-pixel basis. The intracellular free calcium concentration was calculated using the equation, $[Ca^{2+}]_i = K_d \cdot \beta \cdot (R - R_{min}) / (R_{max} - R)$, with $K_d = 224$ nM as the equilibrium dissociation constant for Ca^{2+} and Fura-2. R_{min} is the ratio F_{340}/F_{380} at zero calcium; R_{max} is the ratio F_{340}/F_{380} at saturating calcium; β is the ratio F_{380} (zero calcium)/ F_{380} (saturating calcium), and F is the pixel fluorescence intensity.

Flow Cytometric Analysis of the Percentage of Viable, Apoptotic, and Dead Hepatocytes

The MMP of viable, apoptotic, and dead cells was determined by flow cytometric analysis using ANV, PI, and rhodamine 123 as described earlier (16). Briefly, after addition of ANV, rhodamine 123, and PI the hepatocytes were incubated for 15 min on ice in the dark. The fluorescence of individual cells was analyzed using a FACScalibur flow cytometer (BD Biosciences), using the CellQuest program.

Determination of ATP in the Hepatocytes

Samples of the hepatocytes were snap-frozen in liquid N_2 . Determination of ATP was started by addition of $HClO_4$ to the frozen cells. Subsequently KPO_4 was added, and after 10 min pH was neutralized with KOH. The tubes were centrifuged, and ATP was determined in the supernatant using the Sigma luciferin/luciferase kit.

Statistics

Values are expressed as mean \pm SD. The statistical evaluation was performed with an unpaired two-tailed Student's t test.

RESULTS

Freshly isolated rat hepatocytes were loaded with TMR to visualize effects on the MMP; in addition, ANV labeled with Alexa 488 was added to stain external PS. The hepatocytes were attached to a coverslip mounted at the bottom of the

incubation chamber; after recording of baseline values the A-NK cells were added as a suspension to the chamber, and therefore, it took some time before a contact between the hepatocytes and A-NK cells had been established. Fig. 1 shows that 17 min after the addition of the A-NK cells such a contact was made. At this time point the morphology of the hepatocyte was still normal (Fig. 1, *bottom row*). However, 15 min later (32') the hepatocyte had become apoptotic showing numerous blebs. At this time point at certain sites the MMP was dissipated (Fig. 1, *third row*). This was followed 6 min (38') later by the first staining of PS (Fig. 1, *second row, hardly visible*). These processes, decrease in MMP and externalization of PS, continued progressively. Because TOTO-3 was added the absence of a nuclear staining in the images indicates that until 94 min no secondary necrosis had occurred; the cells were still intact. The merged images of the *green* MMP signal and the *red* PS signal are shown in the *top row*. If these signals co-localize the merged signal becomes *yellow*. The absence of yellow staining indicates that only at the area where mitochondria had lost their MMP externalization of PS occurred.

To acquire more detailed images we used the CLSM. Hepatocytes were incubated under the same conditions as described above. Fig. 2A shows the MMP, and Fig. 2B shows the externalized PS. As can be seen in the merged Fig. 2C the signals do not co-localize confirming the finding that PS only externalize in the vicinity of defective mitochondria. The co-localization was analyzed further using Pearson's correlation, which is discussed at the end of the "Results."

To test the specificity of the response, apoptosis was induced in hepatocytes with cycloheximide, a protein synthesis inhibitor. Fig. 3 shows a CLSM image taken 55 min after the addition

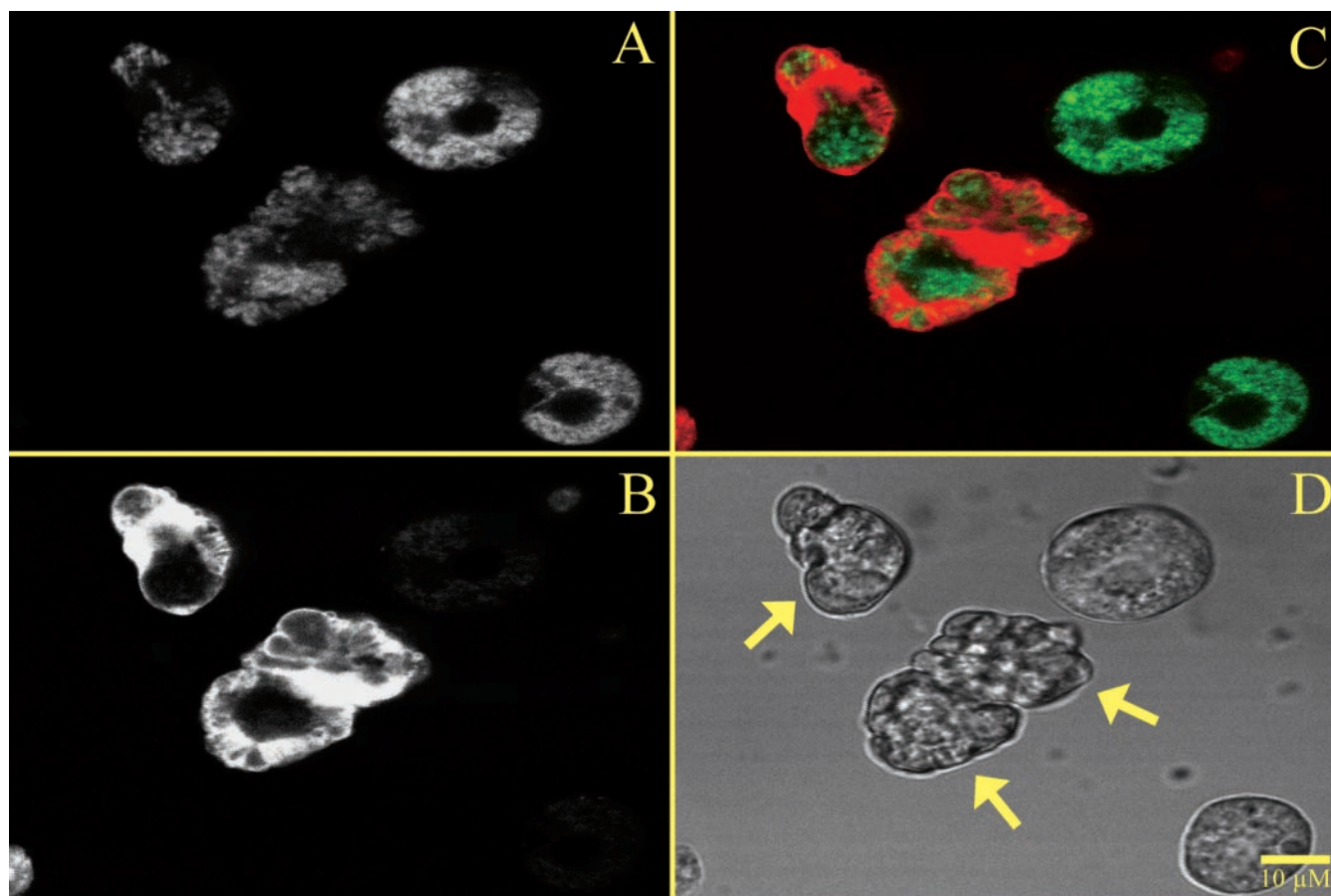


FIG. 4. Confocal laser scan photomicrograph of disruption of the MMP and the externalization of PS in isolated hepatocytes exposed to cycloheximide. Conditions were the same as for Fig. 1. Hepatocytes were incubated with 30 $\mu\text{g}/\text{ml}$ cycloheximide. A, TMR; B, ANV; C, merge; D, morphology is shown. Arrows indicate apoptotic cells. Hepatocytes were exposed to cycloheximide for 55 min.

of cycloheximide. The *arrows* in Fig. 3D point at the apoptotic cells. These cells have a lowered MMP and are high in ANV staining; the merged image again indicates no co-localization of the two signals. Identical results were found when apoptosis was induced using addition of extracellular ATP (Fig. 4). These results show that three completely different apoptotic signals had the same effect: externalization of PS in the near vicinity of defective mitochondria. A possible explanation for this phenomenon could be a local decrease in ATP around defective mitochondria. The enzyme that maintains the asymmetric orientation of PS, aminophospholipid translocase, needs ATP for its activity. The most direct approach to investigate the role of ATP would have been intracellular determination of the concentration using a cell-permeable probe like those used for determination of Ca^{2+} or MMP. Unfortunately such a probe does not yet exist. Therefore, we chose another approach: depletion of ATP without collapse of the MMP by using the mitochondrial inhibitor rotenone. First, in batch incubations of hepatocytes, the effect of rotenone on intracellular ATP and MMP was determined. Cells were incubated, and at different time points two samples were taken. One was immediately snap-frozen for ATP determination. The second was incubated with ANV, rhodamine 123, and PI for flow cytometric analysis as described under "Materials and Methods." Fig. 5 shows that even at 10 μM rotenone ATP levels were less than 15% of control within 30 min. In contrast the MMP remained largely intact for more than 2 h (Fig. 5). Interestingly, addition of fructose prevented the decrease in ATP to about 50% and moreover, delayed the decrease in the MMP. A significant increase in the number of dead cells as a result of rotenone exposure occurred in parallel with the (late) decrease in MMP.

At all time points neither in the control nor in the cells incubated with rotenone more than 1% of the cells were apoptotic, indicating that a decrease in cellular ATP is not sufficient to induce apoptosis. Subsequently hepatocytes were co-incubated with A-NK cells to induce apoptosis, and after 15 min rotenone was added to deplete ATP, and the localization of PS staining and MMP staining was determined in apoptotic cells. Fig. 6A shows the MMP, and Fig. 6B shows the localization of the PS. In Fig. 6C these images were merged; it is evident that both stainings overlap. To further substantiate this the Pearson's correlations of this image and of the image depicted in Fig. 1C were calculated. The average value in Fig. 1C was 0.15 ± 0.05 whereas the value for Fig. 6C was 0.75 ± 0.07 . This indicates that indeed there was much more overlap in staining after treatment with rotenone. Furthermore, comparison of Fig. 1B and Fig. 6B shows that PS staining was patchy in the former whereas in rotenone-treated cells the whole plasma membrane was stained uniformly. When hepatocytes were incubated with rotenone and fructose and A-NK cells similar images were obtained in which MMP and PS staining were co-localized. The prevention of the decrease in ATP was not sufficient to prevent the PS externalization. In the presence of rotenone and fructose or rotenone alone the same percentage of the cells became apoptotic and necrotic. A three-dimensional image of Fig. 6B is available on our web site, www.pharm.leidenuniv.nl/lacdrhomepage/divisions/toxicology/nk2.htm.

To investigate the role of intracellular free calcium hepatocytes were loaded with Fura-2/AM and incubated with the A-NK cells in the presence of ANV. Initially the same magnification was used as in Figs. 2–4. Using video microscopy, in many cells an increase in the intracellular calcium concentra-

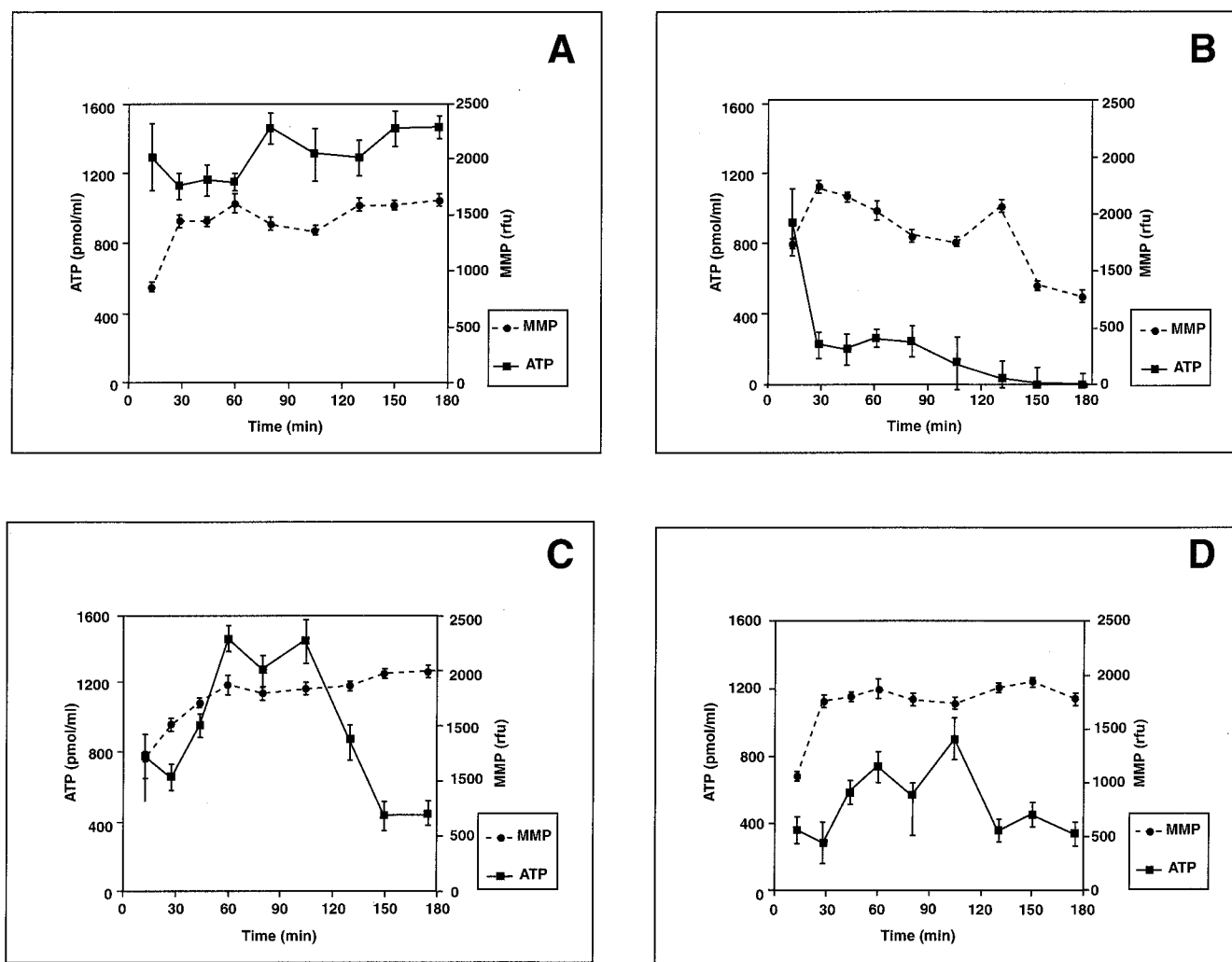


FIG. 5. ATP concentrations in hepatocytes and MMP exposed to fructose and/or rotenone. Hepatocytes were incubated, and samples were drawn. One-half of the sample was immediately frozen in liquid nitrogen. In this sample ATP was determined using the luciferin/luciferase assay. In the other half of the sample the MMP was determined using the flow cytometer. A, control; B, 10 μ M rotenone; C, 3 mM fructose; D, 10 μ M rotenone and 3 mM fructose.

tion was observed, but no shifts in the Fura-2 signal at specific locations inside the cell occurred; the calcium changes were homogeneous within the cells. Therefore, a lower magnification was used to allow us to follow more cells. We found that addition of A-NK resulted in a significant increase of $[Ca^{2+}]_i$ from the resting $[Ca^{2+}]_i$ of 205 ± 102 nM ($n = 56$) to 540 ± 102 nM in 60% (34 of 56) of the observed hepatocytes followed by externalization of the PS. However, in the other 40% no increase in intracellular free calcium occurred before PS were externalized. Moreover, in 17% of the hepatocytes PS were not externalized despite a $[Ca^{2+}]_i$ response.

DISCUSSION

Apoptosis was induced in the hepatocytes after attack by A-NK cells (16) and exposure to ATP (26) or cycloheximide (27). All three conditions resulted in a rapid decrease of the MMP followed by externalization of the PS. As shown in the time-lapse series of the attack by A-NK cells, contact between some hepatocytes and A-NK cells was made after 17 min. Already at 32 min mitochondria in some areas of the hepatocytes had lost their MMP, and at 38 min exposure of PS at sites with affected mitochondria began. As is evident from the photomicrographs externalization of PS took place at those areas or sites of the cell where mitochondria had lost their MMP. The most straightforward explanation for PS externalization at these

sites is that ATP levels in the immediate surroundings of defective mitochondria rapidly decreases resulting in inhibition of the translocase that normally translocates phosphatidylserines to the inner leaflet. Unfortunately no cell-permeable dyes are yet available to determine local changes in ATP within the cell. ATP has been determined in hepatocytes, but this involved microinjection of luciferase and immobilization of the cells in agar, which are both conditions that will probably effect membrane structure (34) and interaction of hepatocytes with A-NK cells. Similarly, microinjection of a vector for luciferase (35) will affect membrane structure, and, in addition, it requires cell culture that certainly affects energy metabolism in hepatocytes. Therefore, to discriminate between effects of a collapse of the MMP itself or the resulting decrease in ATP, we chose the approach to deplete ATP from cells while keeping the MMP intact, by treatment of the cells with rotenone. Alternatively we depleted ATP with rotenone in the presence of fructose to replete it again. Under ATP-depleted conditions the staining for externalized PS co-localized with mitochondrial MMP indicating that PS externalization does not require a collapse in MMP. We found, as reported before (36), that depletion of ATP by itself does not induce apoptosis; an apoptotic signal is needed. Very recently (37) a similar finding was reported; Fas-triggered PS exposure was enhanced by depletion

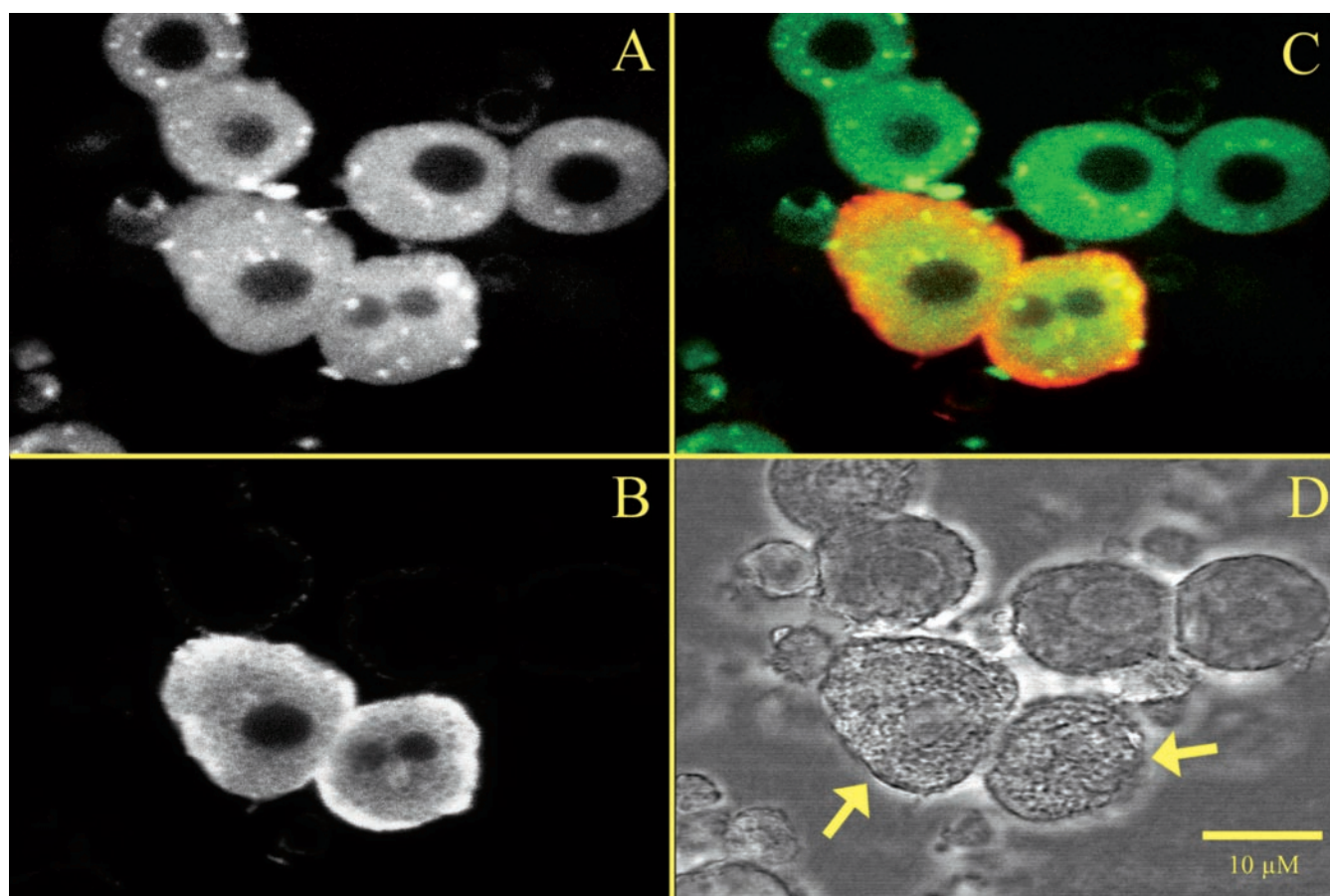


FIG. 6. Confocal laser scan photomicrograph of disruption of the MMP and the externalization of PS in isolated hepatocytes exposed to A-NK cells after addition of rotenone. Conditions were the same as for Fig. 1. TMR was excited with the green laser (A), and ANV was excited with the blue laser (B). These images were pseudo-colored and merged (C). The morphology is shown in D; arrows indicate apoptotic cells. Hepatocytes were exposed to A-NK cells for 70 min. Rotenone was added 20 min after the start of the incubation.

of intracellular ATP. In cells incubated with rotenone in the presence of fructose the ATP level remained partly intact, but, importantly, the MMP was at the same level as in control cells indicating that locally generated ATP from fructose is used for maintenance of the MMP. Such a mechanism has been described in osteosarcoma cells with defective mitochondria and is based on reverse action of the ATP synthase (38). The enzyme that ultimately produces ATP from 3-phosphoglycerol-phosphate, phosphoglyceratekinase, is located throughout the cytosol and often in the direct vicinity of mitochondria (39). Therefore these organelles have direct access to newly formed ATP. We therefore hypothesize that locally produced ATP is used for maintenance of the MMP rather than transported to peripheral regions of the cell.

Only a part of the mitochondrial population in a cell lost its potential in our experiments, resulting in a population of cells that have a relative high overall MMP but do expose PS locally. This is in contrast with the situation in thymocytes and lymphoma cells (6, 40) in which the MMP needs to be fully disrupted before externalization of PS occurs. Above we hypothesized that the link between MMP and PS is a local drop in ATP. The ability of cells to generate ATP by mitochondria or glycolysis and the rate of ATP consumption determine the ultimate local ATP level. In particular, local consumption of ATP by mitochondria and the presence of high ATP consumption by the plasma membrane $\text{Na}^+\text{-K}^+$ - and $\text{Ca}^{2+}\text{-ATPase}$ at the periphery may result in a lower [ATP] in this domain than in the bulk of the cell cytosol (41). This may differ strongly between different cell types and, therefore, influence the coupling between MMP and PS. As the phospholipid translocase is also an ATP-de-

pendent enzyme this may explain why locally, where the mitochondria do not function anymore, PS externalization takes place.

Next to ATP, calcium-dependent processes have also been described to play a role in PS externalization by activation of a calcium-dependent scramblase or synthesis of PS through a calcium-dependent exchange of the polar head group of pre-existing phospholipids. We measured the calcium concentration in hepatocytes loaded with Fura-2 during attack by A-NK cells. ANV and TOTO-3 were added to the medium, and, therefore, we could monitor when cells became apoptotic or necrotic. We found no straightforward relation between elevation of intracellular calcium and PS exposure. In 60% of the hepatocytes calcium was elevated before exposure of PS, but in 40% it was not. Also, in a number of cells, the calcium levels decreased to baseline again before PS exposure occurred. In addition, some hepatocytes had elevated calcium levels but did not expose PS. Therefore, in the experiments with the A-NK cells the activation of a calcium-activated scramblase is probably not essential (but cannot be excluded). Similarly the involvement of calcium-dependent synthesis is doubtful.

In conclusion we show that in hepatocytes during apoptosis mitochondria in certain areas within the cell lose their MMP resulting in local exposure of the PS whereas in other areas mitochondria remain intact. Although the former apparently leads to exposure of PS, which is essential for removal of the apoptotic bodies, the latter could be important for proper assembly of cytochrome *c*, apoptotic protease activating factor-1, procaspase-9, and ATP into an apoptosome. We used in this study A-NK cells that secrete the apoptosis-inducing molecules

granzyme B/perforin and produce FAS ligand and tumor necrosis factor-related apoptosis-inducing ligand. In addition, ATP was used, which induces an immediate influx of calcium, resulting in a loss of MMP (42) and cycloheximide that is a protein synthesis inhibitor. All these stimuli produced the same result, and, therefore, the spatio-temporal relationship between a decrease in MMP and exposure of PS could be a general phenomenon in cells that depend on mitochondria for their ATP supply.

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